Investigation of combinatory drug mix on cell behavior

Arora, Shuchi

2011

Arora, S. (2011). Investigation of combinatory drug mix on cell behavior. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/45594>

<https://doi.org/10.32657/10356/45594>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**INVESTIGATION OF COMBINATORY DRUG MIX ON CELL
BEHAVIOR**

SHUCHI ARORA

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2011

INVESTIGATION OF COMBINATORY DRUG MIX ON CELL BEHAVIOR

SHUCHI ARORA

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

2011

ACKNOWLEDGEMENT

This project and thesis would not have been possible without the contribution and support from a number of people. They have provided invaluable suggestions and criticism as well as encouraged the author on various occasions. Thus, it is a pleasure for the author to extend her gratitude towards them.

The author owes her sincere gratitude to her supervisor and mentor, Associate Professor Lim Chu Sing, Daniel. His continuous support and motivation had deeply inspired the author that led to the completion of this work. His willingness and timeless guidance at all occasions contributed tremendously to this project.

The author would also like to thank Nanyang Technological University, Singapore, for their trust in the author with the award of the Research Student Scholarship.

The author is also indebted to her colleagues and fellow PhD students for their support and help in the laboratory experiments. With special thanks to Mr. Deepak Perumal, Ms. Myintzu Myaing, Ms. Win Mar Soe and Ms. Aneira Pariwano among others.

Lastly, the author would like to extend her gratefulness to her parents, her brother and close friends for their moral and emotional support throughout the course of the project. The author is thankful to Mr. Azfar Khan for his continuous motivation and emotional support especially at times of distress and hardships.

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ABSTRACT

Real-time combinatory drug responses on biological cells have become important for drug screenings with growing threat of diseases. Though rapid progress has been made in the field of biotechnology and pharmacy, the need to understand the effects of drug combinations, such as antibiotics and herbal medicines, on cells have become essential. Drug combinations are used to accelerate and/or increase the efficacy of a treatment. In case of infectious diseases, however, the predicaments of drug resistance prevail in the contemporary scenario. Drug combinations, in these cases, are mainly used to overcome such resistance by the disease causing pathogens.

The present project created a unique system that can simultaneously investigate cell behavior exposed to a combinatory mixture of drugs. The system was aimed to conduct novel studies of combinatory drug performance on various cell types *in vitro*. Protocols for handling different cell types that require different culture conditions were established. The system consisted of a MEMS-based microchannel device and a controlled incubator chamber to accommodate the device that provided an environment for cell sustenance. Together with the control and electronic circuitry, the drug and cell injection systems, the setup was coined as the Combinatory Drug Screening System or CDSS. The system allowed monitoring and tracking of cells inoculated in the culturing chambers of the micro-device, under influence of drug combinations. The combinatory drug response, physiological behavioral changes, such as cell count or optical density, and/or tracking of cell mutations, could be recorded from the system using spectroscopy or microscopy.

As a proof-of-concept, different cell types were initially tested in the system. Mammalian adhesion-type cells and bacterial suspension-type cells were used to establish initial results that closely matched the growth curves of the conventional cell culturing techniques. Subsequently, the system was used to investigate the effects antibiotic, ciprofloxacin in combination with four antibacterial phytochemicals and maggot excretions/secretions (ES),

on *Staphylococcus aureus*. *S. aureus* is one of the most common nosocomial pathogens, causing diseases ranging from superficial dermatological lesions to systemic and life threatening infections such as endocarditis. Due to its rapid adaptive behavior under antibiotic influence, its genome has been termed as the “super-genome” and itself as the “super-bug”. A particular strain of *S. aureus*, methicillin-resistant *S. aureus* or MRSA is known to be resistant to almost all available antibiotics.

The antibiotic-phytochemical combination investigated on *S. aureus* using the CDSS, was shown to delay the occurrence of resistance, which was otherwise encountered after 72 hours of antibiotic exposure. The phytochemicals, therefore, helped to potentiate the activity of ciprofloxacin, when their activity alone was negligible.

The maggot extracts investigated for the first time using maggots of *Lucilia cuprina* blowflies, comprised of an antibacterial compound, phenylacetaldehyde, that led to partial bacterial growth inhibition in gram-positive *S. aureus*. The combination with ciprofloxacin, however, was able to enhance the maggot ES activity.

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1 INTRODUCTION

1.1 Background

Combination therapy is an outcome of a medical situation wherein threat of diseases is constantly increasing. In medical terms, combination therapy refers to – a method of treating disease through the simultaneous use of variety of drugs to eliminate or control the biochemical cause of a disease [1]. Effective drug treatments are required constantly to battle against the spread of diseases. This condition is especially evident in the case of treatment of infectious diseases with antibiotics. Bacterial pathogens are progressively becoming resistant to available antibiotics that are being used to treat them [2]. The rate of emergence of new antibiotics is, however, slower than the rate at which resistant species are being detected around the world [3, 4]. Combination therapy is thus, typically exercised to combat problems and spread of antibiotic resistance [5].

Development of resistance to a drug(s) may cause a treatment to fail and may even lead to failure of future treatments because of sustained resistance to the drug(s) used. Due to this reason, research into alternative therapeutic remedies has been incited in the recent past. Alternative and complementary medicines, such as the ones derived from plants, animals or insects, are of growing interest. Some of these natural medical remedies have been used for centuries for treatment of several diseases and ailments by our ancestors. There has been a trend in the recent past, to understand and isolate specific factors or compounds and their related mechanisms that impart them with medicinal value. Reasonably, the present project also involves antibacterial compounds of plant and insect origin in combination with antibiotics that are known to encounter resistance from the disease causing pathogens. These naturally occurring and ubiquitous antimicrobial compounds enable its host with

intrinsic abilities to fight pathogenic invaders. Moreover, these have been propagated by experts to have tremendous prospects in terms of their medicinal value [6-8].

Combination therapy has also been practiced among the medical folklore to treat several other diseases. It has been used for treating some types of cancers, viral diseases (including retrovirus, HIV), fungal diseases and mental illnesses among others. The rationale to combine drugs for treatment of diseases and disorders is to achieve higher therapeutic outcomes compared to when a single drug is used. The clinical significance of such therapies is usually acceptable as long as the drugs do not interact antagonistically. However, the effects of drug interactions are oftentimes undesired, unoptimized or even harmful. These interactions may increase or decrease the therapeutic effects of one or more drugs, resulting in side effects or failed treatments. This is because, drugs interact in highly non-linear ways that lead to diversified effects and hence, difficult to predict. Optimization of drug combinations that are indeed useful is, therefore, imperative. This can be carried out by the use of combinatorial chemistry algorithms, by performing experiments with random combinations and studying their effect *in-vitro* or *in-vivo*. The *in vitro* systems could comprise of bacterial cells isolated from patient or soil/water samples, mammalian cells like tumor cells, liver cells or blood plasma. These can be experimented upon with two or more drugs and/or medicinal herbs to study drug-herb interactions.

Drug screening experiments and biological assays require large number of samples and chemical reagents to be handled at an instant, together with their multiple dilutions and combinations. As the number of combinations to be tested increases, the conventional methods of large scale testing based on robotic fluid handling and multi-well plates becomes increasingly expensive, impractical and time consuming. In this case, miniaturized fluid handling systems or the microfluidics approach provides a faster and cost effective method. By integrating processes such as mixing, pipetting and cell screening on the same microfluidic device, laborious procedural steps can be reduced and simplified.

Microfluidic technology in the recent past has seen tremendous development in the field of bio-chemical [9-11] and pharmaceutical [12] analysis. These miniaturized fluid handling systems offer several advantages over conventional techniques, such as low sample consumption, ability to carry out separation and detection with high resolution and sensitivity, cost effective production, reduced analysis or experiment time, portability and rapid prototyping [12, 13]. Properly designed microfluidic systems can perform tasks quicker than conventional counterparts and at the same time, consuming smaller amount of reagents. In addition, in the microscopic scale, the low dimensions enable access to a unique physical behavior of fluids in confined spaces that leads to completely new ways of acquiring chemical and biological information.

1.2 Objectives

The main motivation behind the present work was to create a system that would monitor and track combinatory drug effects in order to provide a more effective means of accessing drug-drug effects on cell outcomes.

From the above considerations the following objectives were laid out:

1. To create and optimize a novel system for simultaneous drug-drug investigations on cell outcomes, Combinatory Drug Screening System (CDSS).
2. To validate, calibrate and test the system based on its intended applications, by performing case studies and experiments with different cell types, such as adhesion and suspension cells and related drugs, such as synthetic drugs, natural drugs and herbs along with their combinations.
3. To carry out system characterization and optimization of protocols with respect to various biological and engineering considerations, that include several aspects of its design, layout and fabrication, fluid mechanics and dynamics owing to flow of reagents through the system, mixing issues, structural and functional limitations,

coupling cell culturing procedures with the system requirements, acquiring recordable information and results from the system and data analysis.

1.3 Scope of work

The scope of this project entails the use of MEMS-based microfluidic system that was developed and tested. The device materials, poly-dimethylsiloxane (PDMS) and glass, were used due to their known compatibility with biological samples. For initial tests on the CDSS and microfluidic device, mammalian neuroblastoma cells were used as adhesion-type cells and bacterial *Escherichia coli* cells were used as suspension-type cells. Subsequently, the drug combination studies were focused on *S. aureus* bacterial species that included two strains, methicillin-sensitive and methicillin-resistant *S. aureus* (MSSA and MRSA, respectively). The drugs used in combination were limited to fluoroquinolone antibiotics, ciprofloxacin and ofloxacin with four antibacterial phytochemicals. Out of the four phytochemicals, two were plant flavones, quercetin and rutin; one was a phenolic acid, protocatechuic acid; and one was a tea catechin, ethyl gallate. The antibiotic was also screened in combination with an antibacterial insect extract, extracted from maggots of *Lucilia cuprina* blowflies.

2 LITERATURE REVIEW

2.1 Drug-drug combination therapeutics

2.1.1 Introduction

Combination of two or more drugs has been common in clinical therapeutics. Also referred to as polypharmacy; administration of many drugs or excessive medication may have undesirable consequences and even failed treatment. In addition, treatment based on combination of drugs may not be evidence based. The common consequences of polypharmacy are adverse drug reactions, drug-drug interactions or sometimes higher cost. Each medication has side-effect(s) associated with it. When many drugs are taken together, the risk of side-effects and adverse effects increases exponentially [14]. On the other hand, some drugs may even work synergistically. For single drug treatment, the drug dose can play an important role; it can be therapeutic at one dose and toxic at another. As said by a respected English physician and medical educator, Peter Mere Latham (1789-1875): *“Poisons and medicine are oftentimes the same substance given with different intents”*.

Drugs in combination or polypharmacy can produce enhanced or reduced pharmacological effect of each of its components. They may interact in synergistic, antagonistic or indifferent manner. While the latter does not pose any advantage or disadvantage to the treatment, synergistic and antagonistic drug interactions can have extreme effects. A review of drug-herb interactions and its effects on long term medication was presented by Fugh-Berman [15]. He emphasized that continuous administration of herbs along with drugs may mimic, magnify or oppose the effect of prescribed drugs. Furthermore, over and above the physicians prescribed drug mixture, patients often add over-the-counter medications, such as vitamins, herbal remedies and specific food products, all of which

have a potential to interact once ingested. He also highlighted, some adverse effects associated with drugs and herbs taken together.

Data on true drug-drug interaction studies is not readily available and occasionally found in literature. Specific examples can be cited from past studies that describe interactions between pharmaceutical drugs. For example, American ginseng, while clinically demonstrated to improve immune functions and conditions associated with diabetes, reduces the anticoagulant property of warfarin [16]. These effects can complicate the dosage of long term medications for diseases like cancer or diabetes. In an event of combination therapy requirement for a treatment, the exact dose of each of its component drug must be optimized to reduce the side-effects caused by their combination. Moreover, drug response varies among individuals due to a host of factors, including age, weight, sex, ethnicity, environmental influences, pre-existing disease, and reactions to other drugs and herbs [17].

As modern medicine is moving from targeted medicine to personalized medicines, drug dosages are required to be optimized. The significance of genetic diversity, which underlies many basic biological differences, has been highlighted in number of papers in literature devoted to pharmacogenetics. Pharmacogenetics studies show how genetic make-up of individuals affects their response to medicine with the ultimate aim of improving drug efficacy and safety [18]. Authors of ‘Personalized medicines: hopes and realities’, further asserted that drug therapy should aim to deduce an accurate dosage to produce an optimal patient response, since some dose-response relationships can be quite steep [17]. Given the widespread popularity of complementary and alternative medicines and herbal therapies, cancer and surgical patients are at risk with such treatments, as together with prescribed drugs they can have potentially dangerous or even beneficial interactions [19, 20]. In that case, doctors can only advise patients to avoid concurrent consumption of both substances. Besides, it is unethical to plan clinical studies for the purpose of studying foreseen undesirable and unintended effects of drug-herb or drug-drug

interactions. In the light of emerging personalized medicine, some have suggested that such combination treatments should be encouraged rather than being avoided [21, 22]. As many underlying combinations at optimized doses can be useful and may help to accelerate the treatment. By conducting more studies, it would facilitate to unveil adverse effects for supplementary use of beneficial herbs on diseased patients.

2.1.2 Antimicrobial combination studies

This part of the review would mainly focus on antibacterial combination investigations and its importance for future antimicrobial medicine. The main driving force that led to the study and application of combination therapy for treatment of bacterial infections, was growing bacterial resistance [23] (discussed later in Section 2.5). The other common indications that lead to combination therapy are treatment of life threatening bacterial infection or polymicrobial infections and the search for synergism. Since the discovery of penicillin in the 1940s, that had revolutionized antimicrobial chemotherapy, there has been an increasing number and spread of resistant bacterial pathogens in both hospitals and community settings [24]. Antibiotic-resistance has been, therefore, a major threat to treatment of bacterial infections as many of the community acquired and nosocomial bacteria have developed resistance to many commonly available antibiotics [25]. Due to this changing response pattern of bacterial pathogens to drugs, new treatment strategies need to be established. Tylor *et. al.*, [26] has discussed in his work, the potential of new methods to treat bacterial infection, with emphasis on non-genomic approaches, like modification of bacterial phenotype, photosensitization and bacteriophage therapy. However, immediate actions imperative at this time and that could be applied to antibiotic chemotherapy are - either development of new antibiotics, or revitalization of the old ones.

One of the direct consequence of using antibiotics in combination is that the synergistic or additive combinations are able to reduce the antibiotic dosage to one-fourth or half of its minimum inhibitory concentration (MIC). This helps in reducing the dosages of several

synthetic antibiotics associated with dose related toxicities, such as chloroamphenicol, aminoglycosides, sulphonamides and some quinolones. On the other hand, antimicrobial combination studies also help in eliminating antagonistic combinations, in which the effect of combination is lost or significantly less compared to individual effects of single drugs. It also has the potential to slow the development of resistance, if multi-target therapy is employed [27]. Hence, combination therapy with antibacterial agents is believed to address the problems of growing antibiotic resistance and abuse caused due to antibiotic overuse [23].

With combination therapy gaining popularity due to increasing instances of antibiotic-resistance, statistical models to analyze combination therapy were established to represent and evaluate the data related to it. One such model, known as the Loewe additivity, assumed that the two combining drugs act through similar mechanisms or act on similar targets. In this case, the antibiotic combination effect was constant when one antibiotic was gradually replaced by increasing amounts of the other. Hence, the effect remained same at different combination mixtures also [28]. Another model that dealt with antibiotic combinations was Bliss independence, in which the two drugs act independently. The assumption was that the two drug components could bind to their targets simultaneously and mutually nonexclusively through distinct mechanisms [29]. Apart from application in antibacterial drug combinations, these models have been applied to other combination experiments. However, these models may not be applicable to certain combinations involving potentiating agents, such as plant-derived antibacterials or phytochemicals. Potentiation is a phenomenon in which antibiotic activity is enhanced with addition of a compound that itself is either weak or has low antibacterial activity [23].

In their extensive review on combination therapy by Cottarel *et. al.* [23], the development of useful combinations of antibacterial agents has been classified into four principle modes of actions. These are summarized below:

- The addition of a compound or drug that prevents the degradation or modification of the other (antibiotic)
- The addition of a compound or drug that inhibits the efflux pumps, allowing the accumulation of the other
- The addition of a compound or drug that inhibits the alternative pathways or tolerance mechanism against the other
- The other compound or drug has a different antibacterial mechanism and independently targets similar or different pathway

Combination of antibiotics with other antibiotics has been in practice for many years. Many studies have shown successful synergistic or additive outcomes of two antibiotics in combination [30-33]. Some have even gained commercial success as a single drug in market. For example, Augmentin[®] (manufactured by GlaxoSmithKline), is a combination of amoxicillin (a β -lactum antibiotic) that targets the cell wall and clavulanate (a lactamase inhibitor) that prevents the bacterial β -lactamase enzyme from degrading amoxicillin. Other examples of commercial medications based on combination of β -lactum and lactamase inhibitor include, Timentin[®], Zosyn[®] and Unasyn[®] (manufactured by GlaxoSmithKline, Wyeth and Pfizer, respectively). Bactrim[®] and Septra[®] (manufactured by Roche and King Pharmaceuticals, respectively) are combinations of trimethoprim and sulfamethoxazole, both inhibitors of different steps in folic acid pathway and inhibit a specific step in nucleotide biosynthesis pathway. Likewise, many examples can be quoted from literature and elsewhere that can illustrate the importance of antibacterial combination studies *in-vitro* that has been translated into clinical setting and commercial success.

The introduction of alternative and complementary medicine to antibacterial combination therapy has been recently brought into light. The quest for clinical efficacy and to reduce the antibiotic dosages, thereby reducing toxicity associated with them; combination of

antibiotics with alternative natural antibacterials has been gaining popularity. Even though combination of antibiotics has shown to be effective, the problems of growing resistance still prevail. Hence, increasing demands for compounds or agents that can reverse or delay the development of resistance when treating bacterial infection with an antibiotics, exists. Agents from natural resources, such as, plants, animals or insects could be valuable sources. Traditionally, plant extracts or herbal medicines have been used for centuries for treatment of a number of diseases and disorders including high blood pressure, pain, asthma, depression, viral infection, cancer, diabetes etc. [34-41]. Rich in wide variety of secondary metabolites such as tannins, flavonoids, terpenoids, alkaloids, plant-derived chemicals or phytochemicals, have been shown in the past to possess antimicrobial activity [7, 42-48]. However, with the discovery and popularity of antibiotics in the middle of last century and its rapid effective action against bacterial infections, the use of plant extracts or herbal remedies dropped significantly.

In the recent years, the rate of antibiotic development has reduced significantly compared to the last century [3, 49, 50]. Combination of an antibiotic with plant extracts with known antibacterial properties could help to reduce the antibiotic dosage, as discussed above, as well as harness the benefits of herbal medicines. Plant extracts has potential to modify and/or modulate bacterial internal resistance mechanisms and potentiate the antibiotic activity when used in combination [51]. This, in general, could increase the efficacy of the treatment by inhibiting or delaying the phenomenon of resistance causing elements, like mutation of genes encoding target enzymes, proteins or efflux pumps [51]. Since plant-derived antibacterials are generally weak and have much higher MICs compared to antibiotics ($> 1000 \mu\text{g/mL}$), their clinical relevance is low. With such high MIC values *in-vitro*, the bioavailability of the drug *in-vivo* as effective dose becomes difficult to reach. The rationale to combine them with available antibiotics is to make them utilizable in a practical setting. Many academic researchers have reviewed attractive potential of plant-derived antimicrobials, and have suggested a combinatory outlook to make them clinically

useful [6, 7, 51, 52]. Plant antimicrobials with their narrow spectra of action can target multiple sites in a bacterial cell, many of which are potentiating targets for a selected antibiotic. Their addition as resistance modifying agents can be beneficial when used in combination with a strong antibiotic. This would help in reducing the antibiotic dose as well as dose related toxicity and undesirable side-effects.

Antibiotic potentiating agents are also found abundantly in insects. Like plants, insects also produce secondary metabolites that help them fight against pathogenic invaders; these mainly belong to the class of antimicrobial peptides. Bulet *et. al.* [8, 53], has extensively reviewed the structure and functions of many of these insect-derived antimicrobial compounds. These small molecular weight peptides give insects their intrinsic antimicrobial ability. They are able to produce a series of similar structured peptides to fight against invading pathogens, and may work in synergy, providing insects a powerful defense mechanism. On similar lines, antibiotic substances have been found in maggots of blowflies, used for debridement and healing of chronic wounds [54-56]. Their potential application in antibacterial combination therapy has not yet been harnessed.

2.2 Microfluidic screening systems

2.2.1 Introduction

Microfluidics, by definition, is the science of systems or devices engineered by microfabrication methods to manipulate and handle small amount of fluids (10^{-4} - 10^{-9} L) and their transport process in an in-built microchannel network of characteristic length scale in the micrometer range [13, 57, 58]. They are highly integrated devices capable of incorporating mixing, separating, detecting, monitoring and other useful laboratory processes into a single device, also known as lab-on-chip devices [13, 58]. In the last two decades, the field of microfluidics has witnessed tremendous development, particularly in

the analytical systems for applications in the field of biology, chemistry, biochemistry and pharmaceutical analysis [10, 11, 13].

The expression ‘miniaturized total chemical analysis systems’ was coined by Manz *et. al.* in the early 1990s, with their conceptual work on miniaturized total analysis system (now known as micro-total analysis systems or μ TAS) [59]. Later, he described the fabrication of microfluidic devices using the photolithographic techniques adopted from the microelectronic industry [60-62]. Microfluidic devices and its applications has since then attracted many scientists from across the world because of the numerous advantages that these devices offer. Firstly, the traditional advantage of significantly low volume consumption of sample and reagents due to small size. Correspondingly, low reaction and analysis time, due to high surface to volume ratio. Other advantages include, high sensitivity, amenability to automation, capability of multiprocessing and multi-analyte detection. All the above advantages help to improve process performance and throughput. Moreover, due to the availability of the fabrication methods to fabricate and integrate microfluidic devices with length scales of the order of tens and hundreds of microns, the ability of mass production of these devices also makes them cost effective [63, 64]. At the same time, with the rapid development in biology and biotechnology for which manipulations in the cellular length scale and ability to detect small quantities are required, microfluidics offer advantages [12, 58]. Its applications also include, combinatorial and small quantity syntheses and reactions, high-throughput screening in drug development, macromolecular analysis including DNA and enzyme assays; cellular analysis such as examination and manipulation of single cell, cellular biosensors, cell culturing and high-throughput DNA sequencing [13, 65]. A number of microfluidic systems have also been reported for proteomics and genomics analysis [66].

2.2.2 Fundamentals of microfluidic flow

Microfluidics typically involves handling of fluids in structures of dimensions of the order of 100 nm to 200 μm (10^{-7} to 2×10^{-4} m) [67]. To design a microfluidic system, comprehensive knowledge of fluid dynamics in the microscale regime is important so that the unique characteristics of fluid behavior can be adequately harnessed. Fluid flow in microscale is different in many aspects from that in macroscale. Therefore, studying flow at miniature scale often require different analytical numerical tools. Typically, microchannel flow requires high pressure differences and has very small flow rates.

Classical fluid dynamics or conventional macroscopic flows considers that the fluid properties vary continuously throughout the fluid or are in continuum [68]. For example, properties such as density, velocity, and pressure are assumed to vary continuously in a flow and is defined everywhere in space. It only takes into account the macroscopic properties of the fluid, as a whole. But in the microscopic regime, the fluids behave entirely in a different manner from our everyday perception of fluid behavior. This different behavior of fluids can be attributed to a number of reasons. As the dimension shrink to microscale, the relative importance to surface to volume ratio increases [69]. The fluid flow is governed by viscous forces and pressure gradients with a low moment of inertia. This results in a pure laminar and turbulence free flow. At these scales, surface tension is a powerful force, mixing becomes difficult due to lack or nonexistence of turbulence, diffusion is dominant and only available method for mixing, unless different geometries to introduce turbulence or external/in-built mixers are incorporated. At this scale, even evaporation acts quickly on exposed liquid surfaces. To summarize, the basic differences between microscopic and macroscopic flows lies in the forces that take over in the microfluidic environment, which include, laminar flow, diffusion, surface to volume ratio, surface tension, evaporation and fluidic resistance [65]. These dominant forces are discussed in the following sections.

2.2.2.1 Reynolds number and laminar flow

Flow can be characterized into laminar, transitional or turbulent flow. It can be influenced by a number of fluid properties such as, kinematic properties, which includes velocity, viscosity, acceleration, and vorticity; transport properties, which includes thermal conductivity and diffusivity; and thermodynamic properties, which includes pressure and density [67]. Likewise, a dimensionless parameter, Reynolds number (Re), also evaluates extensively the state of a fluid in motion. Reynolds number relates the inertial forces to the viscous forces and is given by,

$$\text{Re} = \rho \frac{ul}{\eta} \quad (2.1)$$

where, u , l , ρ and η are respectively, fluid speed, length scale (e.g. channel diameter over which changes in the fluid velocity occurs), density and viscosity of the fluid. This parameter is used to characterize the flow regime, whether laminar, transitional or turbulent. The interpretations of Re is often reported differently by different authors and experts in this field. A summary of the differences is given in Table 2.1. There is no fixed agreed upon number for laminar flow characteristics, but usually low Re numbers (≤ 1) in microfluidic channels can be safely considered to be laminar. For example, flow in a microchannel of height 100 μm , of water with shear viscosity $\mu \sim 0.01 \text{ gm/cm}\cdot\text{s}$, at a speed of 1 cm/s, the Re is 1.

Turbulent flow is chaotic and unpredictable flow. It is characterized by rich and complicated flow structure of interacting vortices [70]. Laminar flow, on the other hand, is characterized by high momentum diffusion, low momentum convection, pressure and velocity independent of time. That is, velocity of a particle in a fluid stream is not a random function of time. For the most simple microfluidic channels with characteristic dimensions less than 1 mm and velocities less than a centimeter per second, the flow is considered to be almost always laminar [72]. In laminar flows, the mixing between two

streams flowing in contact with each other will not happen except by diffusion. This is one of the limitations of micro-range flow, where mixing of two fluids becomes difficult and external power or complicated geometry must be incorporated to bring about mixing (discussed in later sections).

Table 2.1: Different interpretations of Re for laminar, turbulent and transitional fluid flow

Re for Laminar Flow	Re for Turbulent Flow	Re for Transitional Flow	Reference
< 1500	> 1500	1500 - 2500	Nguyen and Wereley, 2002 [67]
< 2100	> 4000	2200 - 3900	Saliterman, 2006 [57]
< 2300	> 2300	-	Beebe <i>et. al</i> , 2002 [65]
« 1	> 1000	100-10000	Brody <i>et. al</i> , 1996 [70]
< 2000	> 2000	-	Sharp <i>et. al</i> , 2001 [71]

2.2.2.2 Diffusion in microscale

In microchannels, where the flow is laminar, mixing can only take place by the process of diffusion. Diffusion, by definition, is a process by which particles in a fluid system move by Brownian motion and cause them to spread out over time so that the average concentration of particles is constant throughout the system. The mean square displacement of a particle from its origin is given by,

$$d^2 = 2Dt \quad (2.2)$$

where, d is the displacement of particle in time t and D is the diffusion coefficient. Since, distance varies as a power of 2 with time, diffusion is significant entity in microscale systems. In simple microchannels, pressure flows are laminar and uniaxial, so mixing between the streams is purely diffusive. Moreover, in many microfluidic devices, mixing is usually imperative and with diffusion as the only form of mixing available, other forms of passive or active mixing are incorporated [57].

2.2.2.3 Surface area to volume ratio and surface tension

The surface area to volume ratio increases significantly from macroscale to microscale particularly for microchannels. The million fold increase in surface to volume and its relative mass per unit volume, considerably affects the mass, momentum and energy transfer through the surface [73]. This might be advantageous for some processes but disadvantageous for others. For example, evaporation may increase in open microchannels for some applications where evaporation is not desired.

Similarly, surface tension plays an important role at microscale. As an example, the height water that will travel in a capillary is directly related to surface free energy of water and inversely related to the radius of the capillary. In case of microchannels, that can practically act as small capillaries, the length the liquid will travel based on capillary action alone is important and can be harnessed to pump or create virtual walls in microchannels [65].

Other significant and dominant forces include fluidic resistance and evaporation. These are direct consequences of forces discussed above. The fluidic resistance is inversely proportional to the forth power of radius of the channel (for circular channels), therefore as the dimensions are reduced, the resistance in the fluid increases as a power of 4 [65]. Evaporation at microscale is rapid because of the increase surface to volume ratio. In open channel networks this may be an undesired effect.

2.2.2.4 Fluid dynamics at microscale

Due to a fundamentally different fluid behavior observed at microscale than in macroscale, many reports seek to analyze fluidics in microstructures and geometries through mathematical models, simulations and visualizations [67, 71, 74]. The laws that govern fluid phenomena at the microscale has been explained extensively in a review by Brody *et al.* [70]. They explained the Navier-Stokes equation (the basis for fluid-related

mathematical derivations) for laminar flows, the *Hele-Shaw* flow and the *Poiseuille* flow in their work.

To start with the dynamics of the flow at microscales one must first consider the basic equation that governs incompressible fluid dynamics, the Navier-Stokes equation, given by,

$$\rho \left[\frac{\partial \vec{u}}{\partial t} + (\vec{u} \cdot \nabla) \vec{u} \right] = -\nabla P + \eta \nabla^2 \vec{u} \quad (2.3)$$

where $\vec{u}$ is the fluid velocity, P is the pressure, and η is the fluid viscosity. The equation above is also known as the Newton's law for a fluid, with forces per unit volume on the right hand side, due to pressure gradient, $(-\nabla P)$, and viscosity, $(\eta \nabla^2 \vec{u})$. The left hand side gives the mass per unit volume, ρ -times the acceleration of the fluid, expressed in terms of the convective derivative of the Eulerian representation.

In microfluidics, since flow is assumed to be laminar with low Re number, the fluid flow is dominated by viscous forces (Eq. 2.1), the left hand side of Eq. 2.3 can be effectively ignored. This transforms a nonlinear differential equation into a simple linear one. The flow is determined entirely by pressure distribution with no slip boundary conditions. The governing equation becomes,

$$\eta \nabla^2 \vec{u} = \nabla P \quad (\text{Stokes Equation}) \quad (2.4)$$

This type of flow is usually called “Stokes flow”, “Creeping flow” or “Low Re number flow”. It contains no time derivative, and all motion is symmetric in time. Thus, if the pressure or forces exerted on the fluid are reversed the motion in the fluid is completely reversed [75]. Laminar flow can, therefore, be described by the exact solution of the Stokes equation (Eq. 2.4) with no slip boundary conditions, known as the *Hagen-*

Poiseuille flow or just *Poiseuille* flow. It is seen that at any cross-section the velocity distribution of the fluid is parabolic [57]. The velocity distribution is given by,

$$v_z = \frac{1}{4\eta} \left(\frac{\partial p}{\partial z} \right) (r^2 - R^2) \quad (2.5)$$

where, r is the distance from the center of the channel and R is the radius of the channel (for a circular channel), $\partial p / \partial z$ is the z -component of the pressure gradient, the maximum velocity being at the center of the channel and minimum at the channel wall.

The *Poiseuille's law* or the relationship between the volume rate of flow, Q , passing through the channel and the pressure gradient is given by,

$$Q = \frac{\pi R^4 \Delta p}{8\eta l} \quad (2.6)$$

where, Δp is the pressure drop over length l along a channel of radius R , and η is the fluid viscosity [57]. This result is typically for circular cross-section channel geometry. For rectangular channel geometry, the lubrication theory is considered because the channel width and height are usually much less than its length [69]. For a rectangular channel of height h and width w , pressure drop Δp over a length L is related to the volumetric flow rate Q by:

$$Q = \frac{wh^3 \Delta p}{12\mu L} [1 - O(h/w)] \quad (2.7)$$

where, the approximate form $O(h/w) = \frac{6(2^5)}{\pi^5} \frac{h}{w}$, gives less than 10% error for $h/w \leq 0.7$ [69], which is a modified version of the *Poiseuille's* law.

This understanding of the microfluidic physics is essential for designing, fabricating, optimizing, calibrating and testing of microfluidic devices.

2.2.2.5 Microfluidic mixing principles

Microfluidic devices are mostly popular among areas of analytical chemistry and life sciences. These applications include micro-arrays, DNA sequencing, cell manipulation and detection, drug delivery, biochemical analysis and environmental monitoring. In most of these applications, mixing is essential for analysis. In microfluidic devices, Re is typically less than 100 and hence the corresponding flow is strictly laminar. Having straight laminar flow with dominant viscous forces turbulence is absent and diffusion is the main process for transport and mixing between flowing streams in microchannels (see Sections, 2.2.2.1 and 2.2.2.2). Kockmann [76], has described microfluidic mixing principles based on diffusion, fluid distribution and convection (Figure 2.1).

Mixing in micro-devices can be introduced by passive or active means. A comprehensive review on micromixers, both passive and active, has been covered by Nguyen *et. al.* [77]. Many active micromixers have been fabricated and tested for utilization in microfluidic devices. These include mixers using pressure or temperature as an external disturbance. Other examples of active mixers include, electrohydrodynamic, dielectrophoretic, electrokinetic, magnetohydrodynamic and acoustic mixers. For the purpose of this report, more emphasis is given to passive micromixers.

Figure 2.1: Microfluidic mixing principles (adopted from Kockmann, 2008 [76]).

Several microchannel designs and geometries have been proposed in literature with an aim to introduce rapid and efficient mixing in micro-devices by passive means. These include T and H shaped microchannel geometry, zig-zag and ridged microchannels, 2D and 3D serpentine microchannels, and multi laminators [78-81]. By incorporating complex microchannel geometries, channel wall structures or obstacles in the flow path of the fluid, it is possible to introduce secondary flow effects or vortices to enhance mixing between flow streams [82]. Some examples include, splitting and re-layering the flow streams, which increases the interfacial area and promotes mixing [78]. Similarly, three dimensional serpentine channels can introduce chaotic advection in the channels that enhance mixing [79]. Furthermore, to bring about mixing, grooves and ribs in the channel can cause chaotic advection [81]. Some of the above mentioned microchannel geometries are illustrated in Figure 2.2, adopted from cited literature.

Figure 2.2: Different microchannel geometry adapted to incorporate and enhance microscale mixing by passive means.

(a) Schematic of a 3D serpentine channel, square wave channel and straight channel. The serpentine channel brings about chaotic advection to enhance mixing (adopted from Lui *et. al.*, 2000 [79]). (b) An H-shaped filter, to filter particles based on their diffusivities (adopted from van Leeuwen *et. al.*, 2009 [80]). (c) Ridges on channel walls to allow chaotic flow of fluid over the grooves (adopted from Strook *et. al.*, 2002[81]). (d) A 3D serpentine and laminating micromixer (adopted from Kim *et. al.*, 2005 [83]). (e) A split and recombine channel geometry to introduce chaotic mixing in channels (adopted from Schonfeld *et. al.*, 2004 [84]). (f) T-mixer and 3D T-S-Mixer (adopted from Kockmann, 2008[76]). (g) Y-mixer with square bends and obstructions in fluid flow to enhance mixing (adopted from Nguyen *et. al.*, 2008 [85]). (h) Different T-mixers with fluid obstructions and constricting geometry to induce mixing in the microchannels (adopted from Hsieh *et. al.*, 2008 [82]).

The above examples of incorporating rapid mixing measures into microfluidic devices require complex designs and intricate microscale geometries to be fabricated with the existing methods. Most of the passive mixers may involve two or more masking layers to be patterned on a silicon wafer (detailed fabrication process discussed in the next section).

The active mixers would require external driving force(s) for the mixing to take place. Due to the laborious process involved in fabricating such devices, the device cannot be fabricated by MEMS-based rapid prototyping techniques, using a single masking layer to make disposable and cheap micro-device and hence are not cost effective.

2.2.2.6 Determination of mixing efficiency

The most common method to determine mixing efficiency in micro-mixing structures is by flow visualization, which is done using dilution-type experiments [82]. Flow visualization is achieved with the aid of microscopic-, photo-, video- or high-speed imaging techniques. Hsieh *et. al.* [82], carried out dilution-type experiments by using iodine dye, for qualitative measurement and rhodamine B for quantitative measurement of mixing efficiency. These were visualized through conventional microscopes [82]. Micro-particle image velocimetry (μ PIV) measurements were also used for 2D velocity vectors and stretching calculations. The simple schematic of the visualization system used by them is shown in Figure 2.3.

Figure 2.3: Schematic for visualization of iodine mixing (adopted from Hsieh *et. al.*, 2008 [82]).

To view the mixing of fluids under the microscope, colors must be induced so as to aid in flow visualization. This can be done by using various dyes or coloring agents with water as diluting agent. Besides that, it may also be useful to apply fluorescence in some cases. In experiments involving fluorescent dyes as tracer, visualization is achieved by fluorescent streams; in a commonly applied reaction variant, mixing was proven by quenching of fluorescent streams [86]. The concentration of the mixed product is proportional to the intensity of the image, and the uniformity of the concentration image can then be determined by calculating the standard deviation of pixel intensity values. There are other methods to quantify fluorescent images, such as, by integration of spatial probability density functions over finite regions; and determining the 2D power spectrum of the concentration image [77]. Another study introduced the method of using pH and solvents to differentiate between the developments of two different reactions [86, 87]. Acid–base reactions with a pH-sensitive dye were used that resulted in fast reactions that spontaneously stimulate color changes. The resulting color changes of the fluids when subjected to varying pH and solvents were taken as indication of mixing efficiency.

2.2.3 Microfluidic fabrication techniques and materials

Traditionally, micro-electro-mechanical systems or MEMS and microsystems were fabricated by technology that was used in microelectronics. This included methods of micromachining on silicon or glass by photolithography and etching. These methods, however, posed several disadvantages to analytical microsystem fabrication. Firstly, silicon is a relatively expensive material and due to its opacity to UV/visible light, is not suitable for optical detection methods. The material is also not suitable for devices involving biomolecules such as DNA, cells or proteins because these molecules bind to the silicon surface. Glass, on the other hand, is transparent but amorphous, and etching of vertical side walls is difficult compared to silicon. It is also difficult to achieve desired channel geometry with these materials, such as high aspect ratio channels or structures of

different height. This is due to isotropic etching of silicon, in particular. Micromachining of silicon and glass also involves more number of steps, including, cleaning, resist coating, photolithography, development and wet etching [88]. In addition, all these steps require clean room facilities.

With the advent of growing application of microfluidic systems in biological and analytical chemistry, new materials and fabrication techniques were introduced. Polymer materials were gaining increasing interest and were mainly used as device material for microsystems. In contrast to silicon or glass, polymers are more flexible in terms of ease of use and fabrication. Many of the fabrication techniques do not require clean room facilities and are therefore, cost effective. For the purpose of discussion, this part of the review will mainly focus on poly-dimethylsiloxane (PDMS) polymer material, widely used for biological and combinatorial synthesis applications.

PDMS is an excellent material for fabricating intricate microchannel networks for application in biological micro-devices, involving liquid samples and biological entities. The material properties of PDMS facilitate certain functions useful for biological studies. An overview of fabrication of microfluidic devices in PDMS by soft lithography based on MEMS-based rapid prototyping and replica molding has been explained by McDonald *et. al.* in 2000 and 2002 [89, 90]. They explained the advantages of PDMS over silicon and glass – materials of the microelectronic industry, as well as over other polymers. Some of those advantages are listed below:

- Microchannel patterns on a substrate (master) are reproducible in PDMS by replica molding
- It is a transparent material with low auto-fluorescence and can be used for all kinds of optical detection methods
- It has low curing temperature (60-120 °C)
- It is chemically inert and not easily attacked by acids or alkali

- It is non-cytotoxic, biocompatible, and gas permeable that supports cell growth
- It can seal reversibly or irreversibly, by surface treatment, to itself or glass
- It is autoclavable for sterilization purposes

PDMS microfluidic systems have also found applications in the extensive study of cell biology [91]. Several studies and characterization of the material has been performed previously. Mata *et. al.* [92], carried out PDMS characterization based on several parameters, including, effects of spin speed, chemical immersion, oxygen plasma exposure and sterilization techniques. They reported slight or no variations in the material subjected to various fabrication and chemical processes. Furthermore, the polymer is known to be chemically inert, thermally stable, permeable to gases and simple to handle; making it ideal for biomedical applications. The solvent compatibility of PDMS devices was also carried out by Lee and co-workers [93], with similar findings.

Fabrication of microfluidic devices using polymer materials, mainly PDMS, is done by soft lithography and replication techniques. Soft lithography is a method that uses molding to achieve microstructures, by transfer of patterns from master to an elastomeric material. The master can be made on silicon or glass wafer by SU-8 thin film photolithography or bulk micromachining techniques. This method is also known as rapid prototyping. Negative photoresist SU-8 has been widely used in microfluidic and MEMS applications to achieve thick structures (> 1 mm) with high aspect ratios (> 50) [94]. Rapid prototyping is followed by replica molding, to transfer microstructures from master to the polymer material. These techniques are cost effective, less time consuming compared to silicon micromachining methods and can be performed on bench top [89]. The PDMS final device can then be reversibly or irreversibly bonded to itself, glass, silicon, polystyrene, polyethylene or silicon nitride. Due to its elastomeric nature it can reversibly attach to smooth surfaces via van der Waal forces, without causing harm to the patterned channels. This seal is watertight but cannot stand pressures greater than 5 psi [90]. To achieve an irreversible bond, both surfaces can be treated with air plasma. It is believed that the

silanol groups (Si-OH) on the surface of PDMS are exposed by oxidation of methyl groups and then it condenses with appropriate groups present on the other surface (such as, -OH, -COOH, -O-) when the second oxidized surface is brought in conformal contact with the first [89, 90]. The resultant bond can withstand higher pressures of 30-50 psi.

Untreated PDMS surfaces are hydrophobic, making the channels difficult to wet with aqueous solutions and often forms disruptive bubbles. Exposure to plasma oxidation makes the surface hydrophilic that can last for up to 30 minutes if kept in air. Treating and storing water or polar organic solvents in the channels protects the surface hydrophilicity indefinitely [93].

A schematic showing the process of photolithography and soft lithography is summarized in Figure 2.4.

(a) Photolithography **(b) Soft Lithography** **Figure 2.4: Fabrication steps for photolithography and soft photolithography.**

(a) photolithography with positive (AZ 9620) and negative (SU-8) photoresist and (b) soft photolithography with PDMS.

2.2.4 Microfluidic systems for mixing and screening

The availability of fabrication technology for microfluidic devices and the ability to couple them to monitoring and detection methods, made them useful for making specialized systems for biomedical and chemical analysis, like biological assays and biosensors, drug screening, cell sorting, capillary electrophoresis, combinatorial chemical synthesis and protein crystallization. This part of the review will cover microsystems and specialized microfluidic devices developed for specific applications in combinatorial chemical synthesis, compound mixing, cell culturing, cell and drug screening.

Many laboratory processes have been miniaturized into a microsystem, including, but not limited to, separation, mixing, reaction, pumping, detection, monitoring and cell culturing. However, reducing the process size was not the sole reason for miniaturization, but the emphasis on improving the analytical performance of the process in micrometer range [9]. In addition, the aim was to try and integrate several laboratory procedures, such as sample handling and analysis, into a single platform and at the same time, enabling monitoring and detection of its components. Integration of these active control systems into a microfluidic platform allow to carry out multiplexed bioassays such as, chemotaxis, gene expression, cell sorting and biological separation. In contrast, other submicroliter fluid handling systems such as multi-well plate robotic handling systems poses problems of evaporation and sample wastage due to pipetting errors. These robotic systems may be more expensive and demand high maintenance.

Miniaturization of DNA analysis and polymerase chain reaction (PCR) is one of the classic examples. Woolley and co-workers [95], coupled microfabricated silicon PCR reactors with glass capillary electrophoresis chips to form an integrated DNA analysis system. On similar lines, Jacobson *et. al.* [96], fabricated a monolithic device to perform DNA enzymatic restriction reaction and electrophoretic separation of its fragments based on size (see Figure 2.5). A DNA amplification PDMS-glass hybrid microchip to carry out

PCR was reported by Niu and co-workers [97]. The PDMS base incorporated the microchannels and wells and the glass cover housed the temperature sensors and heaters for precise temperature control. The two were bonded together to make the final device (see Figure 2.5).

Figure 2.5: Examples of DNA amplification and PCR microsystems.

Image on the left shows the micro-device for DNA restriction fragment analysis (adopted from Jacobson *et al.*, 1996 [96]). Image on the right shows the PDMS-glass hybrid microchip for PCR (adopted from Niu *et al.*, 2006 [97]).

Other microfluidic systems or microchips that perform several laboratory procedures in microscale have been reported. A combinatorial synthesis microchip was fabricated to synthesize amides from two different amines and two different acid chlorides. The output was a 2×2 reaction scheme on a single three-dimensional glass microchip [98]. Another combinatorial mixer was presented by Neils *et al.* [99], used for mixing four fluids through a three-dimensional microchannel network that gave sixteen concentration gradients of the input fluids at the output.

Microchips able to generate gradient mixtures of two or more input fluids have also been reported by several groups. A few have been covered in this part of the review. Gradient generator using diffusion mixing through a specialized network of channels was first introduced by Dertinger *et al.* [100]. Parallel laminar streams of different liquids from

different inlets, through a microchannel network, produced step concentration gradient perpendicular to the direction of flow in a single output channel (see Figure 2.6 a). Different gradient shapes were generated, such as parabolic, linear and periodic. A high throughput device with similar gradient generators in multiples together with parallel cell culture chambers was presented by Ye *et. al.* [101]. The device was used to study the response of drug-induced apoptosis in human liver carcinoma cells, cultured in the culture chambers of the device (see Figure 2.6 b).

A three-channel diffusion based gradient generator for bacterial chemotaxis was presented by Diao *et. al.* [102]. A similar work was carried out by Ahmed *et. al.* [103], where they compared different channel alignments to obtain linear and nonlinear gradients for bacterial chemotaxis. Another device capable of generating multiple concentration gradients produced by two input solutions for dose response measurements was described by Pihl *et. al.* [104]. It was typically used for patch-clamp measurements of voltage-gated and ligand-gated receptors in response to multiple dosages at one time.

Similar gradient generators based on controlled diffusion in a single output channel have been presented in the past. They can be used with two or more input fluids and possess the capability of generating gradients perpendicular to the flow of various shapes, linear or nonlinear, based on the specific application of the microchip [105-108]. A universal gradient generator scheme for any output profile was presented by Irimia *et. al.* [109], for two input fluids.

Figure 2.6: Examples of microfluidic systems for gradient generation and mixing.

(a) Gradient mixer (adopted from Dertinger *et al.*, 2001 [100]). (b) Integrated microfluidic device for cell-based screening (adopted from Ye *et al.*, 2007 [101]). (c) Gradient generator showing various levels of diffusion mixing and merged output channel (adopted from Yusuf *et al.*, 2009 [110]). (d) Serial dilution PDMS microchip (adopted from Bang *et al.*, 2004 [111]).

Another comprehensive gradient generator with two input fluids with six or eleven output gradients was reported by Yusuf *et al.* [110] (see Figure 2.6 c). Their design was also based on the original diffusion based gradient generator described by Dertinger *et al.* [100]. Unlike the diffusion based gradient generators discussed above, a serial dilution

microchip was fabricated by Bang *et. al.* [111], capable of mixing two input fluids and produce eight concentration mixtures, that were transferred to 96-well plates for cytotoxicity testing (see Figure 2.6 d).

Apart from gradient generators and mixers, other types of mixing techniques have also been harnessed by various groups. Liu *et. al.* [112], have patented a method of mixing in microfluidic assays by applying sonic frequency, by oscillating the fluid inside the channel using gas pocket micropumps. Yet another microfluidic micromixer was patented by Sklar *et. al.* [113], for mixing of multiple reagents flowing through microchannels. On similar lines, Pugia *et. al.* [114], have proposed and patented a microfluidic mixing device based on moving liquids back and forth from relatively large wells to small capillaries. Other patented mixing devices and techniques include, passive mixing techniques of two fluids in predetermined ratios [115], on-chip dilution system for diluting compounds [116], active mixing by using bellow pumps for mixing particles in suspension at microscale [117], among others.

Although these microfabricated systems discussed above can perform biochemical tests rapidly, they often face problems while handling and applying their output of different concentrations of two or more compounds, to a process. Most of these devices require their mixtures to be transferred to another system or device to be utilized for further analysis. Most of the gradient generators produce the gradient in a single output channel perpendicular to the flow. Their output must hence, be transferred to other devices or isolated chambers for analysis on biological entities. Many similar microreactors and combinatorial chemical synthesis microchips have been fabricated for dedicated and specific applications. They have been reviewed in details in many journals and reports. Pihl *et. al.* and Dittrich *et. al.* reviewed such micro-devices and microchip based systems that find application in the process of drug discovery and screening [12, 58].

Microfluidic devices that incorporate chambers and/or incubators for the process of cell culturing, cell detection or cell sorting, have also been fabricated and reported in literature. *In-vitro* cell culturing is a significant and one of the basic steps in modern biological research. These have been widely accepted for safety testing, since control variables can be handled with ease, the experiments are less time consuming compared to animal testing and are cost effective. Cell culturing at microscale have allowed precise control of the microenvironment of the cells, such as cell-cell interaction and cell-media interactions [118]. Microscale cell culturing platforms offer other advantages, such as increased fluid control, greater fluid-to-cell ratio and improved culture efficiency. Some of the gradient generators discussed in the previous paragraphs also incorporate cells for analysis of gradients on cell systems, for cytotoxicity, chemotaxis or pharmacological profiling [101-103, 107, 108, 119].

Kim *et. al.* [118], have comprehensively described design, fabrication and operation related issues for microfluidic perfusion culture systems for adherent cells. An extensive review of microfluidic devices and microsystems that can integrate the process of cell culturing, treatment, cell selection, lysis, component separation and their biochemical analysis, has been done by El-Ali *et. al.* [91].

Microfluidic systems have been used extensively for cell culture and screening that provides and support high-throughput investigation for drug testing or for studying complex biological processes of the cell (such as differentiation and cell division). However, the focus of this part of literature review will remain on micro-devices specially designed and developed for the process of *in-vitro* cell culturing, detection and monitoring.

Christen and co-workers [120], fabricated and tested a hybrid CMOS/PDMS microsystem for cell culture and incubation. The device was capable of sustaining a culture of BHK-21 cells (baby hamster kidney cells) for a three day period at room temperature in an on-chip cell culture vessel having a volume of 12 μL (see Figure 2.7). On similar lines, a PDMS-

silicon hybrid microchip for bacterial culture was presented by Chang *et. al.* [121]. The micro-chamber of the device was used for sustaining long term batch cultures of *Listeria innocua* and *Escherichia coli*. The micro-chamber (5.3 nL volume) and the microchannels were fabricated on a silicon wafer by bulk micromachining, whereas the three-dimensional microchannel network for injection and extraction of media and fluids was made using PDMS and Teflon tubing (see Figure 2.7). The chamber was capable of holding liquid for up to 22 hours and batch cultures of the two types of bacteria, *L. innocua* and *E. coli*. Another complex microfluidic device designed for cell culturing and screening was fabricated by Gomez-Sjoberg and co-workers [122] (see Figure 2.7). The device was fully automated with 96 independent culture chambers capable of maintaining cell viability for weeks. The chambers could be customized in terms of cell seeding density, culture media composition, and replenishment schedule, providing versatility to the entire system. The chambers could also be monitored at periodic intervals under a microscope. A microbioreactor reported by Lee *et. al.* [123], was able to perform quantitative high-throughput cell analysis. A C-shaped array unit enclosed the nano-liter cell culturing and monitoring chamber (see Figure 2.7).

Figure 2.7: Examples of microfluidic cell culture and monitoring platforms. (a) Hybrid CMOS/PDMS microsystem (adopted from Christen *et. al.*, 2007 [120]). (b) PDMS-silicon hybrid microchip for bacterial culture (adopted from Chang *et. al.*, 2003[121]). (c) Nanoliter microbioreactor for quantitative cellular analysis with cell culturing chambers (adopted from Lee *et. al.*, 2003 [123]). (d) Fully automated microfluidic cell culture system with 96 culture chambers (adopted from Gomez- Sjoberg *et. al.*, 2007 [122]).

Several microfluidic culturing and detection systems have also been patented. Lowe *et. al.* [124], developed a microfluidic device to monitor cell metabolism with an incorporated sensor, providing a method to investigate whole cell systems in an array of such devices. Others include, dedicated cell culturing device with reservoirs and monitoring chambers, such as, the microbioreactor patented by Zhang *et. al.* [125], consisting of microfermentors for chemical process development, cell culture device proposed by Takayama *et. al.* and Graefing *et. al.* [126, 127], and a microfluidic monitoring system proposed and developed by Beebe *et. al.* [128], to monitor microenvironment of the cells within the device.

2.3 Cell systems

In vitro cell systems used in biological research areas have been proved to be highly productive, in terms of solving and revealing many complex biological processes and

pathways. These are being increasingly employed in pharmaceutical research areas as well, such as, in cancer diagnostics, drug screening and toxicity testing. Experimental classification of *in vitro* cell systems can be made into adhesion and suspension cell types. Adhesion cells require a surface to attach in order to grow, in the presence of a liquid nutrient medium. They attach to the bottom surfaces that are appropriately treated glass or polystyrene flasks and dishes. These can be directly monitored under the microscope, centrifuged to get dry weight of the cells or its components or by measurement of metabolites. Examples of adhesion cells include most mammalian cells and solid tissue cells. Suspension cells, on the other hand, grow suspended in a liquid nutrient medium. The simplest way to monitor growth in a suspension is by measuring optical density of transmitted light through the liquid media. Examples include most bacterial cells and naturally occurring blood. For the purpose of this report, in the adhesion cell category, mammalian neuroblastoma cells of mouse (N2A cell lines) would be discussed in the following sections. In the suspension cell category, bacterial cells of gram negative *Escherichia coli* and gram positive *Staphylococcus aureus* would be discussed.

2.3.1 Adhesion cells: Mammalian cells

Neuroblastoma cells or N2A cells lines are mouse brain tumor cells that are commercially available for the purpose of research and development. Generally, brain tumors can be classified on the basis of their tissue origin. Tumors that begin in brain tissue are known as primary brain tumors. Most common brain tumors in humans are gliomas that begin in the glial cells or supportive tissue of the brain. There are several types of gliomas based on their origin; astrocytomas, brain stem gliomas, ependymomas and oligodendrogliomas. Out of which astrocytomas are the most commonly occurring tumors in humans. Most of their therapies are targeted to destroy the tumor mass cells. The tumor also consists of heterogeneous populations of cancer cells that differ in their ability to proliferate and form new tumors. These cells are known as the cancer stem cells (CSC). They can be a result of

sequential mutation occurring due to genetic instability and/or environmental factors. The major cell mass of a tumor has limited ability to divide but a population of these CSCs has an exclusive ability to extensively proliferate and form new tumors and they can be identified by marker expressions [129]. They have the ability to self-renew, similar to those of stem cells. These cells can drive the formation and growth of tumors.

The drugs used to treat the main tumor mass cells mostly work antagonistically for the CSCs and sometimes even facilitate their growth *in vivo*, as they may also be resistant to these drugs. Evidence also suggests that pathways that regulate the self-renewal of normal stem cells are deregulated in CSCs resulting in continuous expansion of self renewing cancer cells and tumor formation [130]. This suggests that agents that target the defective self-renewal pathways in cancer cells might lead to improved outcomes in the treatment. The identification of brain tumor stem cells provides a powerful tool to investigate the tumorigenic process in the central nervous system and to develop therapies targeted to those cells. A hypothesis proposed by Singh *et al.* [130], that the cell origin of brain tumor arise from the dedifferentiation of a normal brain cell or from the transformation of a normal neural stem cell (NSC). This is illustrated in Figure 2.8.

These cells can be identified based on marker expression, the CD133, which is a cell surface antigen found in the cancer stem cells for the brain tumor. Singh *et al.* [131], isolated a CD133⁺ cell subpopulation from human brain tumors that exhibited stem cell properties *in vitro*. Therefore, the patient must be treated with a combination of drugs that can target the heterogeneous tumor cell population to prevent its reoccurrence, consisting of at least one drug that targets the main tumor mass and one that targets the CSCs. However, each tumor may be of unique cell type and of unique basis (for example, arising due to mutations in the progenitor cells, due to radiation or similar external factors). Hence, a particular combination may not treat all types of tumors in various individuals. Thus, the main aim should focus on isolating the CD133⁺ subpopulation from the individual tumor and optimizing the chemosensitivity of these cells *in vitro*.

Figure 2.8: Dedifferentiation of a normal brain cells and transformation of a normal neural stem cell (NSC)

The normal neural stem cells on the left, divide and renew in a regulated manner. They regenerate themselves and also produce progenitor cells and then differentiate into the three main neural lineages, neurons, astrocytes and oligodendrocytes. On the right, multiple mutational events target a stem cell, progenitor cell or mature brain cell results in the formation of a brain tumor stem cells. These cells has self-renewal and proliferative capacity and also differentiates aberrantly into other cells in the tumor that have limited proliferative capacity (adopted from Singh *et. al.*, 2004 [130]).

2.3.2 Suspension cells: Bacterial cells

Unlike mammalian cells, most bacterial cells are suspension cell types. Bacteria usually replicate and increase in number by the process of binary fission and hence, grow exponentially. Their growth involves, increasing cell mass, duplication of chromosomes, synthesis of new cell-wall and membrane, partitioning of two chromosomes, septum formation and division. Once a new cell is formed, it can grow and produce more cells similarly if conditions are favorable. Hence, one cell can give rise to a large population. They can grow on solid media (nutrient agar plates) or liquid media (nutrient broth). On

solid media, one viable cell can give rise to a group of cells of same genotype and phenotype, known as a colony, and the single cell is known as the colony forming unit (CFU). Each colony has a characteristic shape, color and size and is organism specific. In liquid media, they grow in number and remain suspended in the media as they grow. As the concentration of cell increases, the liquid media becomes more turbid. The turbidity of the liquid media can be used to quantify bacterial growth by spectrometric analysis also known as turbidimetric analysis. Turbidity measurements employ a spectrometer to determine the amount of light scattered by a suspension of cells. Particulate objects such as bacteria scatter light in proportion to their numbers. The turbidity or optical density of a suspension of cells is directly related to cell number, after construction and calibration of a standard curve [132].

Other methods of measurement of bacterial growth include, physical measurements (by measuring dry weight of the bacterial cells), chemical measurement (by measuring a chemical component of the suspension, like DNA content), or metabolic activity measurement. The cell number can also be used to quantify bacterial growth in a suspension. There are several methods to count bacteria in a suspension, like direct microscopic counting using counting chambers (such as, hemacytometer) or indirect counting of viable colonies by plating on nutrient agar medium. A bacterial growth curve is a measurement tool used to illustrate and compare bacterial growth in liquid media. A typical bacterial growth curve is shown in Figure 2.9 for *E. coli* in liquid nutrient broth. Bacterial concentration for this graph was calculated by indirect counting method, by plating and counting on nutrient agar medium. The growth curve is typically divided into lag phase, log phase, stationary phase and death phase.

2.3.2.1 *Escherichia coli*

Escherichia coli (*E. coli*) is a gram negative bacterium belonging to the enterobacteriaceae family. The members of this family are non-spore forming bacilli that grow both

aerobically and anaerobically on ordinary laboratory media. Individual enterobacterial bacilli are typically $2\text{-}4\ \mu\text{m} \times 0.6\ \mu\text{m}$ in size [133]. They grow as 2-3 mm diameter, low convex, grey smooth or mucoid colonies on nutrient agar medium. *E. coli* is also a microbial symbiote found in the colon and large intestine of most warm-blooded animals and plays a critical role in vertebrate metabolism. It is a facultative anaerobe and is the predominant organism in the human gastrointestinal (GI) tract also.

Wild-type *E. coli* has no special growth factor requirements and it can transform glucose into all the macromolecular components needed to make up a cell. It is widely used for cloning genes because of its ubiquity and simplicity in handling, for example, to produce human insulin using recombinant DNA technology [134]. It is routinely used for experiments in areas of microbiology and biotechnology. The strain MC 1061 is the most commonly used *E. coli* in the biotechnology laboratories, because of its simplicity of handling and non-pathogenic nature. It is a streptomycin resistant strain. It has no antibody selection marker in its outer membrane, making them sensitive to broad-spectrum penicillins (e.g. ampicillin). They grow on simple laboratory media containing glucose as the main carbon source. Their optimal growth temperature and pH is 36-37 °C and 6.0-7.0 respectively.

Figure 2.9: Bacterial growth curve.

A typical growth curve for *E. coli* in Luria Bertani broth media in air at 37 °C for 48 hours. The curve is divided into growth phases, viz. lag, log, stationary and death phase.

2.3.2.2 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a gram-positive bacteria that belong to the family *Staphylococcaceae*. They are facultative anaerobes that grow by aerobic respiration or by fermentation, yielding lactic acid. Staphylococci are perfectly spherical cells of diameter about 1 μm . They grow in clusters because the cells divide successively in three perpendicular planes with the daughter cells remaining attached to one another following each successive division. They form distinct round yellow colonies on nutrient agar medium. They typically thrive in the human nose. But on entrance through the mucus membranes, it can cause major infection to tissues and organs of the body, mainly infections of the skin, respiratory tract and urinary tract. It is the most common cause of nosocomial infections in immunocompromised patients and general patients due to continuous use of catheters, injections or due to surgical wounds. *S. aureus* is also known for its capability of acquiring resistance to many antimicrobial agents. As early as in 1940s, after the discovery of penicillin and later methicillin, *S. aureus* had developed resistance to both β -lactams shortly after their usage. This strain was termed as the methicillin-resistant *S. aureus* or MRSA. Recently, vancomycin resistant strains of *S. aureus* have also been isolated from patients [135]. Vancomycin was introduced to treat infection caused mainly due to MRSA. It was, therefore, termed as the “super-bug” because of its widespread extents of infections in hospitals, multi-drug resistant nature, “super-genome” characteristics and hard-to-eradicate pathogen [136, 137].

2.4 Antimicrobial Drugs

As discussed in Section 2.1.2 on Antimicrobial combinations studies, the main focus in this review would remain on antibacterial drugs of synthetic, natural, plant and insect origin. The major classes of natural, synthetic and semisynthetic antibiotics include: β -lactams, aminoglycosides, cephalosporins, tetracyclines, macrolides, glycopeptides,

quinolones, sulfonamides, lincosamides, and phenicols. Many antibiotics belonging to the above major classes have encountered resistance by several multi-drug resistant (MDR) pathogenic bacteria. Plant-derived secondary metabolites or phytochemicals with antimicrobial properties are known to modulate and/or delay the process of development of resistance in bacteria and for potentiating antibiotic activity. On the other hand, insect antimicrobial substances include small molecular weight peptides, forming a part of their intrinsic mechanism to fight antimicrobial invaders.

2.4.1 Antibiotics

In this part of the review, antibiotics belonging to the family of β -lactams, aminoglycoside and quinolones would be particularly discussed. β -lactams, e.g. penicillin, acts on the cell wall of bacteria. Their mechanism of action involves weakening of the cell wall by binding to the transpeptidase enzyme, also known as PBP (penicillin binding protein). This inactivates the PBP by forming stable acyl-enzymes and prevents cross-linking. This inhibition of the glycan cross-linking leads to a weakened cell wall which eventually ruptures due to osmotic pressure. These antibiotics are also thought to depolarize the membrane potential which in turn act as a signal to induce autolysis [138]. Ampicillin is a semi-synthetic penicillin (see Figure 2.10 a), created by replacement of the α -carbon of the hydrophobic side chain at position 6 of the benzylpenicillin by an amino group. This substitution helps in extension of the antibiotic spectrum as well as penetration through the outer cell membrane of gram-negative bacteria.

Aminoglycosides, on the other hand, interfere with the bacterial protein synthesis. These antibiotics (e.g., kanamycin) contain two or more amino sugar residues, linked to a central 6-membered aminocyclitol ring by glycosidic bonds. The mechanism of action of aminoglycoside involves binding to the 30S ribosomal subunit in the bacterial cell leading to decrease in protein synthesis. They act as polycations that binds to the polyanionic 16S rRNA on the 30S ribosome at the A-site for aminoacyl-tRNA binding. This blocks the

transformational transition during the peptide-bond formation by forming translocation step in protein synthesis, bringing the process to a halt.

As mentioned in Section 2.1.2, combinations of β -lactams and aminoglycosides have been previously reported to be synergistically effective against gram-negative bacteria [31]. Many commercial antibiotics are also combination of the two antibiotic classes.

Fluoroquinolones are bactericidal antibiotics that inhibit type II prokaryotic DNA topoisomerase enzymes, important for DNA replication and cell division. They interact with either or both of two topoisomerases; DNA gyrase and topoisomerase IV. Both enzymes have a pair of subunits; in DNA gyrase there are GyrA and GyrB subunits encoded by *gyrA* and *gyrB* genes respectively. Similarly topoisomerase IV has two subunits, GrlA and GrlB encoded by *grlA* and *grlB* genes respectively. DNA gyrase is a vital bacterial enzyme, responsible for producing negative supercoils in circular DNA ahead of the transcription process. Subunit A, GyrA catalyzes the step involving the passage of a segment of DNA through a double-stranded DNA break held open by the enzyme and then reseals the break. Subunit B, GyrB is responsible for ATP hydrolysis required for catalytic supercoiling by gyrase. Topoisomerase IV is involved in decatenation of DNA, by separation of interlocked DNA strands to allow separation of daughter chromosomes to daughter cells and removes the positive and negative supercoils. At the time of transcription initiation, an enzyme reaction intermediate is formed, where both DNA strands are cleaved and covalently linked to the enzyme subunits. Quinolones bind to one of the subunits of the enzyme and inhibit further transcription by stabilizing this DNA-enzyme complex. This prevents the completion of DNA replication and eventually leads to cell death.

Figure 2.10: Chemical structures of antibiotics.

(a) ampicillin, (b) ciprofloxacin and (c) ofloxacin

Fluoroquinolones have a fluorine atom on the C-6 position, that enhances the DNA gyrase inhibition as well as penetration through the cell wall [139]. The carboxylic acid and ketone groups help in the formation of hydrogen bonds essential for its antibacterial and penetration activity. The ringed structure at the C-7 position is also known to enhance the gyrase inhibition activity [139]. A commonly used fluoroquinolone to treat bacterial infections, ciprofloxacin, has an amine substituted with a cyclopropyl group which increases its lipophilicity. They exhibit higher activity against gram-positive bacteria due to their lipophilicity. Another antibiotic that belongs to the fluoroquinolone class, ofloxacin, has an oxygen molecule substituted at the C-8 position. The chemical structures of ciprofloxacin and ofloxacin are shown in Figure 2.10 b, c.

2.4.2 Naturally occurring antimicrobials

2.4.2.1 Phytochemicals

The use of plant extracts for antimicrobial therapy and related research greatly increased in the 1990s, when the antibiotics were progressively becoming ineffective against MDR bacterial pathogens [6, 42]. Plants produce a rich variety of phytochemicals and many of

these are in response to external stress stimulus or related to their self-defense mechanism. Many of these phytochemicals also possess antimicrobial activity [52]. The major classes of phytochemicals with recognized antibacterial activity have been extensively studied in literature; these include, phenols, phenolic acids, flavones, flavonols, tannins, alkaloids, polyamides, glycosides, quinones, terpenoids, essential oils and sulfides among others. For the purpose of this report, the following phytochemicals would be discussed in details in this section; quercetin and rutin belonging to the class – flavonoids, protocatechuic acid – one of plants phenolic acids and ethyl gallate – one of the major tea catechins.

Flavonoids are phytochemicals found in most photosynthesizing cells and hence, are found in a variety of fruits, vegetables, stems and flowers. They are, therefore, a common component of the human diet too. Besides their antimicrobial properties, flavonoids have other structural and functional attributes in plants. They provide pigmentation (red/blue color) to various parts of the plant, such as color to flower petals, fruits and vegetables, which also help in attracting plant pollinators. They play a role in protecting the plant from microbial invaders (including bacteria and fungus) and ultra violet radiations of the sun. Apart from these basic functions, they are involved in other plant physiological activities as well, such as, photosensitization, energy transfer, actions of plant growth regulators and hormones, respiration, photosynthesis, morphogenesis and sex determination [44].

The basic flavonoid structure is based on the flavone backbone or 2-phenyl-1,4-benzopyrane (see Figure 2.11 a). Quercetin is typically a flavonol, a sub-class of flavonoids and rutin is a glycosylated flavanol, also known as quercetin-3-rutinoside. Rutin has a disaccharide hydrolyzed to one of the hydroxyl groups of quercetin (at position C-3, see Figure 2.11 c). In general, flavonoids are known to possess antibacterial activity. Quercetin and rutin in particular, act against DNA gyrase and topoisomerase IV of bacteria, and interfere in DNA replication similar to quinolones. Quercetin is also reported to cause bacterial membrane function damage by increasing the membrane permeability, dissipating the membrane potential and inhibiting bacterial motility. It has

also been suggested that this may decrease the bacterial resistance to other antibiotics [140]. Like fluoroquinolones and quercetin, rutin too is a bacterial topoisomerase type II inhibitor.

Naturally occurring quercetin is found in many fruits and vegetables including evening primrose leaf, mayapple, onion bulb, tea leaf and neem flowers (> 1000 ppm [141, 142]). Fruits and vegetables like buckwheat, citrus fruits, asparagus, berries and tomato are rich in rutin (>10,000 ppm [141, 142]).

Figure 2.11: Chemical structure of flavonoids.
(a) flavone backbone, (b) quercetin and (c) rutin

Ethyl gallate is one of the major constituent of tea catechins. These have been reported to possess significant antibacterial activity against gram-positive and gram-negative bacteria [143, 144]. In general, alkyl gallates have an amphipathic molecular structure with a hydrophobic alkyl part (tail) and a hydrophilic pyrogallol moiety (head) (see Figure 2.12 a). They are known to disrupt the fluidity of the lipid bilayer of the bacterial cell membrane. As explained by Kubo *et. al.* [143], the antibacterial activity of alkyl gallates does not entirely depend on the alkyl chain length, but is responsible for its bactericidal

activity. Due to their molecular structure, they act as surfactants and inhibit the electron transport chain of the membrane, thereby inhibiting the respiratory system of the bacteria. Since they do not have to enter the cell for their action, they are not usually affected by resistance causing mechanisms of the bacteria.

Protocatechuic acid is a plant phenolic antioxidant and anti-inflammatory (see Figure 2.12 b). It is one of the major components of Roselle calyx (*Hibiscus sabdariffa*) and barks of *Cananga odorata* medicinal plants, and has been shown to possess antibacterial action against most gram-positive and gram-negative bacteria [145, 146]. It is also abundantly found in onion and leafy vegetables like parsley, cilantro and coriander (> 1000 ppm [141, 142]). The molecular mechanism of action of protocatechuic acid and related compounds against bacteria is not completely understood, but their *in vitro* antibacterial properties have been demonstrated by many groups earlier [145, 146].

Figure 2.12: Chemical structures of phytochemicals.
(a) ethyl gallate and (b) protocatechuic acid or 3,4-dihydroxybenzoic acid

2.4.2.2 Maggot-extracted antimicrobial substances

Maggot therapy or biosurgery has been traditionally practiced for debridement of necrotic wounds as well as for curing bacterial infection at the wounds site [54, 147]. It has shown to have advantages over the conventional methods in terms of being more efficient and cost-effective for wound care and healing [148-152]. The basic process of wound healing in biosurgery is carried out by three main actions on the wound; debridement, disinfection and simulation of wound healing [150, 152]. The effective disinfection action on the

wound and the antiseptic activity of maggots led many researchers to investigate specialized antibacterial properties possessed by them. The antibacterial activity of maggot extracts was demonstrated in the 1930s by Simmons *et. al.* followed by Pavillard *et. al.*, in which they described the extraction techniques and bactericidal activity of excretions and secretions (ES) extracted from maggots of *Lucilia sericata* [153, 154]. Initially, some suggested the antimicrobial action by the maggots on the wound was due to antibacterial factor(s) in the ES of the maggots [56, 153-155], while others reported ingestion and gut activity [156]. Beasley and Hirst [157], explained the disinfection action of maggots; that is caused by various methods. They emphasized that it is either one or a combination of these actions performed by the maggots on the wound that disinfects it. These include, movement and irrigation by larvae, ingestion of the necrotic tissues of the wound leading to dilution of the bacterial concentration, secretion of antibacterial compounds, such as, phenylacetaldehyde and phenylacetic acid. They also bring about increase in the pH of the wound by excretion of ammonia and calcium carbonate that indirectly inhibit microbial growth.

For this reason, ES extracted from many species of blowfly maggots have been studied for *in vitro* antibacterial activity against a variety of gram-negative and gram-positive bacteria [55, 155, 158, 159]. The majority of these investigations were carried out using maggots of *Lucilia sericata*, used for maggot therapy or biosurgery in European countries. Their ES or clarified whole body extracts have been shown to be bactericidal, especially against gram-positive bacteria. *Lucilia cuprina*, on the other hand, has been shown to have partial growth inhibition of gram-positive *S. aureus* [160]. Recently, the ES of *L. cuprina* maggots have shown to have potentiating effect on ciprofloxacin activity against *S. aureus* [161].

2.5 Antibiotic resistance

Antimicrobial resistance is not a new phenomenon, but has been in nature even before the discovery of antibiotics. Bacteria have been thriving on earth for more than 3 billion years and have developed exceptional surviving skills. They have been exposed to natural antimicrobial agents of plants, animals and even humans as a part of their defense mechanisms against pathogenic invaders. They have, in a way, evolved and developed extremely wide range of resistance mechanisms to survive under selective environmental pressures. This phenomenon has only experienced an increased rate since the discovery of commercial antibiotics as clinical medicine in the last century. The alarming increase in antibiotic resistance has become a major health issue and continuously confronts the present antimicrobial medicinal research [24, 162]. This part of the review would focus on the basic antimicrobial resistance mechanisms and their spread, a review of resistance in *S. aureus* and in particular to ciprofloxacin.

2.5.1 Mechanisms of antibiotic resistance

In general, the druggable targets of bacteria are cell wall, bacterial proteins, folic acid synthesis pathways, nucleic acid synthesis pathways and bacterial membranes [163]. Most classes of present antibiotics inhibit or disrupt one of these targets resulting in either inhibiting bacterial growth and reproduction (bacteriostatic antibiotics) or causing cell lysis or killing (bactericidal antibiotics). Clearly, resistance is a consequence of use and overuse of antimicrobial drugs. The nature of the drug itself induces and promotes evolution and hence the development of resistance [164]. The antibiotic selects organisms that are slow-dividing or non-dividing and are capable of growing in a specific environment by undergoing a series of mutations, transformations, or plasmid acquisition. These types of adaptive mutations help a particular sub-population to thrive in such adverse environments and persist even with long term antibiotic pressure. As more and

more antibiotics are used, this vicious circle of drugs resulting in resistance would accelerate.

The development of resistance can be attributed to a number of factors, and they are usually independent of each other. The mechanism of action of the antibiotic, the rate at which it brings about control in a bacterial population, the rate and duration of exposure, drug potency and dosage, are some of the factors involved. Some have even suggested that drugs having single target experience resistance more rapidly as compared to multi-targeted treatment [165].

Antibiotic resistance can be classified as:

- Intrinsic resistance (or natural): for example, target inaccessible to the drug due to permeability barrier etc.
- Mutational resistance (or genetic): for example, altered target, decreased permeability, alternative pathways etc.
- Acquired resistance: for example, extrachromosomal (plasmids) or transferred from other bacteria, causing similar resistance mechanisms, like altered targets etc.

The nature of resistance to a drug may be any of the above; there are a number of mechanisms of resistance that a bacterial cell can undergoes upon exposure to antibiotics. These are explained in the following paragraphs.

Since the profiling of complete genomes of pathogenic bacteria during the 1990s, the understanding of genes involved in antibacterial resistance has greatly increased. The knowledge of specific gene expressions and changes after antibiotic exposure has led scientists to comprehend the molecular mechanisms of resistance. These mechanisms can be broadly classified into three main mechanisms [163, 166]:

- Expulsion of drug by efflux pumps

- Inactivation or destruction of drug molecule
- Alteration in target or pathways involving the target

The action of antibiotics depends on the accumulation of the drug molecule at its target site, whether outside or inside the bacterial cell. For targets inside the cell, membrane permeability plays an important role in the drug efficacy. Efflux pumps help the bacteria to reduce the concentration of drug at the target site inside the cell, by actively and continuously pumping out drug molecule out of the cell. This results in reduced concentration of the drug around its target and hence its efficacy and renders the bacteria resistant to that drug. Drug efflux pumps are usually variants of the existing transporter proteins of the cell membrane, but are modified to extrude the drug out of the cell at a faster rate than its uptake. These could be multi-drug transporters or single drug transporters. Examples of efflux pump mediated resistance include, resistance to tetracyclines by most gram-positive and gram-negative bacteria - by over production of membrane proteins; and multi-drug transporters in *S. aureus* like QacA [167, 168].

Another mechanism by which bacteria confers antibiotic resistance is by inactivation or destruction of the drug itself. A typical case is one of that of β -lactamase, an enzyme that hydrolyzes β -lactam class antibiotics including penicillin. In other cases, for example with aminoglycosides, the drug is modified so that its affinity to the target is reduced and hence the drug efficacy.

Altered drug target is also a mechanism by which bacteria acquires resistance to antibiotics. As a consequence, the drug molecule then has a lower or no affinity towards the new altered target. This has been observed widely in erythromycin-resistant species, where the target RNA subunit is methylated once or twice by an enzyme called Erm, a methyl transferase enzyme [169]. This enzyme is known to impart immunity to erythromycin producing organism and also observed in MDR *S. aureus* species. A series of genetic mutational events can also lead to expression of an altered target, making the

bacteria resistant to the drug. In some cases, it has been observed that an alternative pathway is adopted minus the target or by-pass the steps involving the target itself. For example, *S. aureus* can develop resistance to β -lactams by acquiring *mecA* gene that produces a low affinity penicillin-binding protein (PBP) called PBP2` and gives rise to the MDR methicillin-resistant *S. aureus* or MRSA specie [165].

It has been continuously emphasized in literature that continuous use of antimicrobial agents is one of the major causes of development and spread of resistance in bacterial pathogens. It not only makes them resistant to that antibiotic but to a number of other structurally unrelated antibiotics as well [24]. Levy and Marshall [24], in their review suggested that, there may be a link between the resistant genes acquired by gene transfer through plasmids or transposons from other bacterial cells, as a result of over use of one antibiotics, that eventually makes the receiving bacteria resistant to several other antibiotics of different class and mechanisms of action. This plasmid may carry a number of other genes that makes the pathogen resistant and hence are MDR. This ensures the maintenance of resistance as long as the population survives or transferred to another species by a similar mechanism as it was acquired. In addition to plasmid and transposon mediated gene transfer, there are other mechanisms by which resistance can be spread, these include, by bacteriophages, by uptake of naked DNA and by direct person to person transfer. Figure 2.13 summarizes the bacterial resistance mechanisms discussed above and methods that lead to its spread.

Figure 2.13: Mechanisms of antibacterial resistance and spread.

Resistance mechanism (left) and mechanisms of spread of antibacterial resistance (right) (Adopted from Levy and Marshall, 2004 [24])

2.5.2 Resistance in *Staphylococcus* species

S. aureus among others is the most common nosocomial infection causing pathogen. Initially, it was only common in a hospital setting due to continuous antibiotic usage and patient to patient transfer, but more recently it has spread into a community setting causing severe diseases through minor skin infections and wounds. The major concern in this rapid spread is that, in the process it is becoming increasingly resistant to antibiotics. The first penicillin resistant strain of *S. aureus* or MRSA was reported soon after its discovery in the 1960s. Its rapid spread was then seen in the late 1980s until now when they are endemic both in hospitals and community and difficult to eradicate [170]. In addition to methicillin resistance, MRSA strains have shown resistance to almost all classes of antibiotics, including vancomycin. Resistance to vancomycin was reported recently, when strains with reduced susceptibility to the drug were isolated from patients, and was termed as vancomycin-resistant *S. aureus* or VRSA [135, 171, 172]. MRSA has also been termed as the “super-bug” due to its multi-drug resistant nature.

Several attempts have been made to understand the genome to gain insights into the pathogenesis and development of resistance to antimicrobial drugs in *S. aureus* strains [137]. Lindsay *et. al.* [137], studied the genomes of five *S. aureus* strains and using comparative analysis of their chromosomes illustrated some useful conclusions (refer to manuscript). The auxiliary or accessory genome is known to possess mobile genes capable of horizontal transfer between stains and species. Many of its constituent genes are mainly for virulence and resistance expressions in the organism. They include elements like bacteriophages, plasmids, chromosomal cassettes, transposons, pathogenicity and genomic islands. *S. aureus* strains isolated from hospitals carried one or more of these plasmids or bacteriophages. Plasmids carry antibiotic resistance genes that impart the bacteria with multi-drug resistance to several antibiotic classes at once. *S. aureus* species are also capable of acquiring these plasmids from closely related species by horizontal transfer. For example, VRSA isolates were found to carry the *vanA* resistance gene from vancomycin resistant enterococci [173]. It was obtained by horizontal transfer by a pheromone response from *S. aureus* that triggered the transfer from enterococci.

Another part of the accessory genome that plays a role in conferring MRSA with multi-drug resistance is the staphylococcal cassette chromosome or SCC, a mobile genetic element in methicillin-sensitive *S. aureus* or MSSA. The SCC element in MRSA that impart resistance to most β -lactam antibiotics is the SCC*mec* element. It carries the *mecA* gene which encodes an altered penicillin binding protein, PBP2A, which has low affinity towards the β -lactam antibiotics, making them resistant. SCC*mec* is integrated at the same site on the *S. aureus* chromosome towards the origin of replication, for a possible immediate use in the bacterial cell [170]. They also carry genes that allow their mobilization, called the cassette chromosome recombinases or *ccrs*, for insertion into the host chromosome. They are believed to be acquired from coagulase-negative staphylococci (CoNS) species. They are found in many types in different hospital

associated or community-acquired MRSA (type I to V) and is mobilized largely by horizontal transfer mechanisms from one strain to another [137].

2.5.2.1 Resistance to ciprofloxacin

Fluoroquinolones has been a preferred choice of antibiotics for treatment of *S. aureus* infections, and among them most commonly prescribed is ciprofloxacin [174, 175], commercially available as Baycip[®], ClioXan[®], Cipro[®] and Ciproxin[®] among others. However, extensive use of ciprofloxacin and other quinolones have increased resistance among *S. aureus* species. There are two principle mechanisms by which *S. aureus* attains ciprofloxacin resistance; by alteration of the target, type II topoisomerase enzymes or by expulsion of the drug out of the cell, thereby reducing the drug concentration around the target [176]. The alteration of DNA gyrase and topoisomerase IV enzymes takes place by changes in their amino acid sequence, by spontaneous mutations in regions of genes encoding the enzyme subunits called the quinolone-resistance-determining-regions (QRDR). Consequently, ciprofloxacin has less affinity to the altered target and hence makes the bacteria resistant to the drug. In *S. aureus*, the primary target for ciprofloxacin is topoisomerase IV and hence mutation in QRDR regions of GrlA subunits is more commonly observed in the resistant isolates [176]. In these even single changes, like point mutations are sufficient for clinical resistance to ciprofloxacin [177]. Mutation in *gyrA* gene is less common in ciprofloxacin resistant *S. aureus* and is silent in the absence of mutations in the *grlA* gene. However, simultaneous mutation in *grlA* gene and alteration in GyrA subunit enhances the level of ciprofloxacin resistance [178]. Few or no reports on mutations in *gyrB* and *grlB* genes that accounts for ciprofloxacin resistance were found in literature.

Another mechanism that leads to reduced susceptibility of *S. aureus* to fluoroquinolones involves the reduction of drug concentration near the target. For its action, the drug molecule must pass the peptidoglycan cell wall and bacterial membrane, to reach its

intracellular targets. Bacterial cell membranes have transporter proteins for the transport of selective molecules in and out the cell. As discussed in Section 2.5.1, efflux pumps are found in almost all bacteria to transport substances in and out of the cell. The expression of these efflux pumps however, is genetically controlled and can be increased in response to a drug attack. These pumps are called MDR pumps that are capable of pumping out several antibiotics. In *S. aureus*, ciprofloxacin resistance can occur by increased expression of an MDR pump - NorA by mutations in the *norA* gene promoter causing increased stability of *norA* mRNA [176].

Resistance by other mechanisms such as acquiring genes or plasmids from other strains or species is less commonly observed in ciprofloxacin resistant *S. aureus*. It is more commonly a combined effect of spontaneous mutational events that lead to alterations in the target and/or over expressed efflux mechanisms by MDR pumps.

2.6 Summary

Combination therapeutics and screening of combinations on cell systems or *in vivo* models are increasingly becoming more important with the advent of personalized medicine and growing necessity for long term effective treatment solutions. Microfluidic systems and platforms have emerged as an unmatched screening and analysis tool for the abovementioned practice. The multi-well plate and robotic fluid handling systems are prone to problems of evaporation, experimental pipetting errors, time consuming and not economical. In spite of numerous microfluidic devices that have been reported, little or no work has been published on cell screening outcomes under influence of different combinations of drugs or drug and herbs on a single platform. The gradient generators are capable of producing stable gradients but are restricted to single channel outputs or suitable with mammalian type adherent cellular analysis. There is therefore, a scientific gap identified, and has been partially proposed in this study. Numerous micro-devices or portable devices employs various culturing, detection and monitoring modalities; the

present project proposes a Combinatory Drug Screening System or CDSS that can generate serial concentrations on a microfluidic device, from small quantities of two substances and perform *in-vitro* studies on cells without having to transfer or dispense the solutions into the 96-well plates or well systems containing cultured cells [179, 180]. The micro-device designed and fabricated, was free from complex geometry for mixing and involved no moving parts. The mixing process was carried out in the centrally aligned wells by disturbance in the flow pattern of the incoming liquids. From the microchannel separating circuitry, the fluids were forced into wells of higher dimension (of the order of 1 mm and higher), which was responsible for mixing of the fluids.

The initial tests on the system were carried out by using different cell types to ensure biocompatibility of the materials of the system for cell growth and maintenance. The cell types chosen, as also discussed in the review, were adhesion and suspension types. The materials used to construct the proposed micro-device in the present project, glass and PDMS, have been extensively used for applications in cellular, biochemical and chemical analysis. Numerous devices have been reported in literature having PDMS platforms that allow cell growth [121, 181-183]. There are several devices also reported aiming to culture adherent mammalian cells [118]. All these devices aim to support microscale cell culture for high-throughput applications. With the objectives and application of the system in mind the micro-device was modulated to allow certain amount of volumes to be generated in the wells to accommodate both short term and long term monitoring of wells.

3 EXPERIMENTAL METHODOLOGY AND MATERIALS

As stated in the objectives of this work in Chapter 1, microchannel devices were designed and tested for their intended application as a cell culturing platform and drug mixing. A system was designed and assembled to carry out sustenance and monitoring of the long term behavior of cells. The system was referred to as the Combinatory Drug Screening System (CDSS) along with the micro-device. The CDSS was tested by performing case studies on different types of cells belonging to the adhesion and suspension categories with selected drug combinations. For the purpose of this report, the microfluidic device designed and tested in this study, has been termed as “microchip” or “micro-device” at many instances for simplicity and linguistic ease.

Following this, investigations were performed on *Staphylococcus aureus* (*S. aureus*) with drug and phytochemical combinations on the CDSS. The development of adaptive resistance was monitored for several days and the effects of phytochemical addition were established. The results were compared and validated against those obtained from the conventional methods of antibiotic susceptibility testing. Combination of drug and maggot extracts was also investigated on *S. aureus* and its survivability.

3.1 Experimental system

3.1.1 Microchip design, fabrication, calibration and testing

As discussed in the previous chapter, the low Reynolds number results in a turbulence-free flow in microfluidics, with the consequence that mixing becomes difficult at microscale.

Mixing at microscale is purely diffusive. This is the reason why many microscale mixing approaches introduce complex designs that induce time dependent diffusion between flow layers [184], use three dimensional microchannel features [98], incorporate ridges in the microchannel to generate chaotic stirring, or use additional instruments or active mixers that require external energy to make mixing occur rapidly [185, 186]. The mixing techniques published by others and discussed in the previous chapter, require complex designs and microscale geometries to be fabricated (Section 2.2.2.5 – Microfluidic mixing principles). This makes the fabrication process complex, involving two or more masking layers to be patterned on the silicon wafer and the device cannot be fabricated by rapid prototyping to make disposable and cheap microchips.

The microchips presented in this project have been made free from any such complex geometry for mixing and involve no moving parts. They were fabricated in a single step of standard contact photolithography process using a single mask. Mixing typically occurred at the wells of the microchip that were exposed to atmospheric pressure. The mixing of the two input fluids occurred by creating disturbances in the flow patterns, from microchannels to wells of higher dimension. The mixing, hence, took place passively requiring no external power source. As the wells were designed to be of higher dimension (~4 mm in diameter), the problems of microscale mixing were not encountered because the microfluidic phenomenon no longer existed at the level of the wells.

Three layout designs for the microchips were fabricated and tested and their working principle has been explained in the following sections. The devices were capable of integrating the process of separating, mixing, screening and cell culturing on the same platform. The design of a microfluidic system requires a comprehensive knowledge of fluid dynamics in the micro regime so that the advantages of the unique fluid behavior at that level can be adequately harnessed (discussed in Section 2.2.2 – Fundamentals of microfluidic flow).

3.1.1.1 Microchip designs

The layouts of the microchip were designed on L-edit software portal which is an easy-to-use layout editor program to construct the plastic mask for photolithography process. Several criteria were imposed on the designs according to the intended application of the final device. Firstly, it must be able to achieve the basic function of separating two fluids into serial concentration in increment of 20% and mix the complementary portion of each fluid completely in an enclosed chamber. Secondly, it must allow cells to be continuously cultured *in situ*. Based on the preliminary results, one of the designs was modified at a later stage to ensure better performance and accuracy.

Design I: The layout of the Design I is shown in Figure 3.1. The mechanism was based on the number of sub-microchannels with the same flow rate and pressure that determined the total amount of liquid that fed each well. The layout was designed in such a way that from the inlets (inlet A and inlet B, width 1.15 mm, Figure 3.2) the channel split into ten daughter channels (100 μm each), two of which acted as inputs to each smaller sub-system of microchannels and wells from both the inlet sides. In each sub-system the main channels that acted as input further divided into ten microchannels (100 μm each). Fixed number of channels out of these ten daughter channels fed each well (4 mm diameter each) accordingly to achieve desired concentration ratio. Figure 3.2 shows the details of the layout, the channel divisions at the inlets marked as Inlet A and Inlet B (Figure 3.2 a and b), and details of one of the sub-systems, having the ratio 9:1 and 1:9 (Figure 3.2c).

Figure 3.1: L-Edit Layout for Design I or D-I.
The circular outline shows the size of the standard 4 inch silicon wafer.

Figure 3.2: The details of the layout for Design I.

- (a) The overall layout with the ratio of the fluid mixes denoted as A:B from inlets A and B respectively. (b) Area outlined by the dotted box in (a), shows the division of the main inlet channel into 10 daughter channels. The arrows show the direction of fluid flow. (c) Area outlined by the dotted box in (a), shows the details of one of the sub-systems, producing the ratio 1:9 and 9:1 (A:B).

Design I was later modified to incorporate symmetry and curvaceous microchannels due to discrepancies encountered in the results of calibration and testing with dye solutions (calibration tests discussed in details in the next chapter). The basic principle of the modified Design I was the same, with changes made to the inlet design and channel distribution. Earlier, the channels were divided into ten channels before they fed the sub-systems. In the modified design, the main inlet was bifurcated at two levels (see Figure

3.3a). The four outlets at level 2 had equal fluid volume, flow rate and pressure drop, due to symmetry. The two levels of channel bifurcations are indicated in Figure 3.3 a as level 1 and level 2, symmetrical from either inlet. Figure 3.3 b and c explains the channel distribution at level 2 outlet, (outlined by dotted lines in Figure 3.3a) which is similar to original Design I channel distribution for the sub-systems. The concentrations are designated at the wells as the ratio of Fluid A: Fluid B.

Figure 3.3: The details of the layout for modified Design I.

- (a) The overall layout with the ratio of the fluid mixes denoted as A:B from inlets A and B respectively. (b) Area outlined by the dotted box in (a), shows one of the sub-systems, producing the ratio 2:8 and 8:2 (A:B). (c) Area outlined by the dotted box in (a), shows the details of the sub-system producing the 100% of each fluid, where the channels divide but do not split.

Design II: The L-edit layout for Design II is shown in Figure 3.4. In this design the daughter channels had equal cross-section and were positioned symmetrically with respect to the parent channel upstream. Therefore, each of the daughter channels received the same amount of fluid and the flow rate was effectively halved.

Consider the channel network that lies between Inlet A and the row of wells (Figure 3.5a). Let the initial volume of fluid entering the two inlets be $16X$ and $16Y$ ($X = Y$, for understanding two different variables are selected). At level 1, (indicated by dotted line in Figure 3.5a) the main inlet channel bifurcates and fluid entering each horizontal channel is $8X$ and $8Y$ respectively from inlet A and B. At level 2, the two channels further divide so that the fluid entering the subsequent four channels is now $4X$ and $4Y$. At level 3, each $4X$ and $4Y$ volume is again halved to give $2X$ and $2Y$, respectively. Finally at level 4, out of the four bifurcations at level 3 that gave rise to eight channels with $2X$ and $2Y$ fluid volume, four of them further bifurcates to yield X and Y volume of fluid. The middle two bifurcations still contain $2X$ and $2Y$ fluid volume that directly fills the wells without dividing further.

Figure 3.4: L-Edit Layout for Design II.

The circular outline shows the size of the standard 4" silicon wafer.

Figure 3.5: The details of the layout for Design II.

(a) The overall layout with the ratio of the fluid mixes denoted as $xX + yY$ ($x \neq y$, $X = Y$). Volume of $16X$ from inlets A and $16Y$ from B enters the microchip. (b) Area outlined by the dotted box in (a), explaining the principle of derivation of the ratios in the wells.

By selectively splitting in certain branches and calculated recombinations into the wells, the frequency of splitting and redundancy was minimized. In this case, the right and left extreme wells contain residual fluids of volume X or Y which is one fifth of the total volume of each well. The ratio of fluid A and B is indicated as the sum of $xX + yY$ ($x \neq y$, $X=Y$) in each well (see Figure 3.5a). The details of an area outlined by dotted lines in Figure 3.5a is shown in 3.5b, demonstrating the ratio derivation for the wells. Rounded bends were also incorporated to improve the fluid flow in the microchannels where the Reynolds number is small. The microchannel width was $200\ \mu\text{m}$ and the well diameter was $4\ \text{mm}$.

Design III: The layout of Design III is shown in Figure 3.6 in the L-Edit layout format. The principle of Design III was based on the distance traveled by a fixed amount of fluid from the parent channel to the daughter channel, for a particular period of time that determined the volume entering into the wells. Each well was filled by each of the opposite two channels as shown in Figure 3.7, at the same time.

The circular outline shows the size of the standard 4" silicon wafer.

To understand the working of the microchip, fluids A and B are considered to enter the inlets with a same flow rate and pressure for 10 unit time. Each vertical channel is assumed to have a length of Y ; therefore, fluid advanced the channel by a distance of $Y/10$ in each unit time. Given that flowing time and velocity are constant in each channel, the relative position of the well in the channels would determine the duration of the fluid collection (filling time) from each inlet. In the first channel from the left (see Figure 3.7), for the time fluid A takes to travel distance Y (to reach the first well from the left) fluid B would have filled the same well. The same is true for the other channels and wells. For example, consider the second channel from the left, fluid B would have filled it for an extra 6-unit time by the time fluid A reached the well, so a concentration of 20-80% (A-B ratio) would be obtained.

No rounded bends were required as the channels were larger compared to the previous two designs. Vertical channels were of width 1 mm and length 30 mm (excluding wells);

horizontal channels were of width 2 mm and length 31.5 mm, while the inlets were of width 2 mm and length 5 mm. With a depth of 150 μm , the total volume of each vertical channel was 4.5 mm^3 . It was matched with 4.5 μL of fluid in each well. The well diameter was 4 mm.

Figure 3.7: The layout for Design III with annotations.
The percentage mixtures are indicated on the wells as A-B% of fluid volume.

The plastic transparencies for the four designs used in photolithography with positive and negative photoresist are attached in Appendix C.

3.1.1.2 Microchip fabrication

The device was fabricated in two major steps, standard photolithography followed by soft lithography using PDMS [89, 90]. The layout was generated in L-Edit software portal, and was printed on a plastic transparency sheet using a commercial high resolution image setter by an external agency (IGI, Singapore). This transparency served as a photomask for contact photolithography that was used to produce a positive relief of photoresist or etched silicon on standard 500 μm thick and 101.6 mm in diameter silicon wafers (JA Associates).

This positive relief is known as the silicon master. The silicon master was prepared by two techniques, by etching the silicon wafer by deep-reactive ion etching (DRIE) using a positive photoresist, AZ 9620 and by lithography using a negative photocurable epoxy resin, SU-8. The process of photolithography and soft lithography that include rapid prototyping and replica molding techniques was explained in the previous chapter and summarized in Figure 2.2. The details of standard photolithography process using positive and negative photoresists has also been described in many books [57, 67]. The detailed fabrication process with process parameters are discussed in the following sections.

Before the lithography process, silicon substrates <100>, p-type, with 0.1-10 Ω -cm resistivity and 100 mm in diameter (J.A. Associates, Singapore) were cleaned in piranha solution for 20 min at 120 °C to remove organic contaminants. The wafers were dried in nitrogen air blast at 500-3000 rpm for five minutes.

Fabrication of DRIE etched silicon master: Clean and dried silicon wafers were coated with a layer of hexamethyldisilazane (HMDS). This coating process was done to promote the adhesion of photoresist to the inorganic silicon wafer. It was performed at 100 °C for 90 s in an HMDS chamber, and the process is known as HMDS priming of wafer. A 10 μ m thick AZ 9620 photoresist was spun-coated on the silicon wafers. The plastic film mask having the desired design was used to transfer the patterns onto the photoresist by exposing the wafer to UV radiations through the mask on a Mask Aligner (Karl Suss Mask-Aligner, Germany). The wafer was exposed for 60 s to UV light intensity of 9.3 mW/cm², on hard contact mode to improve the photoresist sidewall profile. The exposed resist layer was developed in AZ 421 developer with manual agitation for 90 s followed by drying in nitrogen air dryer.

The dried wafer was exposed to oxygen plasma for 1 min to ensure the complete removal of photoresist at the bottom of trenches and sharp corners. Plasma power and oxygen flow rate were 300 W and 10 sccm, respectively. This wafer was temporarily bonded with

another support wafer using 5 μm thick AZ 9620, to avoid chances of wafer breaking during the etching process. The wafers were etched in Surface Technology System (STS) multiplex inductively coupled plasma (ICP) system for various etching time ranging from 1-2 hours to get variable etching depths. The total etching area was about 80-90% of the wafer area (due to negative mask). Brief details of these parameters are given in Table 3.1. The chamber pressure was set at 15 mTorr by automatic pressure control. The substrate temperature was set at 20 $^{\circ}\text{C}$ by the helium cooling system. The typical etch rate was found to be 1.2-1.5 $\mu\text{m}/\text{min}$. After the DRIE process, the wafers were separated from the support wafer by soaking them in acetone for an hour. The photoresist residuals were once again removed by oxygen plasma in the reactive-ion etching (RIE) system. To ensure the easy removal of PDMS from narrow etched lines in silicon, a thin layer of polytetrafluoroethylene (PTFE or C_4F_8 , a teflon-like material) was deposited on the DRIE etched silicon master. This deposited material have shown to possess super hydrophobic characteristics and widely used as an anti-stiction material in MEMS-based systems [187]. C_4F_8 gas plasma was used for 2 min to deposit a thin layer. The gas flow rate and the coil power were at 100 sccm and 800 W, respectively. This layer facilitated the peeling of PDMS after its curing.

Table 3.1: Brief details of DRIE process parameters

DRIE Process Parameters	Experimental value
SF_6 flow rate (sccm)	130
O_2 flow rate (sccm)	13
C_4F_8 flow rate (sccm)	100
Etch time (s)	14
Passivation time (s)	8
Coil power (W)	800
Platen power (W)	140
Automatic Pressure Control (APC) ($^{\circ}$)	70
Substrate temperature ($^{\circ}\text{C}$)	20

Fabrication of SU-8 mold on Si wafer: Negative tone photoresist SU-8 100 (MicroChem, USA) was used as the mold material. A 150 μm thick SU-8 layer was coated at a spin speed of 2000 rpm on a clean and dried silicon wafer. The coated wafer was pre-baked at 65 °C and 95 °C for 20 min and 50 min, respectively, as per manufacturer guidelines. The wafer then was exposed on the mask aligner (UV intensity 9.3 mW/cm²) for 60 s. After the exposure, the wafer was post-baked at 65 °C and 95 °C for 1 min and 12 min, respectively. The exposed and baked wafer was developed in an ultrasonic agitator for 20 min and then rinsed by iso-propanol (IPA) to ensure proper removal of the resist. The developed wafer was dried and exposed to C₄F₈ gas plasma to increase the hydrophobicity and thereby facilitate PDMS peeling.

Soft lithography using PDMS: The final micro-device was fabricated in PDMS elastomer by soft lithography process. Negative replicas of the silicon master with embossed channels were fabricated by casting liquid PDMS polymer against it. The PDMS polymer solution was prepared by mixing the commercially available prepolymer and catalyzer or curing agent (Slygard 184 kit, Dow Corning, USA) in a ratio of 10:1 (w/w). The mixture was degassed in vacuum to eliminate bubbles formed during mixing. The prepolymer mixture was casted on the master and placed under vacuum once again to remove bubbles created by pouring. The wafer and PDMS was then baked over a contact hotplate set at 120 °C for 1 h. After cooling and demolding, the PDMS mold was kept in 30% HCl bath for 4 h to render the surface characteristics hydrophilic [188]. It was then rinsed and dried thoroughly in deionized (DI) water. The PDMS mold was then cut into chip size and holes were punched to make the wells using a cork borer and rinsed in DI water. The mold was then bonded to glass plate to form the final microchip assembly. To form an irreversible seal, the PDMS mold and a clean glass plate were exposed to oxygen plasma for 18 s (Pressure: 75 mtorr, RF power: 99 W) in an RIE system. Immediately after removal from the RIE chamber the two substrates were brought to conformal contact and an irreversible seal was formed simultaneously. The holes were then drilled to form

the inlets using an 18 gauge blunt needle (Becton and Dickinson, Singapore). The final device and its specifications are discussed in the next chapter (Chapter 4 – Experimental Results).

3.1.1.3 Microchip calibration and testing

The integrity of the microchip was confirmed with tests using dye solutions (trypan blue, food color blue and yellow aqueous solutions). A control set was prepared in 96-well plates by conventional mixing using pipettes. The color concentrations were compared with those of the microchip well concentrations obtained by injecting dyes of different colors from the two inlets. By comparing their corresponding absorbance values at the peak wavelength of light depending on the color, the microchips were calibrated.

Materials: General food coloring agents (blue and yellow) were obtained from stores and dissolved in DI water in the ratio of 1:1000 (v/v) to get the 100% color solution. This color concentration was kept constant for all the experiments throughout the study. Trypan blue dye and DI water were also used for color experiments. A 100% trypan blue solution was prepared by diluting the original trypan blue dye with DI water in 2:8 (v/v) ratio. Other concentrations for the control and test experiments were diluted from the 100% solutions accordingly. Ethanol (99.9%, Sigma) and acetic acid (10% v/v, Sigma) was used for flow visualization experiments on the microchips. 20 ml luer-lok[®] tip syringes (Becton and Dickinson, Singapore) were mounted on programmable syringe pumps (SyringePump.com) with respective solutions. 70% Ethanol and DI water were used for washing and rinsing the microchip channels and wells after every use.

Experiments: To prepare the control set, the two colored solutions or colored dye and DI water, were pipetted by parts into a 96-well plate in repetitions of five, to a final volume of 50 μ L for each combination obtained from microchip wells. The combinations prepared were; 100% of all colored solutions and DI water, 20-80 (v/v)% of blue and yellow colors

or trypan blue and DI water, respectively, 40-60%, 50-50%, 60-40%, and 80-20% (Figure 3.8). The spectral signature of each concentration mixture was read by a MicroQuant spectrometer plate reader (BioTek, Singapore) at a range of 300-700 nm wavelengths.

The microchip test was carried out by injecting the dye solutions or dye solution and DI water from the two inlets using the two programmable syringe pumps (Figure 3.8). For the initial testing, all color solutions were dissolved in DI water. The syringe pumps were loaded with 20 mL syringes containing the desired solutions and connected via 1 mm internal diameter silicone tubing to the inlets of the microchip to be tested. For modified Design I, the total volume to be injected from both syringes was set to 250 μ L including residual in the channels. For Design II it was set to 170 μ L. In both cases the final volume of each well was 50 μ L. Similarly, for Design III 100 μ L of dyes were injected from the two inlets to fill up the wells up to 25 μ L each. For primary concentration tests, the flow rate for both injections was kept at 10 μ L/min since both were aqueous solutions. The reference signal was taken as the absorbance of plain DI water.

To obtain the spectral signatures from the microchip wells, the experimental set up shown in Figure 3.9 was used in a dark room. This set up was also used to obtain the absorbance values for all the subsequent testing with the microchips. An optical fiber was connected to a white light source (Analytical Instrument System Inc., USA) at one end, that supplied light to the wells of the microchip at the other end. Viewing chambers were aligned with the hole in the center of a 1 cm thick opaque plastic sheet (see Figure 3.9), so that the light would travel through the samples to reach the optical fiber on the opposite side that led to the detector. The transmitted light was detected by the USB 2000 spectrometer (Ocean Optics, USA) and the data was collected by a computer with OOI software portal (Ocean Optics, USA) for data analysis and storage. The absorbance values at peak wavelengths and full spectrum in the visible range were collected for all the wells, one at a time.

Intensity of absorbance obtained at the peak wavelengths were plotted against the concentration of the mixtures. The spectral signatures as intensity against wavelengths were also plotted for each well. This test would also prove the compatibility of the microchip with optical detection systems. The tests with dyes for each microchip were carried out five times on different chips, to get an average spectral signature and absorbance value at the peak wavelength.

Figure 3.8: Testing and calibration of the microchips.

(a) Control in 96-well plates, concentrations prepared using pipettes. (b) Modified Design I with yellow and blue solutions from the two inlets. (c) Design II with yellow and blue solutions from the inlets. (d) Design II with DI water and trypan blue from the inlets.

Following every experiment the microchips were cleansed by pumping 70% ethanol to maintain the hydrophilicity of the channels, followed by DI water several times from both inlets. The residual water was pumped out by blowing high-pressure air through the inlets. The microchips were then kept in a blow dryer maintained at 60 °C for 24 hours for subsequent usage. The velocity of the flow was measured by videotaping the flow from

inlets to the wells through the microchannel network. Re was calculated using the formula given in Equation 2.1, by substituting the viscosity and density of DI water.

Figure 3.9: Experimental setup to study the performance of the microchip.

This setup was used to collect full spectrum in the visible range and absorbance value at the peak wavelength. (The box around the microchip was the incubator chamber of the CDSS). (The setup is shown in Appendix H)

Further experiments with the microchips were carried out using colored solutions dissolved in liquids of different viscosities by flow visualization. Yellow color was dissolved in DI water and blue color in ethanol and acetic acid in the ratio 1:1000 (v/v) that aided the viewing of the mixing process under the microscope. One of the syringe pumps was mounted with a syringe filled with yellow colored water and the other was mounted with a syringe containing blue ethanol or blue acetic acid. The microchip was placed under the microscope (Nikon Fluorescence Microscope, Model T-DH, TE2-PS100W) as shown in Figure 3.10 with the inlets connected to the respective syringe pumps. The microscope was connected with an inbuilt digital camera (Nikon) to capture the frames of the visual area, focused at the middle of each well. Increasing flow rates

were employed on the syringe pumps and frames were captured every 200 ms for the first one second after the fluid entered the microchip, followed by 10 s and 1 min. The camera was programmed to take the snap-shots of the center of the microchip well that was focused under the microscope at 10× magnification. The pumping was started simultaneously to dispense a final volume of 250 μ L for Design I and 170 μ L for Design II at varying flow rates. For the first experiment with yellow DI water and blue DI water, the flow rates for both pumps was kept same since both liquids had equal viscosities. The flow rates chosen for the experiments were 8 mL/min, 10 mL/min and 16 mL/min.

Subsequently, combination of liquids was used with varying flow rates and viscosities. Yellow water was used in combination with blue ethanol, with flow rates ranging from 2 mL/min, 8 mL/min and 16 mL/min for both syringe pumps. Since the viscosity of ethanol was greater than the viscosity of water, the flow rates of the syringe pumps were kept lower for water and higher for ethanol. Two combinations rates were employed, 2 mL/min – 10m/min for water and ethanol, respectively and 10 mL/min – 16 mL/min for water and ethanol, respectively. The same procedure was repeated with yellow water and blue vinegar with varying flow rates. The pictures were similarly taken after 1 s, 10 s and 1 min after pumping.

Figure 3.10: Experimental setup to study the wells of the microchip under the microscope. Microchip connected to the respective syringe pumps placed on the stage of an inverted microscope to study flow and mixing studies. (The box around the microchip is an incubator chamber connected to a microcontroller circuitry, see Figure 3.11)

3.1.2 Combinatory Drug Screening System (CDSS)

A bioreactor system was designed and assembled to hold the microchip and provide favorable growth conditions for living cells. It was also used for real-time monitoring of the wells of the chip under the microscope or spectrometer (see Figure 3.10, 3.11). The block diagram of the system is illustrated in Figure 3.11a. It consisted of a box incubator to house the microchip, integrated with two plate heaters (PTC Heaters, European Thermodynamics Ltd., UK) and two temperature sensors (LM35, precision celsius temperature sensors, National Semiconductors, USA) placed at the front and rear of the box for temperature control. The microchip was inserted into the box incubator from the cover head shown in the figure. This could be placed either on the stage of an inverted microscope for imaging the wells (see Figure 3.10 and 3.11b), or on the opaque stage of the set-up shown in Figure 3.9 for spectrometric readings and analysis.

The electronic and control circuitry of the system consisted of an 8-bit microcontroller (ATmega16, Atmel, USA) and AVR[®] SKT500 starter kit and development system (Atmel, USA) for user interface and programming. The pin diagram, block diagram and general features of the microcontroller-ATmega16 used in the system is given in Appendix A. The microcontroller was programmed via RS-232 interface of the computer using the SKT500 board that held the microcontroller IC onto its 40-pin socket. The code was written in C++ language in the user interface dialogue box of the AVR Studio software and was stored in the 16-Kbyte programmable flash memory of the microcontroller (the C++ code is given in Appendix B). While programming, all the ports of the microcontroller were initialized and assigned specific tasks. Port A was enabled as the analog to digital converter (A/D converter), which received the analog current input from the temperature sensors and converted them into digital signal as output. A relay was connected to Port B of the microcontroller. The temperature was controlled and maintained by a relay circuit that acted as a diode switch connected to the microcontroller output, that switched the heater inside the box 'on' or 'off' as the temperature detected by the sensors decreased below or increased above 37 °C, respectively. When the temperature reached 37 °C, the relay would cut off the power supply to the heater and after some time lapse, toggle between on and off to maintain a constant temperature. Port C of the microcontroller was connected to a 2-line digital display device that read the temperature of the front and rear temperature sensors, and updated it every ten seconds. The microcontroller and the SKT500 board were powered by a 5 V DC supply and the relay circuit was powered by a 12 V DC supply, as per manufacturer recommendations.

(a)

(b)

Figure 3.11: Schematic of CDSS

(a) Block diagram of CDSS, (b) the box incubator connected to the temperature control and display circuitry on the stage of an inverted microscope (also illustrated in the schematic of Figure 3.10)

3.2 Initial tests on CDSS

The initial tests on the CDSS were carried out using different cell types belonging to the adhesion and suspension categories. Two cell lines from each type were chosen for preliminary investigations on the system. The scope of these tests was to determine the capability of the system to allow growth and reproduction of living cells, ability to monitor them overtime and to perform case studies with drug combinations. The tests were carried out with bacterial *E. coli* and *S. aureus* cells (strain MC 1061 and MSSA ATCC 29213, respectively) with and without drugs, mammalian neuroblastoma cells (N2A cell lines) and smooth muscle cells of pig intestine without drugs.

3.2.1 Case studies on suspension cells: *E. coli* and *S. aureus*

Standard protocols for bacterial growth and storage were followed in the experiments involving bacteria [189]. Unlike standard procedures the sub-cultures of bacteria were performed in the wells of the microchips. Before the experiments the microchips were sterilized by autoclaving at 120 °C for 20 minutes and dried overnight at 60 °C.

Luria-Bertani (LB) and Iso-sensitist (IS) broth and agar dehydrated powdered mixtures acquired from Sigma-Aldrich (Singapore) were used as liquid and solid media for cultures of *E. coli* and *S. aureus*, respectively. The media was prepared by dissolving the powders according to the manufacturer recommendations in DI water and autoclaved at 120 °C for 20 minutes. After sterilization the media was allowed to cool to a temperature of ~ 50 °C. Liquid agar was poured into petri-dishes (Greiner, Dimension: 94 x 16 mm) (20 mL each) and stored along with broth at 4 °C for up to 6 weeks. The *E. coli* MC1061 and MSSA ATCC 29213 stocks were stored at -80 °C in LB broth containing 40% glycerol (v/v). Stocks were streaked using a sterile loops on sterile LB or IS agar plates and allowed to incubate for 18-24 hours at 37 °C. These plates could then be stored at 4 °C for future use. One day before the experiments, sub-cultures were prepared by separating a healthy and

isolated colony of bacteria from the culture dish stored at 4 °C, using a sterile wire loop and streaked onto a fresh sterile agar plate followed by incubation at 37 °C for 18-24 hours. On the day of experiment, 4-5 well isolated colonies were picked up from the overnight cultures using a wire loop and swirled into 5 mL broth. The resultant turbidity of the culture was adjusted according to 0.5 McFarland standards [190], to an absorbance value of 0.08 measured at 600 nm wavelength. It was further diluted 100 times to get the final bacterial inoculum. 320 µL of the prepared culture was injected into a sterile microchip with Design II, from both the inlets simultaneously using 1 mL syringes (160 µL in each syringe). The volume in each well was 50 µL. The inoculated microchip was placed in the box incubator of the CDSS shown in Figure 3.11. The wells were monitored under the microscope, by measuring optical density (OD) of the culture at 600 nm and by plate counting after specific time intervals (0, 3, 6, 9, 12, 15 and 18 hours). 10 µL aliquots of bacterial broth were extracted from the wells of the inoculated microchip and diluted in plain sterile broth or sterile phosphate buffer saline (PBS, Sigma). The 10-fold dilutions were plated on agar plates and incubated at 37 °C for 24 hours. The experiment was repeated three times using other sterile microchips to get averaged results. The colonies were counted on a colony counter (Stuart SC6) and the average cell number was determined for every time interval. The cell number was calculated by using the formula given below,

$$\frac{N}{V \times D} = CFU / ml \quad (3.1)$$

where, N is the viable colonies counted for each dilution (colony forming units or CFU), V is the volume plated (mL) and D is the dilution factor. Graphs of cell number versus time (CFU/mL vs. Time or Log_{10} of CFU/mL vs. Time) and OD versus cell number were obtained. Growth and sterility controls were set up separately in 15 mL test tubes and monitored in the same way as the microchip to compare the growth curves obtained in

both cases. The detailed procedures of media preparations, bacterial inocula preparations and monitoring methods are explained in later sections of this chapter.

Besides *E. coli* and *S. aureus* growth monitoring, as case studies, *E. coli* was further investigated under single drug and combinatory drug influences. Antibiotics kanamycin and ampicillin were selected, belonging to the aminoglycoside and β -lactam antibiotic family, respectively (details explained in Section 2.4.1). After the inoculation of the microchip with bacteria as explained in the previous paragraphs, drug solutions at a concentration equal to their minimum inhibitory concentration (MIC) were injected from the inlets of the microchip, either in combination of two or with DI water from the other inlet (Figure 3.12). MIC of the drugs was determined by broth microdilution method (explained in the later sections [191]).

Ampicillin and kanamycin powders were procured from Sigma-Aldrich with 96% and 99% purity, respectively. Stock solutions of antibiotics were prepared using the formula given below,

$$\frac{1000}{P} \times V \times C = W \quad (3.2)$$

where, P is the antibiotic potency given by the manufacturer ($\mu\text{g}/\text{mg}$), V is the volume of the solution required (mL), C is the final concentration of the antibiotic stock (mg/L) and W is the weight of the antibiotic powder (mg) to be dissolved in volume V . 1 M NaOH was used to aid dissolution of ampicillin in DI water (20% of 1 M NaOH v/v) and kanamycin was dissolved directly in DI water. The solutions were sterilized using a syringe filter of pore size 0.2 μm , aliquoted and stored at $-20\text{ }^{\circ}\text{C}$ for up to three months. Working solutions for experiments were prepared by dissolving required amount of stock solution in sterile DI water in aseptic conditions using the formula given below,

$$M_1 \cdot V_1 = M_2 \cdot V_2 \quad (3.3)$$

where, M_1 and V_1 are the concentration (mg/L) and volume (mL) of stock solution required to prepare working solution of concentration M_2 (mg/L) and volume V_2 (mL).

Figure 3.12: The well markings on the microchip for experiments with antibiotics.

The arrowheads show the injection of DI water and drug from the two inlets. (well 2 = 100% DI water, well 3 = 20% antibiotic, well 4 = 40% antibiotic, well 5 = 60% antibiotic, well 6 = 80% antibiotic, well 7 = 100% antibiotic = MIC of antibiotic against the test organism)

Case studies were performed with *E. coli* and antibiotics to study the effects of the drug at various dilutions (starting concentration = MIC of antibiotic) on bacterial growth [179]. The diluted overnight broth was similarly injected from the two inlets of a sterile microchip having Design II (160 μ L per syringe). The inoculated microchip was placed in the box incubator of the CDSS for incubation at 37 °C. The bacteria were allowed to grow in the new environment for 6 h before the drugs were injected. The antibiotic working solution was prepared and filled in 100 μ L precision syringe (Hamilton). Another precision syringe was filled with sterile DI water. Both syringes were then mounted on a syringe pump and connected to separate inlets of the microchip. Subsequently, 16 μ L each, of drug and DI water were simultaneously injected. Readings were taken every three hours for 18 hours by extracting 2 μ L aliquots of growing bacteria followed by diluting in PBS and plating. Plate counts were performed after 24 hours. The OD at 600 nm was also determined after every 3 hours by placing the incubator on the stage of setup shown in Figure 3.9. Some images were also then taken at 100 $\times$ magnification under the microscope (Figure 3.10, 3.11).

Similar case studies were carried out with kanamycin and DI water to study the effects of its dilutions on growth of *E. coli*. Subsequently, ampicillin and kanamycin were used together to study the effects of their combination in various concentration mixtures on the growth of *E. coli*. In this case, ampicillin and kanamycin were injected (16 μ L each) from the inlets of the microchip (Design II), incubated and monitored in the CDSS, as explained previously. Cell number and OD versus time were plotted with the measured values. A combination test was also carried out using conventional methods prescribed by the Clinical and Laboratory Standards Institute (CLSI) [189, 192] to determine the fractional inhibitory concentration index (FICI) for ampicillin and kanamycin combination. Time-kill plots for the combination that had OD < 0.05 at the end of 24 hours from the CDSS recording were also performed (the detailed procedures for combination tests are explained in later sections). Each of the case studies was repeated three times with different microchips of same design. The sample dilutions were plated in duplicates to get statistical average of cell number and OD was taken from more than three points in each well to get an average.

3.2.2 Experiments with adhesion cells: mammalian N2A and smooth muscle cells

Experiments were carried out using cells of mouse neuroblastoma or N2A (mouse brain tumor cells obtained from Brenner's Center, Singapore) and smooth muscle cells lining the small intestine of pig. Unlike bacterial suspensions, these cells were adhesion cell types that adhered to the base of their growing chamber. Sterile microchips (Design II) were used for the experiments. For N2A culturing, the cells were taken directly from the vials stored in nitrogen tanks and kept at 30 °C water bath for half hour. The cells were then transferred to tubes with fresh media and centrifuged at high speed. The media was separated from the pellet and supernatant was discarded. Fresh media was added and cells were again suspended by constant pipetting in and out. These were planted in the wells of

a sterile microchip by injecting 160 μL of the media + cells solution from the two inlets. A control was also set up by pipetting 40 μL of the same solution per well into another microchip and in standard flasks to compare the growth and adhesion properties. Both test and controls were incubated at 37 °C for 24 hours. Images were taken at 0, 2 and 24 hours. With a similar protocol, the swine smooth muscle cells were fixed in the wells of a sterile microchip.

3.3 Drug combination studies on *S. aureus*

The preliminary studies were carried out to study the biocompatibility and feasibility of the CDSS along with the microchip, for cell growth, maintenance and screening. Two cell categories were used and protocols were established. Drug combination case studies were performed with gram-negative *E. coli* as a proof of concept. However, *S. aureus* was selected for further studies as it possesses a highly adaptive genome [137] (also discussed in detail in Section 2.3.2.2 *Staphylococcus aureus*). It is known to readily and rapidly undergo adaptive mutation and resistance under antibiotic pressure, sometimes even within as few as four days of exposure. The antibiotic ciprofloxacin was also appropriately selected for a similar reason, as *S. aureus* has been previously shown to develop resistance to ciprofloxacin in four days of continuous exposure at sub-MIC levels [193, 194].

The subsequent sections of this chapter would focus on *in vitro* studies performed on *S. aureus* with drug combinations, aimed to delay or modulate the process of development of resistance and/or mutation. The antibiotic susceptibility testing protocols recommended by Clinical Laboratory Standards Institute (CLSI) and British Society for Antimicrobial Chemotherapy (BSAC) were followed, to determine various parameters relating to antibiotics and their combinations [192, 195-198]. In the following section, the preparation of materials that was used in the subsequent experiments has been described with their storage recommendations.

3.3.1 Material Preparation

3.3.1.1 Preparation of growth media

The growth media, both liquid and solid, used to carry out experiments with *S. aureus* was procured as dehydrated powdered mixture in the form of Iso-Sensitest (IS) broth and agar from Oxid, Biomedica. This media is recommended by BSAC for antibiotic susceptibility testing on *Staphylococcus* species.

Antibiotic-free broth and agar: For broth preparation, 23.4 g of IS broth powder was dissolved in 1 liter of DI water with vigorous shaking followed by autoclaving at 121 °C for 20 min. The sterilized broth was kept in water bath at 37 °C with the lid closed tightly, until its temperature was cooled to room temperature (tested by holding the bottle with hands for 10 s or above). The cooled media was dispensed aseptically into clean test tubes in the Biological safety cabinet Class II (Gelman, BH Class II series) and stored at 4 °C for not more than three weeks.

Agar media was prepared by dissolving 31.4 g of IS agar powder in 1 liter of DI water with vigorous shaking followed by autoclaving at 121 °C for 20 min. The media was allowed to cool down to a temperature of 50 °C and poured into 94 mm diameter petri-dishes up to a height of 2-3 mm (or 20 mL liquid agar in each plate). The plates were allowed to dry in the biosafety cabinet for 15 min. They were individually named, dated, packed in sterile packets and stored at 4 °C for not more than three weeks. Before use, the plates were allowed to open dry in the biosafety cabinet for 15 min or until the condensation over the plates disappeared.

Antibiotic containing agar: Antibiotic containing agar plates were made by dissolving the IS agar powder in DI water (31.4 g/L). After autoclaving and cooling to a temperature approximately 45-50 °C or cool enough to be hand held for 10 s, the antibiotic solution was added in the desired concentration prepared using the antibiotic stock (explained in

the next sections). The bottle was then gently swirled and inverted for 5-10 s and immediately poured into 94 mm petri-dishes previously named and dated. The plates were allowed to dry in the biosafety cabinet for 15 min, sealed and stored at 4 °C for not more than three weeks. The whole process was carried out aseptically in the biosafety cabinet.

3.3.1.2 Bacterial strains and preparation of stocks

Reference bacterial strains were used in this study to eliminate the need for characterization of the strains. *S. aureus* American type culture collection (ATCC) 29213 and ATCC 43300 for methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA), respectively, purchased from Biogen were used for the experiments. Both strains are recommended by CLSI for quality control. The bacteria were kept suspended in separate vials containing Luria-Bertani (LB) broth containing 40% glycerol (v/v) and stored at -80 °C. The bacterial stocks were prepared on IS agar plates. A sterile wire loop was swirled in the vials maintained at -80 °C and streaked across a fresh IS agar plate followed by incubation at 37 °C for 24 hours. Subsequently, the plates with MSSA and MRSA stocks were sealed and stored at 4 °C for up to four weeks and refreshed from time to time.

3.3.1.3 Preparation of bacterial inoculum

Bacterial inoculum or working bacterial culture was prepared from stored stocks before carrying out the experiments to maintain the purity of the strain. For *Staphylococcus* species, both BSAC and CLSI recommend the direct colony suspension method to prepare the inocula. The turbidity of the suspension was calibrated to McFarland turbidity standards, usually 0.5 McFarland turbidity. It is a standard particulate suspension solution for comparing the turbidity of the bacterial inocula. This particulate suspension is of barium sulphate and is prepared by adding 0.5 mL of 0.048 M BaCl₂ (1.17% w/v BaCl₂·H₂O) to 99.5 mL of 0.18 M H₂SO₄ (1% v/v) followed by constant stirring. It is then

distributed in 5 mL aliquots and stored at 4 °C in sealed capped glass tubes. Alternatively, a commercial standard barium sulphate solution having 0.5 McFarland turbidity was purchased from Becton Dickinson.

24 hours before the experiment, bacteria from the stocks were sub-cultured on a fresh agar plate by streaking one isolated colony from the stock plate followed by incubation for 24 hours at 37 °C. On the day of experiment, inoculum was prepared by direct colony suspension. Four or five morphologically similar colonies were touched with a sterile loop and swirled in a tube containing IS broth or PBS (5 mL). The resultant suspension was compared with the 0.5 McFarland standard turbidity tube. Prior to comparison, both tubes were vigorously agitated on a vortex mixer. A visual comparison was made against a white background with contrasting thick black lines in a brightly lit room. Alternatively, the optical density of the suspension was measured at 600 nm wavelength that should be in the range of 0.08-0.10 for it to possess standard turbidity. The turbidity of the suspension was made either greater than or equal to the standard, by adjusting with sterile DI water or fresh broth addition. The resultant inocula, generally contained 10^7 - 10^8 CFU/mL bacteria. Subsequently, for most of the procedures, the 0.5 McFarland standard inocula were diluted further 100× in broth or PBS, thence, containing 10^5 - 10^6 CFU/mL bacteria.

3.3.1.4 Preparation of antimicrobial stock and working solutions

Antibiotic powders of ciprofloxacin and ofloxacin were procured from Sigma with 98% and 99% purity, respectively. Purified plant extract powders were also obtained from Sigma; viz., quercetin (98% purity), rutin (94% purity), protocatechuic acid (or 3,4-dihydroxybenzoic acid, 97% purity) and ethyl gallate (96% purity). The concentration of the stock solutions was selected as 10 mg/mL for antibiotics and 102.4 mg/mL for the plant extracts. The weight of the powder was determined by using the formula given in Equation 3.2, and weighed in milligrams on a precision balance (Fisher Scientific,

Singapore). The powders were transferred to separate tubes and dissolved in their respective diluents. Different diluents were used to aid the dissolution of the powders. For ciprofloxacin, ofloxacin and rutin, 0.1 M NaOH was used to aid dissolution and remaining volume was topped with sterile DI water. For quercetin 1 M NaOH was used to aid dissolution. Similarly, for protocatechuic acid and ethyl gallate, 99.9% ethanol (Biotechnology grade, Sigma) was used. The resultant stock solutions were filtered using syringe filters of pore size 0.2 μm , distributed in aliquots of 1 mL in vials and stored at -20 °C for not more than three months.

To prepare the working antibacterial solution, the stocks were thawed aseptically at room temperature and diluted in required volume in sterile DI water, using the formula given in Equation 3.3. Unused thawed stocks were discarded after use, as re-freezing could affect the potency of the drug.

3.3.2 Experimental procedures

3.3.2.1 Determination of minimum inhibitory concentration (MIC)

The procedure for MIC determination was adopted from the one described by Andrews [191]. She emphasized that MIC(s) of antibiotics are considered gold standards in all types of susceptibility tests and are used very often even to confirm unusual resistance where other methods of susceptibility fails to provide a conclusive answer. MIC is defined as the minimum concentration of an antimicrobial agent required to inhibit visible growth of a test organism. It can be determined by agar dilution or broth dilution methods. In the present study, MICs were determined by the broth microdilution method [138]. The method was carried out on sterile 96-well plates (Greiner Bio One). The antimicrobial working solution of desired concentration was prepared from the stocks as explained earlier. In one of the rows of the titer plate a dilution range of the antimicrobial agent of interest was prepared. For the antibiotic this range was from 0.0125 to 128 $\mu\text{g/mL}$, in 2-

fold dilutions, and for plant extracts this range was from 8 to 8192 $\mu\text{g/mL}$, also in 2-folds. Subsequently, the bacterial inoculum for which the MIC was to be determined was prepared as explained in the previous section. The tests were performed in duplicate rows for each organism and antimicrobial agent for consistency of results. Briefly, 50 μL of IS broth was dispensed in each well of a row (12 wells), followed by 50 μL of antibiotic working solution in the first two wells of the row. From the second well onwards, 50 μL of solution in the well was diluted into the next well and so on, so that each successive well had half the concentration compared to the previous well and a dilution range was achieved. 50 μL of prepared inoculum was then added to each well. The same process was repeated for the subsequent rows and duplicate tests. The titer plate was covered and incubated at 37 °C for 24 hours. Control wells were also prepared in wells of empty rows. A sterility control with 100 μL IS broth and a growth control with drug-free bacterial inoculum was prepared. The results were read as the MIC value at concentration in which no visible growth was observed as seen against light or measured by spectrometer plate reader ($\text{OD} < 0.05$ at 600 nm, MicroQuant Plate Reader, Biotek).

3.3.2.2 Determination of fractional inhibitory concentration and index (FIC and FICI)

The FIC was determined when drugs were used in combination, therefore, for each drug an FIC value was first established followed by calculation of the FICI value for the combination. Based on the FICI value, the combination was rated as useful or not useful. The FICI value is oftentimes used to qualify antimicrobial combinations during routine susceptibility testing. It has been emphasized at many instances that antimicrobial combinations are more useful compared to single drug treatments, with its broad spectrum coverage, synergistic outcome or treatment of resistant pathogens [5] (see Section 2.1.2 - Antibacterial combination studies). Antibiotics in combination can interact in many ways. To quantify their interaction certain criteria have been set for universal acceptance and

validation. A positive interaction between two antimicrobials is termed as synergism, in which their combined effect is significantly greater than the effect when the drugs are used separately. On the other hand, antagonism is a negative effect, in which the combined effect of the two drugs is significantly less than the effect of each drug used separately. Additivity and indifference are other kinds of interactions that can occur in antimicrobial combinations. The former describes an interaction in which the combined effect is equal to the sum of effects of the two drugs used separately, while indifference occurs when the drugs do not interact and is usually the effect of the more active drug alone. These two interactions are generally not reported as they are not significant interactions. There is yet another important interaction that is observed when drugs are used in combination. Potentiation occurs when the antibiotic activity is enhanced in combination with a compound, which on its own has little or no antimicrobial activity. It is usually observed when antibiotics are combined with antibacterials derived from natural sources, such as plants or insects [199, 200].

The FIC for a drug is defined as the MIC of the drug in combination divided by the MIC of the drug used alone. The FICI is then calculated as the sum of FICs for each drug. The checkerboard titration method is most commonly used method for assessment of drug interactions. Similar to the MIC assay, the method used to determine FIC was also performed on a titer plate. However, a prerequisite for the test was knowledge of MICs of the drugs to be tested for interaction. The concentrations range for each drug was then selected in the range of four-five dilutions lower than the MIC and three-four dilutions higher than the MIC. Similar to the MIC assay, two fold dilutions of the antimicrobial agents were prepared. The dilutions were made in such a way that, each row had constant concentration of one drug and serially increasing (2-fold) dilutions of the other. Similarly each column had a constant concentration of one drug and serially decreasing concentration of the other. The process was adopted from Antimicrobial Susceptibility Testing Protocols [190], and briefly described below.

The antibiotic working solutions were prepared from the stocks (explained in Section 3.3.1.4) at concentrations four times the value of MIC of the drugs for the test organism. For one organism two titer plates were prepared and labeled as “1” and “2”. Plate 1 was used to prepare serial dilutions of drug A and plate 2 was used to prepare serial dilutions for drug B. For plate 1, each well was first filled by 50 μ L IS broth leaving out columns 1 and 12 of the titer plate. Additional 50 μ L of broth was added to well G12 (row G and column 12). Drug A was added to wells of last two columns of the plate (wells A11-H11 and A12-G12). Serial dilutions were made from each well of column 11 to each well of column 2, discarding the additional 50 μ L from column 2 to maintain the final volume in each well as 50 μ L. Plate 2 was prepared by dispensing 100 μ L of IS broth into all wells leaving row A and H. 100 μ L of drug B was added to wells of row H and G except well H12. Serial dilutions were carried out from each well of row G to each of row B, discarding additional 100 μ L from each well of row B to maintain the final volume in each well as 100 μ L. Subsequently, 50 μ L from row B (B1-B11) from plate 2 was transferred to corresponding wells of plate 1. Similarly, the rest of the wells of plate 2 were transferred to the corresponding wells of plate 1. Row A and column 1 had exclusively the dilutions of one drug, drug A and drug B, respectively. Also, each well contained a unique concentration mixture of the two drugs in 2-fold dilutions. The inoculum was prepared as described in Section 3.3.1.3 and 50 μ L of it was added to each well of the plate (except well H12). The well A1 and H12 were the growth and sterility control wells, respectively. Plate 2 was discarded and plate 1 was covered with a sterile lid and incubated at 37 °C for 24 hours.

For the interpretation of the results, growth control and sterility controls were observed for maximum turbidity and optical clarity, respectively. The MIC for each drug could be reconfirmed from wells A2-A12 (for drug A) and from wells B-H in column 1 (for drug B). The rest of the plate was observed against light and a magnifying lens for growth and no growth. Isobolograms were drawn to interpret results of the checkerboard titration. The

FIC was calculated for each drug as the minimum concentration of the drug necessary to inhibit growth as seen in an optically clear well, divided by the MIC of that drug for the test organism. The FICI was then determined for the combination as the sum of FIC for both drugs as given in the formula below,

$$\frac{(A)}{MIC_A} + \frac{(B)}{MIC_B} = FIC_A + FIC_B = FICI \quad (3.4)$$

where, (A) is the lowest inhibitory concentration of drug A in its row, MIC_A and FIC_A are the MIC and FIC of drug A, respectively. (B) is the lowest inhibitory concentration of drug B in its column, MIC_B and FIC_B are the MIC and FIC of drug B, respectively. The FICI values obtained can be interpreted as follows [138, 190]:

- $FICI \leq 0.5 \rightarrow$ Synergism,
- $FICI = 1.0 \rightarrow$ Additivity,
- $< FICI \leq 4.0 \rightarrow$ Antagonism, and
- $FICI > 4.0 \rightarrow$ Indifference

As emphasized by Pillai *et. al.* that checkerboard method is one way of interpreting drug interactions and one must not depend on it entirely for the feasibility of a drug combination. Some limitations of the test were listed out. An erroneous assumption in the isobolograms exists, that for all antimicrobials the dose-response curve is linear. The results are only based on growth or no growth response, and hence incapable of measuring a graded or timely response. The results are observed only at the end point (after 24 hours), hence the results remain static and does not give dynamic response of the drug interactions [5]. Despite of the above mentioned limitations, this method is widely used as the first step to assess drug interactions. Determination of a time-kill plot is usually followed by this method to provide a dynamic view of the drug interactions studied over a stipulated period of time (usually 24 hours) (explained in the next section).

3.3.2.3 Time-kill assays

This method is also used to evaluate drug interactions and to study the efficacy of a drug on an organism over time. Over the checkerboard method, it has an advantage of a dynamic and time-based response of the organism, when a drug combination is used. It can also be used to determine bactericidal activity of a drug. The method has been adopted from Antimicrobial Susceptibility Testing Protocols [190], and briefly described in this section.

An array of 15 mL tubes were labeled with the combination concentrations to be tested. Usually, according to the stipulated experimental procedures, the combinations that are tested include:

- $\frac{1}{4}\text{MIC}_A + \frac{1}{4}\text{MIC}_B$ (for FICI = 0.5)
- $\frac{1}{2}\text{MIC}_A + \frac{1}{2}\text{MIC}_B$ (for FICI = 1)
- $\text{MIC}_A + \text{MIC}_B$ (for FICI = 2)
- MIC_A
- MIC_B

where, MIC_A and MIC_B are the MICs determined previously for drug A and B, respectively. These are the combinations that are a direct outcome of the isobologram concentration and FICI values, hence, have a limited scope. The antibiotics of desired concentration ($=100\times\text{MIC}$) were prepared in their working solutions as explained earlier. Each tube was filled with 9.7 mL (for drug combination) or 9.8 mL (for single drug) IS broth followed by 100 μL of each of drug A and/or B. For the concentration lower than MIC e.g. $\frac{1}{4}$ or $\frac{1}{2}$ MIC, 25 μL or 50 μL of drug working solution was added and remaining was topped up to 100 μL with sterile DI water or broth. Inoculum containing 10^7 - 10^8 CFU/mL was prepared and 100 mL was added to each tube. Growth and sterility controls were also set up. The growth control consisted of 9.9 mL IS broth and 100 μL bacterial

inoculum and the sterility control contained 10 mL broth. The final volume in each tube was 10 mL. The tubes were thoroughly mixed on a vortex mixer and arranged on a tube rack. From the growth control 10 μ L aliquot was removed and rest were placed on a shaker at a speed of 150 rpm/min in an incubator at 37 °C with their caps loosened.

The 10 μ L aliquot from the growth control was diluted and plated to give the '0' hour reading. 10-fold serial dilutions were made with PBS by adding the 10 μ L aliquot to 90 μ L PBS on a sterile titer plate well. The further dilutions were made with successive wells containing 90 μ L PBS. Dilutions were made up to 10^{-8} . 10 μ L of each dilution was then plated on a drug-free IS agar plate as 8 small drops. The plate was allowed to dry followed by incubation at 37 °C for 24 hours. Following the same procedure of dilution, aliquots of 10 μ L were removed from each tube after 4, 6, 8 and 24 hours and plated on agar plates followed by 24 hour incubation. After a period of 48 hours following the start of experiment the colonies on the plates were counted on a colony counter for each tube and each dilution. Viable colonies were counted in a range of 30-300, colonies less than 30 were considered too small and colonies more than 300 were considered too many to count. The viable bacterial number in the tubes was determined using the formula given in Equation 3.1 to get the number in CFU/mL. The values were converted to Log_{10} values and plotted against time for interpretation of results.

The results were interpreted as effect of combination compared to the most active drug in combination and the control. Synergism was defined when a 100-fold increase was observed with the combination compared to the most active drug or ≥ 2 - log_{10} decrease in bacteria number (CFU/mL or $\text{Log}_{10}\text{CFU/mL}$) compared to the most active drug and ≥ 3 - log_{10} decrease from the initial inoculum concentration. The opposite was observed for antagonism.

3.3.2.4 Population analysis profiling (PAP)

The PAP test was performed to determine the heterogeneity of a bacterial population in terms of antibiotic resistance, since MIC value only described the apparent antibiotic resistance. PAP tests also provide insights about homogeneous or heterogeneous reduced susceptibilities of a bacterial isolate. The method was adopted from Wootton *et. al.*, as the modified PAP test, which they used to determine heterogeneity in MRSA strains for reduced susceptibility to vancomycin [201]. In this project, the antibiotic of interest was ciprofloxacin for which resistance needed to be established; therefore the procedure was accordingly modified. Inoculum of isolates, in which resistance to the antibiotic of interest was to be determined, were prepared having 10^3 - 10^4 CFU/mL. 10 μ L of this inoculum was plated on IS agar plates containing antibiotic having the following concentrations; 0, 0.0625, 0.125, 0.25, 0.5, 1 and 2 times its MIC. The plates were incubated at 37 °C for 48-72 hours. The colonies were counted and plotted as CFU/mL (and Log₁₀ CFU/mL) and relative percentage growth (compared to growth on drug-free agar) against the concentration of the antibiotic. The area under curve (AUC) was calculated for the graphs of viable counts versus antibiotic concentration for each strain and compared with the control strain, by dividing the AUC of test strain by AUC of control strain [201, 202]. The values close to unity (ratios from 0.9 to 1.2) suggested similarity to the control strain with little or no heterogeneous resistance. Higher ratios values suggested heterogeneous populations and further tests to confirm resistant phenotypes were carried out by selecting resistant mutants on agar plates with higher concentration of the antibiotic ($\geq 2 \times$ MIC).

3.3.2.5 Disk-diffusion test

Disk diffusion tests were also used to determine resistance to antibiotic of interest in isolates under study. The test is based on the diffusion of antibiotic from the disks through solid agar medium. The procedure is adopted from Antimicrobial Susceptibility Testing Protocols [190], and is briefly described in this section. Drug-free IS agar plates were used

with a controlled depth of 4 mm agar in each plate, as the depth of agar greatly affects the outcome of the test. The inoculum of the test isolate was prepared as explained in Section 3.3.1.3, up to 0.5 McFarland standard turbidity (with no further dilution in the final inoculum) and final inoculum concentration was 10^7 - 10^8 CFU/mL. 50 μ L of inoculum was spread on agar plate with the help of a sterile wire loop and allowed to dry for 10 min under the biosafety cabinet. Standard antibiotic disks purchased from Becton and Dickinson (Singapore) (ciprofloxacin, 5 μ g per disk and oxacillin 1 μ g per disk) were placed on the inoculated agar using a sterile forceps and tapped gently to avoid movement of the disk. After the placement of disks the antibiotic immediately starts to diffuse through the agar medium from the edge of the disks, therefore movement of the disk after placement was not recommended. The plates were then incubated for 20-24 hours at 37 °C. Zone of inhibitions were formed around the disks after incubation as a result of contest between diffusion of the drug and growth of bacteria. The zone diameters including the disks at the center were measured on an opaque dark surface with reflected light using a scale to the nearest millimeter. The diameters were then compared with those given in the manufacturer database of zone diameters for different bacterial species and antibiotic disks. The results based on zone diameter measurements were then interpreted as 'susceptible', 'intermediate' or 'resistant' bacteria.

3.3.2.6 Determination of mutation frequency and mutation prevention concentration

The mutation frequency is defined as the number of mutant bacteria in a culture. In the experiments with *S. aureus* under selective pressure of antibiotics and antibiotic combinations for extended time periods (discussed in later sections), the mutation frequency was determined and mutants were selected with the antibiotic of interest at 2 $\times$ and 4 $\times$ MIC. The frequency was then used to establish if the antibiotic combination was able to modulate the occurrence of mutational events compared to single antibiotic

(ciprofloxacin) in long term drug exposure. This was also followed by molecular analysis of the mutants. The experimental procedures have been adopted from Drugeon *et. al.* and Khan *et. al* [199, 203], and briefly described in this section. A bacterial suspension of the strain in which the mutation frequency is to be determined was prepared containing 10^8 - 10^9 CFU/mL. 100 μ L of this suspension was plated onto agar medium containing antibiotic of interest at concentrations equal to 2 $\times$ and 4 $\times$ MIC (MIC of the parent strain). The frequency of antibiotic resistant mutants was calculated by dividing the total number of colonies counted after 48 hours of incubation at 37 °C on the drug containing plate by the total number of CFU plated initially.

Mutation prevention concentration (MPC) was determined for the parent strain (*S. aureus* ATCC 29213). It is defined as the concentration (μ g/mL) of an antimicrobial agent which prevents the emergence of resistant mutants in a bacterial population selected at concentrations higher than the MIC of antibacterial agent. A suspension of parent strain was prepared containing 10^8 - 10^9 CFU/mL. 100 μ L of this suspension was plated in duplicates onto agar medium containing the antibiotic at concentrations equal to 2 $\times$, 4 $\times$, 8 $\times$, 16 $\times$ and 32 $\times$ MIC. The plates were incubated for 72 hours at 37 °C in air. The MPC was read as the concentration at which there were no apparent colonies observed after 72 hours. The mutation selection window was then determined with minimum concentration equal to the MIC and maximum concentration equal to the MPC. MPC and the mutation selection window are important measurement of antibiotic potency together with MIC that gives insights into its ability to restrict the emergence of resistant mutants upon long term treatment with it. It has been emphasized by Khan *et. al.* that for it to be a clinically useful measurement, the MPC must be lower than the maximum concentration of drug in serum (C_{max}) or at the site of infection [199]. When combined with other pharmacokinetic parameters it can provide useful information on the ability of the antimicrobial agent to select resistant mutants *in vivo*. Hence, if the MPC of an antibiotic can be achieved *in vivo*

then the development of resistance to it is less likely and treatment with it is more likely to be successful.

3.3.3 Control tests

For the subsequent experiments to study drug combinations on the microchip inoculated with bacterial suspensions of standard inoculum size, controls and calibration curves were established to standardize bacterial numbers based on the optical density of the suspension. The initial conditions of all experiments including the tests were normalized. This includes the inoculum preparation (explained in Section 3.3.1.3), test and control volumes, media, OD wavelength and incubation conditions (duration and temperature), assuming all other factors were kept constant. Controls were prepared in two volume sizes. One to compare the growth and drug effects in the microchip (final volume = 50 μ L) and another to compare growth and drug effects in standard 15 mL test tubes (final volume = 10 mL). Sterility controls were also setup for every experiment. The controls were repeated five times for each volume preparation to get a statistical average.

For the microchip control, a sterile microchip was inoculated by a bacterial suspension containing 10^5 - 10^6 CFU/mL bacteria, by simultaneously injecting the suspension from both the inlets. The microchip was placed in the box incubator of the CDSS for 24 hours. 10 μ L aliquots were removed from one of the wells and diluted in 10-folds in PBS for plating. 10 μ L of each dilution was then plated in duplicates on agar plates previously divided using marker pen for each dilution. The dilution and plating was repeated after 2, 4, 8 and 24 hours after inoculation. The first reading was taken immediately after inoculation (at '0' hour). Two wells of the microchip were used to take OD on the setup shown in Figure 3.9 at the same time intervals of 0, 2, 4, 8 and 24 hours. The plates were counted for viable colonies after 24 hour incubation at 37 °C, and average cell number was calculated for each time interval. The averaged OD was then plotted against cell number at each time interval and a calibration curve was established using linear

regression models. The curve allowed to predict cell number based on the OD values obtained during an experiment.

Growth controls were also prepared in standard 15 mL sterile tubes with standard inoculum size (Section 3.3.1.3) and volume 10 mL. The tubes were incubated on a shaker at a speed of 150 rpm/min at 37 °C. 10 µL aliquots were removed from the tubes after 0, 4, 8, and 24 hours after inoculation for dilution and plating. The cell counts obtained after 24 hours were converted to Log₁₀ values and plotted against time. These served as control to the time-kill assays. All control experiments were performed in triplicates to get averaged values.

3.3.4 Drug combination studies on CDSS

The microbial tests were performed on the experimental system separately with MSSA and MRSA with ciprofloxacin and ofloxacin in combination with one of each of the phytochemicals, quercetin (quer), rutin (rut), protocatechuic acid (PCA) and ethyl gallate (EG). A total of eight combinations were tested on both strains, viz. ciprofloxacin with quercetin at starting concentrations equal to the MICs determined earlier (abbreviated as Cip1-Quer512, numbers indicating the MIC in µg/mL), ciprofloxacin with rutin (Cip1-Rut4096), ciprofloxacin with PCA (Cip1-PCA4096), ciprofloxacin with EG (Cip1-EG1024), ofloxacin with quercetin (Of11-Quer512), ofloxacin with rutin (Of11-Rut4096), ofloxacin with PCA (Of11-PCA4096) and ofloxacin with EG (Of11-EG1024). All experiments were performed in triplicates.

Overnight cultures of bacteria in IS broth containing 10⁵-10⁶ CFU/mL, were injected from the two inlets of a sterile microchip (Design II) to fill the wells up to 45 µL. Subsequently, an antibiotic (ciprofloxacin or ofloxacin) and a phytochemical (quercetin, rutin, PCA or EG) solution in concentrations equal to their respective MICs for the test organism, were simultaneously injected from the microchip inlets, so that 5 µL of total solution feed each

well containing bacterial suspension. The final solution in the wells hence consisted of six concoctions of the two drugs, including the original drug concentration (wells 2 to 7, Figure 3.12). The resulting volume after drug injection in each well became 50 μ L. The wells, therefore, had decreasing concentration of antibiotic at 20% steps below its MIC and vice versa for the phytochemical. The following combinations were obtained, 20% antibiotic with 80% phytochemical (v/v), denoted as Ant20-Phy80 (Ant = Cip or Ofi and Phy = quer, rut, PCA or EG), 40% antibiotic with 60% phytochemical (Ant40-Phy60), 60% antibiotic with 40% phytochemical (Ant60-Phy40), 80% antibiotic with 20% phytochemical (Ant80-Phy20) and 100% (= MIC, Ant100 or Phy100). The inoculated and drugged microchip was incubated at 37 °C in the incubation chamber of the CDSS for 24 hours. Bacterial growth was monitored by directly reading the optical density (OD) of the wells at 600 nm wavelength after 2, 4, 6, 8 and 24 hours. Identical method was used with MRSA. For comparison of combination efficacy another experiment was performed on both strains with antibiotic injected from one inlet and sterile DI water from the other. In this case, the output wells had antibiotic concentrations less than the MIC at 20% step reduction.

The combinations indicating maximum bacterial growth reduction or inhibition, with OD < 0.055 or cell number < 2.5×10^7 CFU/mL bacteria (MSSA), after 24 hours (or at T₂₄) were then tested with the standard methods to obtain the corresponding time-kill plots [138]. The procedure is explained in Section 3.3.2.3. Growth and sterility controls were prepared to obtain control curves. The time-kill assays were performed as combination of antibiotic with phytochemicals in their working concentrations established from the microchip results along with corresponding concentrations of the antibiotic alone. This was done for comparison and quantification of the results based on log differences between the final activity of combination and final activity of the antibiotic alone (antibiotic being the more active drug in the combination, Section 3.3.2.3).

After the selection and confirmation of combination mixtures of antibiotic and phytochemicals that resulted in maximum growth inhibition or killing, a 15 day long study was conducted both on the system and by standard techniques. Five sterile microchips (Design II) were inoculated with MSSA suspension ($\sim 1.4 \times 10^8$ CFU/mL) by injecting simultaneously from both inlets. Antibiotic was injected from one inlet and one of the phytochemicals or DI water from the other inlet at concentration equal to their respective MICs. The zero hour reading or T_0 reading was taken at the start of the experiment on day 1. The inoculated devices were allowed to incubate at 37 °C for 24 hours in the incubation chamber of the system. On the following day at T_{24} 80% of the fluid in the wells (~ 40 μ L) was extracted directly from the wells for analysis and fresh drug combination and media was added from the inlets (up to total volume ~ 40 μ L each well). The extracted fluid was used to run a disk diffusion test with 5 μ g ciprofloxacin disks (Becton and Dickinson, Singapore) following the procedure as previously explained [190] and in Section 3.3.2.5. Each sample was analyzed for ciprofloxacin susceptibility using standard manufacturer guidelines regarding the zone diameters measured in millimeters. This process was repeated after every 24 hours. A part of the extracted well sample at T_{72} , T_{144} , T_{216} and T_{360} was diluted in sterile PBS and plated onto drug-free agar plates for further analysis. Following an incubation period of 24 hours at 37 °C four-five random colonies were picked up from the plates and streaked onto another sterile agar plate using a sterile wire loop. These samples were consecutively sub-cultured seven times on drug free agar before a population analysis profiling (PAP) was carried out to study the heterogeneity of the bacterial population after drug exposure for the respective time period (72, 144, 216 and 360 hours). The PAP was carried out using the method explained in Section 3.3.2.4 with IS agar plates drugged with ciprofloxacin at concentration ranging from 0 to 2 μ g/mL (0, 0.0625, 0.125, 0.25, 0.5, 1 and 2 μ g/mL). Together with the disk diffusion and PAP test, the susceptibility of ciprofloxacin was established for each sample after continuous exposure to the antibiotic at MIC and sub-MIC levels and to its combination with phytochemicals.

3.3.5 Detection of mutation in *S. aureus*

In addition to the PAP and disk diffusion tests, the T₃₆₀ sample was also used for further analysis and tests to calculate the mutation frequency and mutation prevention concentration (MPC) of ciprofloxacin after continuous drug exposure for 360 hours (procedure explained in Section 3.3.2.6). This would provide valuable information on whether the addition of the phytochemicals potentiates the antibiotic activity by either delaying the emergence of resistant mutants of *S. aureus* or by modulating the common mechanisms of resistance with continuous antibiotic external pressure. The MPC window of mutation selection (window: minimum-MIC and maximum-MPC) for ciprofloxacin on *S. aureus* would also elucidate on the antibiotic-phytochemical interaction after continuous drug exposure. The mutants selected at 2× and 4× MIC were also assessed for their susceptibility with ciprofloxacin by establishing MICs for the respective strains obtained. As mutants can undergo further mutations after releasing the antibiotic pressure, the stability of their resistance must, therefore, be assessed. Four-five random colonies were touched and streaked on drug free agar at least seven times followed by incubation periods of 24 hours before their MICs were determined again. The MIC microdilution assay was carried out in triplicates to get consistent MIC values for all the mutants selected. The strain with 8-fold or higher increase in MIC from the parent MSSA ATCC 29213 strain were scrutinized further by biomolecular analysis to understand the mutations or genetic resistance, if any. However, according to CLSI guidelines, strains susceptible to ciprofloxacin have MIC ≤ 1 µg/mL and resistant if their MIC is ≥ 4 µg/mL, MIC of 2 µg/mL usually fall in the intermediate category with mostly phenotypic resistance (NCCLS M100-S14 (M7-A6) [197]).

Ciprofloxacin resistance was confirmed by carrying out biomolecular studies on strains with 8-fold or higher MIC increase from the parent strain. Amplification of quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *grlA* and *grlB* genes and *norA*

promoter were performed using PCR [193]. The parent strain MSSA ATCC 29213 was included as the reference strain. Tube and slide coagulase test was performed to ensure all isolated were *S. aureus* [204]. Molecular typing of the mutants and reference strain indicated that they were all same isolates. Chromosomal DNA was extracted from the cells and a polymerase chain reaction (PCR) amplification of topoisomerase QRDRs and *norA* promoter (*flqB*) regions was performed using primers listed in Table 3.2 [205].

Table 3.2: Primers used in PCR performed on QRDR and *norA* promoter regions

Gene amplification (nucleotide positions)	Primer	Sequence (5' to 3')
<i>grlA</i> (2347 to 2870) ¹	GrlAF2	AATGATCAATTTGATGAGGAGG
	GrlAR2	CACTAGTAAGTTAGGAAATCT
<i>grlB</i> (1562 to 1877) ¹	GrlBF2	GTGAAGATGCTCGTTCAGG
	GrlBR2	ACGATTATAATTACTATCTT
<i>gyrA</i> (2152 to 2658) ²	GyrAF1	ATGGCTGAATTACCTCAATCAAG
	GyrAR1	GGCTAATAAGTTAGGGAATCGA
<i>gyrB</i> (1379 to 1686) ²	GyrBF1	GTGAAGTAACACGTCGTA
	GyrBR1	TTTGTGATATCTTGCTTTCGC
<i>flqB</i> (129 to 546) ³	NorAL2	AACGTCATCACATGCACCA
	NorAL3	TATTACTAAACCGATACC
¹ Nucleotide positions are indicated according to GenBank sequence number D67075.		
² Nucleotide positions are indicated according to GenBank sequence number D10489.		
³ Nucleotide positions are indicated according to GenBank sequence number D90119.		

DNA extraction was performed by using QIAamp DNA mini-kit (Qiagen, Research Biolabs). PCR was performed by initial melting at 95 °C for 5 min followed by 25 cycles of melting (95 °C for 30 s), annealing (55 °C for 30 s) and elongation (72 °C for 1 min). Final elongation was set at 72 °C for 7 min. Agarose gel electrophoresis (SeaKem LE agarose 1.5% gel) was used to identify the PCR products, which was purified using the Qiagen PCR purification kit (Qiagen, Research Biolabs, Singapore). The purified product was loaded on 1% agarose gel to check the quality and quantity of the product followed by comparison with 100 bp ladder marker. This product was sequenced in ABI genetic Analyzer 3100 (Applied Biosystems), which was outsourced to a commercial laboratory (Research Biolabs, Singapore).

The electropherogram of the sequence was analyzed with FinchTV software (Geospiza) and ambiguous nucleotide was assumed. The nucleotide sequence was then analyzed by Sequence Manipulation Suite 2 [206]. ClustalX v1.81 [207] was used to perform both nucleotide and protein alignment. GenBank accession numbers D67075, D10489 and D90119 were used to obtain the coding sequence and amino acid sequence for DNA topoisomerase IV, DNA gyrase and *norA* gene [205, 208]. These sequences were used for sequence alignment as well.

3.4 Combination studies with maggot extracts

Antibiotic ciprofloxacin was also used in combination with excretions/secretions (ES) extracted from maggots, against *S. aureus*. It has been demonstrated earlier that ES extracted from maggots of *Lucilia/Phaenicia sericata* possess antibacterial properties and contain antibacterial factors of low molecular weights or peptides [55, 56, 155, 158]. For the present study, maggots of *Lucilia cuprina* were used to extract the ES and body fluids to analyze their antibacterial activity, if any. Maggot extractions were made by five different methods, some based on previous ES studies and others on previously proposed principles of antibacterial activity of maggots.

3.4.1 Preparation of maggots for metabolites/serum extraction

The common starting step for all the methods for extracting ES from maggots was the rearing of the larvae of *L. cuprina* blowflies to late second or early third-instar stage of their life cycle (see Figure 3.13). They were harvested at the Medifly Laboratory, Singapore. The flies were fed with raw pork in plexi-glass cages maintained at a constant temperature (25 ± 1 °C) and controlled humidity. The blowfly eggs laid on the meat were collected periodically using forceps from the cages and washed with 70% ethanol and sterile DI water successively three times.

Part of the treated eggs were aseptically placed on sheep blood agar plates and allowed to incubate for 2-3 days at 35 °C in air. The rest were placed on raw pork meat for 2-3 days and were allowed to hatch and grow into maggots. Late second-instar or early third-instar maggots from the above mentioned setups were separately transferred to flat petri-dishes and aseptically washed with 70% ethanol and sterile DI water three times and soaked in Wattman filter paper. These were then used for extracting the maggot ES. Initial trials with both types of maggot rearing methods showed that maggots cultivated on agar and meat had similar results based on their antimicrobial activity. For all further studies, maggots were reared on meat.

Figure 3.13: Life cycle of *L. Cuprina*.

(a) Life cycle of *L. cuprina* blowfly. (b) Maggots reared on sheep blood agar plates (stage: late second-instar)

3.4.2 Excretions/secretions/gaseous metabolites extraction and analysis

The antibacterial activity of maggot extracts was demonstrated in early 1930s by Simmons *et. al.* followed by Pavillard *et. al.*, in which they described the extraction techniques and bactericidal activity of ES from maggots of *L. sericata* [153, 154]. Later, the mechanism of antibacterial activity of these extractions was studied by different research groups. Some suggested it was due to antibacterial factors in the excretions or secretions (ES) of maggots [56, 153-155], while others reported ingestion and gut activity [156]. Based on the suggested mechanisms of disinfection by maggots, the present study with maggots investigated five methods to collect ES and maggot whole body extracts. For the collection of ES, PBS was added to maggots during incubation. The gaseous excreta or metabolites (gEM) were collected using a specialized setup assembled to carry out direct antibacterial screening on *S. aureus* and *E. coli*. These methods are explained in the following sections.

3.4.2.1 Method A: Extraction of maggot body fluids

As suggested in previous literature on disinfection action by maggots during wound healing, their inhibitory action on microbes was partly due to larval ingestion and gut activity [56, 156]. This is due to the presence of antibacterial factor(s) in its gut. In this method, the maggots were excised into half using a sterile blade so as to expose their gut secretions. Together, its body parts were pulverized and collected in a test tube (see Figure 3.14).

Two hundred maggots were used to get the debris which was transferred to a sterile centrifuge tube and centrifuged at 10,000g for 5 min at 25 °C. The supernatant was collected and sterilized for antibacterial screening.

Figure 3.14: Maggot ES extraction: Method A.

(a) Excised and pulverized maggots in a petri-dish. (b) Maggot debris centrifuged at 10,000g for 5 min at 25 °C, the supernatant was used for antibacterial screening

3.4.2.2 Method B: Incubation of live maggots with bacterial suspension in broth media

This method was a modification of Method A and adapted from Cazander *et. al.* [209]. Live maggots were allowed to incubate in the bacterial suspension in nutrient media at 37 °C. From stock bacterial cultures of both MSSA and *E. coli*, inocula of 0.5 McFarland standards were prepared in the respective liquid media (10^8 CFU/mL, see Section 3.3.1.3). Two hundred live maggots were added to each tube containing 10 mL of inoculum. The maggot number was varied from 50-200 for comparison of results. A control was set up without maggots to compare bacterial growth after 24 hours of incubation at 37 °C.

This method was slightly amended (modified method B) to include nutrient for maggots together with bacterial nutrient media. Sterile crushed sheep blood agar was added to the tube containing bacterial suspension in LB or IS broth and incubated for 24 hours. Samples from both test and control experiments were quantified and compared.

3.4.2.3 Method C: Maggot ES extraction in PBS (overnight incubation)

The subsequent methods were adapted and modified from previous antibacterial studies done on maggot ES [155, 158, 159].

In this method, 500 maggots were transferred to a sterile 200 mL conical flask with 2 mL of PBS (Figure 3.15). The covered flask was placed in an aseptic environment and ambient temperature for 24 hours. The resultant liquid in the flask was then extracted using a pipette and centrifuged at 10,000g for 5 min at 25 °C. The supernatant was collected and sterilized for antibacterial screening.

Figure 3.15: Maggot ES extraction: Method C.
500 maggots in 2 mL PBS

3.4.2.4 Method D: Maggot ES extraction in PBS and nutrients (overnight incubation)

With a slight modification in Method C, sheep blood agar or meat was provided as nutrients to the maggots during the incubation period. Late second-instar maggots were allowed to grow in sheep blood agar plates with 2 mL of PBS overnight at 37 °C (Figure 3.16). The ES was then collected from the plates with a pipette and centrifuged at 10,000g for 5 minutes at 25 °C, followed by sterilization for antibacterial screening.

Figure 3.16: Maggot ES extraction: Method D.

(a) Late second-instar maggots with sheep blood agar and 2 mL PBS. (b) Late second-instar maggots with raw pork meat and 2 mL PBS

3.4.2.5 Method E: Maggot ES extraction with PBS (1 hour incubation)

This method is the most widely used method to extract ES from maggots of *L. sericata*. With some modifications in the existing method, maggots were transferred to sterile tubes to provide a density of 100 larvae in 200 μ L of PBS and allowed to incubate in dark at room temperature (25 $^{\circ}$ C) for 1 hour. Resultant liquid obtained was transferred to another tube using a pipette and sterilized. Meanwhile, overnight cultures of bacteria were sub-cultured in respective media to a standard inoculum of 0.5 McFarland turbidity. 50 μ L of each inocula was added to resultant ES separately and vortexed thoroughly. The resultant 250 μ L of ES plus bacteria was incubated at 37 $^{\circ}$ C for 24 hours and directly screened for antibacterial activity.

For comparison of results of ES analysis, in the 1 hour incubation period, raw meat was added and the ES was collected in a separate tube, sterilized and used for similar testing.

ES collected by all methods were sterilized using 0.2 μ m syringe filter or autoclaved at 121 $^{\circ}$ C for 20 minutes. The sterile ES was then assayed for MIC (see Section 3.3.2.1) against both MSSA and *E. coli* by the broth microdilution method, except for Methods B and B (modified). For these methods, the resultant 24 hour assay was investigated by taking OD of the bacterial suspension and by plating and counting viable colonies. For the

MIC test assay on 96-well plates, clear wells presented antibacterial activity whereas turbid wells were considered as no observed antibacterial action. The summary of the methods is presented in Table 3.3.

Table 3.3: Summary of maggot ES collection and screening methods

Method	A	B	C	D	E
Principle	Larval ingestion and gut activity	Larval ingestion	Excretion/secretion of antibacterial factor(s)	Excretion/secretion of antibacterial factor(s)	Excretion/secretion of antibacterial factor(s)
Pre-processing of maggots	Larvae excised and pulverized	Maggots washed successively three times with 70% ethanol and DI water	Maggots incubated in PBS for 18-24 hours at 25 °C	Maggots with nutrients, meat or blood agar, incubated in PBS for 18-24 hours at 25 °C	Maggots incubated in PBS for 1 hour in dark at 25 °C
Collection of serum/ES	Centrifugation of debris. Supernatant collected and sterilized by autoclaving	Live maggots allowed to incubate in nutrient media with bacterial inoculum (with and without maggot nutrient, blood agar).	Resultant liquid, collected filtered and sterilized by autoclaving	Resultant liquid, collected filtered and sterilized by autoclaving	Resultant liquid, collected filtered and sterilized by autoclaving
Antibacterial screening	MIC assay using sterilized serum	Samples analyzed after 0,8 and 24 hours post maggot addition	MIC assay using sterilized ES	MIC assay using sterilized ES	Bacteria added to the ES and assayed by MIC method

Controls were also prepared for methods A-D to compare growth of bacteria in media without ES or maggots and were investigated by measuring OD and plate counting. Control for Method E was prepared separately in PBS only, as media for bacteria. The collected ES of Methods A, C, D and E were also made to undergo gas-chromatography and mass spectrometry (GC-MS) analysis for the presence of previously suggested antibacterial compound, phenylacetaldehyde, in the ES [157]. The analysis was performed on a gas chromatograph (GC 7890A, Agilent, Singapore) and mass spectrometer (MS, 5975C MSD, Agilent, Singapore) using a HP-5MS analytical column. The GC was fitted with a manual splitless injector and the injector temperature was maintained at 250 °C.

The complete parameters of the GC are summarized in Table 3.4a. The mass spectrometry scanning was done across the range of m/z 35-450. The temperature at the inlet was maintained at 280 °C to prevent condensation of the analytes. The parameters for the MSD are summarized in Table 3.4b. The compounds detected by the mass spectrometer were compared in structure to those in the Nist98 database for mass spectral peaks and standard solution peaks.

Table 3.4 (a) Gas Chromatograph control parameters.

Gas Chromatograph	Agilent 7890A		
Analytical Column	HP-5MS (30m × 0.25 mm × 0.25 mm)		
Injection Port Type	Manual Split/Splitless		
Injector Temperature	250 °C		
Pressure	11.567 psi		
Injector Type	Split		
Split Ratio	200:1		
Split Flow	200 mL/min		
Gas saver	OFF		
Total flow	204 mL/min		
Carrier Gas	Helium		
Oven program	Temperature	Hold Time	Rate
	40 °C	1 min	
	280 °C	5min	10 °C/min

Table 3.4 (b): Mass spectrometer control parameters.

Mass Spectrometer	5975C MSD
GC inlet line temperature	280 °C
MS quad temperature	150 °C
Full scan range	m/z 35-450
Solvent delay	0.1 min

The pH of the samples was also measured using a bench top pH meter (Fisher Scientific, Singapore) against PBS reference and standards of pH 7 and 10. Subsequently, the collected ES that gave maximum bacterial inhibition compared to the control after statistical averaging of the experimental results, and the one that showed the presence of phenylacetaldehyde, was chosen for further antibacterial analysis and tests (discussed later, in Section 3.4.3).

3.4.2.6 Gaseous excreta/metabolite (gEM) collection

gEM was collected in a specialized setup shown in Figure 3.17. Late second-instar maggots were placed in a petri-dish with sheep blood agar and sealed airtight (petri-dish B). The plate was connected via silicone tubing to another petri-dish (A) with overnight bacterial cultures of MSSA or *E. coli* streaked on respective agar mediums. The maggot plate was also connected through filters (0.2 μm pore size) for fresh air replenishment. A negative pressure maintained at the bacterial petri-dish made the air flow into it for its action. This setup was maintained for 24 hours at ambient temperature. A control was setup for comparison, by replacing petri-dish B with a sterile sheep blood agar plate without maggots.

Figure 3.17: Setup to collect gEM of maggots.

For analysis of the gaseous metabolites of the maggots, petri-dish A was replaced with a syringe to collect the gases from the maggot plate. After they were allowed to incubate for 24 hours, gases were collected in the syringe and subsequently used for GC-MS analysis of the gaseous mixture obtained. The analysis was carried out similarly on the gas

chromatograph and the mass spectrometer. The parameters set on both the equipments for the measurements are summarized in Table 3.5 a and b.

Table 3.5 (a): Gas Chromatograph control parameters.

Gas Chromatograph	Agilent 7890A		
Analytical Column	HP-5MS (30m × 0.25 mm × 0.25 μm)		
Injection Port Type	Manual Split/Splitless		
Injector Temperature	315 °C		
Pressure	11.4 psi		
Injector Type	Pulsed Splitless		
Split Ratio	50:1		
Split Flow	50 mL/min		
Gas saver	20 mL/min after 2 min		
Total flow	54.5 mL/min		
Carrier Gas	Helium		
Oven program	Temperature	Hold Time	Rate
	35 °C	5 min	
	180 °C	2 min	5 °C/min
	315 °C	Hold	

Table 3.5 (b): Mass spectrometer control parameters.

Mass Spectrometer	5975C MSD
GC inlet line temperature	230 °C
MS quad temperature	150 °C
Full scan range	m/z 15-300
Solvent delay	0 min

3.4.3 ES and ciprofloxacin combinations investigation on CDSS

The ES-inoculum mixture of *S. aureus* (MSSA) prepared using Method E (Section 3.4.2.5) was injected into a sterile microchips from both inlets to fill each well up to 25 μL. Subsequently, ciprofloxacin at a working concentration equal to the MIC for MSSA was injected from one inlet and sterile PBS from the other, simultaneously to fill each well further with 25 μL, to a total volume of 50 μL. The resultant antibiotic concentration in the wells of the test microchip was then 100%, 80%, 60%, 40%, 20% and 0% of the MIC (starting concentration) of ciprofloxacin (Figure 3.18), with previously added maggot ES and bacterial suspension in PBS. The microchip was incubated at 37 °C for 24 hours in the

incubation chamber of the CDSS. OD was measured at 0, 8 and 24 hours after adding the ES, inoculum and antibiotic. The starting bacterial concentration was determined by performing plate counts of viable colonies at the start of the experiment and then after 24 hours. The OD at 600 nm (OD_{600}) was plotted against time to seek a combination of the antibiotic and ES that show enhanced antibacterial activity compared when to ciprofloxacin was used alone.

Another microchip was inoculated with only PBS and bacteria without the ES, with same inoculum concentration. The antibiotic and PBS were similarly injected form the inlets of the chip simultaneously, so that each well had similar concentrations explained in Figure 3.18, without the ES. OD and plate counts were recorded at the same time intervals as for the previous experiment and results were compared. Plate counts at 24 hours were calculated as relative reduction of total viable bacteria number (CFU/mL) after incubation with drugs for 24 hours. The results were then compared to each other and with decreasing concentration of ciprofloxacin only. The growth control in this case was the well with 0% ciprofloxacin containing bacterial suspension in only PBS. Sterility control was separately prepared.

Figure 3.18: Well markings for maggot ES and ciprofloxacin combination experiment.

Well marked 2 contains Maggot ES with bacterial suspension obtained by method E in Section 3.4.2.5 (25 μ L) plus 100% ciprofloxacin or cipro (=MIC) (25 μ L). Total volume in each well was 50 μ L. Similarly, well 3 contains Maggot ES with bacterial suspension (25 μ L) and 80% ciprofloxacin (25 μ L), and so on. Finally well 7 contain Maggot ES with bacterial suspension and PBS up to a total volume of 50 μ L.

3.5 Summary

A system to investigate drug combination effects on cell behavior was designed, fabricated, implemented and tested. As a proof-of-concept, initial tests were performed with two broadly classified cell types, comprising of mammalian and bacterial cells. Case studies were performed with drug combinations on the CDSS. Subsequently, extensive drug and phytochemical/maggot ES combination investigations were performed on *S. aureus*, due to its readily detectable mutational behavior, that was studied under combination influence.

Phytochemical antibacterial substances were incorporated in the combinations to study their possible potentiating effects on *S. aureus* killing or inhibition. Consequently, their effect on mutation of *S. aureus*, that cause resistance to the antibiotic, ciprofloxacin was studied. This was verified by molecular analysis of the selected mutants, as a result of continuous drug exposure. Similar combination investigations were carried out with maggot ES extracts. Firstly, the ES extracted from maggots was screened for antimicrobial properties on *E. coli* and *S. aureus* as test organisms. Later, these extracts were combined with antibiotic, ciprofloxacin to study their effects on bacterial killing or inhibition.

4 RESULTS AND OBSERVATIONS

4.1 Experimental system

4.1.1 Microchip device

The design and fabrication of the microfluidic chip was carried out in three major steps,

- Layout design
- Contact photolithography (using positive and negative photoresist)
- Soft lithography

While the layout design and principles have been covered in the last chapter, the outcomes of the photolithography and soft lithography processes are outlined here. Positive photoresist AZ 9620 was used for micromachining silicon wafers by DRIE process and negative photoresist SU-8 was used to prepare the microchannel pattern on the silicon wafer (transparencies of the layouts for positive and negative photoresists are attached in Appendix C). The wafers obtained by both methods are shown in Figure 4.1 and 4.2.

It can be noted from Figures 4.1 and 4.2, that the SU-8 patterned wafers were much smoother and clean compared to the DRIE etched wafers shown in Figure 4.1. The roughness observed in the DRIE etched wafer was due to differential etch-rates experienced by the micro-patterned structures on the silicon wafer. The wells (4 mm diameter) and microchannels (200 μm width) had different dimensions due to which the etch-rates were different resulting in uneven etching at the microscopic level. Moreover, masters in SU-8 photoresist are considered more durable, when using for PDMS casting. It can be used indefinitely and failure may only occur from breakage of the wafer or resist

release from the wafer [89]. Therefore, all further replica molding procedures were carried out using SU-8 photoresist for preparation of the silicon masters.

After PDMS pouring, curing and peeling the final device was assembled by bonding the polymer mold to glass by exposing both surfaces to oxygen plasma in an RIE chamber. The PDMS mold and final devices are shown in Figure 4.3. The inlets were made by piercing a channel through the PDMS mold with 18 gauge needle and wells were made using 4 mm diameter cork borer.

All the channels had rectangular cross-section. The depth of the channels was measured using a profilometer as 150 μm for both deep reactive ion etched wafer and SU-8 photoresist patterned coated wafer. However, in some of the DRIE wafers the wells were of greater depth than the channels due to differential etch rates explained above. The wells had diameter of 4 mm with a volume of approximately 50 μL . As the final fluid yield was only limited by the well height (since the channel dimensions were fixed), fluid loss in channels could be reduced by increasing the material thickness and decreasing the channel height, both the parameters were process dependent and could be easily manipulated during the fabrication process. For the present case, the channel height was set to 150 μm and the PDMS thickness and the height of the wells was 4 mm. The device dimensions were 10 cm $\times$ 6 cm $\times$ 0.7 cm for Design I and II and 6 cm $\times$ 7.5 cm $\times$ 0.7 cm (width $\times$ length $\times$ height) for Design III.

Figure 4.1: Silicon masters prepared using positive photoresist and DRIE etching.

(a) DRIE process on silicon wafer with Design III patterned using positive photoresist AZ 9620, (b) DRIE etched silicon wafer with patterned Design III, (c) Design II and (d) Design I and PDMS molded on it. The impressions at the edge of the wafer were marks of the DRIE chamber plugs that held the wafer in place while etching. (e) SEM micrograph of the etched regions on the wafer with Design II. The DRIE etched surface was rough due to differential etch rates for the wells and channels of different dimensions, therefore not ideal for PDMS casting and molding.

Figure 4.2: Silicon wafers patterned with negative photoresist (SU-8) and development.

(a) Design I, (b) Design II and (c) Design III. Design II wafer shown in (b) was further coated with thin layer of poly-tetrafluoroethylene using the C_4F_8 gas plasma to facilitate PDMS peeling. (e) SEM micrographs showing SU-8 deposited and developed on silicon wafer. The top 3 micrographs are of Design II, the middle 3 are of Design I and Design I modified, and the bottom 2 are of channels of Design I modified. The surface and vertical side walls were smoother compared to those of the DRIE etched wafers, hence more suitable for PDMS casting and molding.

(a)

(b)

(c)

(d)

(e)

Figure 4.3: Final micro-device assemblies.

(a) Final micro-device assembly for Design I and Design I (modified). (b) Design III (left) and Design II (right), size compared to that of a standard 1 ml syringe. Device dimensions for Design I and I (modified) – 10 cm × 6 cm × 0.7 cm, Design II – 10 cm × 6 cm × 0.7 cm and for Design III – 6 cm × 7.5 cm × 0.7 cm (width × length × height). (c), (d), and (e) Channels and wells in PDMS for Design I, Design I (modified) and Design II, respectively.

4.1.2 Microchip calibration tests

The integrity of the microchip was confirmed by performing tests using dye solutions (aqueous tryphan blue, aqueous food color - blue and yellow). Colored dyes were also dissolved in ethanol and acetic acid which were injected from the inlets of the microchips to visualize flow through the device and consequent mixing in the wells. Spectral signatures and microscopic mixing of the fluids were recorded from each well over time to optimize the flow rates of the fluids with different viscosities other than water.

The initial studies carried out using the three microchip designs were compared to the manual controls prepared by pipetting the desired dye concentrations. The graphs plotted were those of OD or absorbance (at peak wavelengths) versus dye concentration obtained from the microchip wells and from control well plates. The data was compared using statistical tools suggesting the microchip(s) that exactly fit or approximated the control values. Two statistical tools were used to compare the results, R^2 values (or coefficient of determination) and area under curve (AUC). R^2 was used to determine the proportion of variability in a data set whilst AUC was used to establish coherence with the control (i.e. the smaller the difference of AUC between test and the control graph, the more coherent the results). In statistics, R^2 is defined as the proportion of variability in a data set that is accounted for by a statistical model; in this case, linear regression was used to model absorbance values versus concentration of dye. Variability is defined as the sum of squares of errors or deviation from the model used to relate the data set. The position and slope of the linear regression line was calculated, the R^2 value would then give information about how well the trend line approximated the actual data points [210].

The spectrum of tryphan blue dye when used in 100% prepared concentration peaked at 590 nm wavelength. It was diluted with DI water to concentrations of 80, 60, 50, 40, 20 and 0% tryphan blue (v/v). The absorbance spectrum for each concentration of control and those for the microchip wells were plotted and grouped for each set - control and three

microchips. The absorbance values at the peak wavelength were plotted against the concentration of the dye for all sets to compare results. The experimental process was repeated five times for each set to get statistical average and standard deviation. The absorbance spectrum graph for microchip Design II is shown in Figure 4.4, using trypan blue dye and DI water as the diluent. The reference signal for all measurement was the absorbance of pure DI water, to minimize the effects of diffraction and refraction by the microchip material. The absorbance spectrum for control and other microchips is not shown here.

Figure 4.4: Absorbance spectrum with trypan blue dye on microchip Design II.

The graph shows the absorbance spectrum obtained from eight wells of microchip Design II. The peak was observed at 590 nm wavelength.

Graphical plots of absorbance values or OD versus concentration of trypan blue are shown in Figure 4.5. The regression lines were drawn using linear regression modeling in Sigma Plot[®] software. Table 4.1 gives the comparison of AUC for the trend lines and AUC for the curves shown in Figure 4.5 and the respective R^2 values for the trend lines.

Figure 4.5: Absorbance vs. concentration of trypan blue dye for control, Design I (D-I), Design II (D-II) and Design III (D-III) microchips.
The R^2 and AUC values are given in Table 4.1

Table 4.1: Comparison of area under curve (AUC) and regression coefficient of determination (R^2) for control, Design I, II and III microchips.

Experiment	AUC (curve) Sq. units	AUC (regression line) Sq. units	Absolute area difference (curve) Sq. units	Absolute area difference (line) Sq. units	R^2 value for regression line
Control	168.57	167.52	0.00	0.00	0.99
Design I	104.03	103.45	64.54	64.07	0.91
Design II	156.06	152.28	12.50	15.24	0.98
Design III	68.66	68.60	99.91	98.92	0.94

The AUC calculations shows that the difference in area of Design II with that of the control was the least (represented in bold in Table 4.1) followed by Design I and III respectively. In addition, the R^2 value for Design II, 0.98, suggested a close approximation of a linear relation between absorbance and concentration of dye and also presented the closest match with the control line among the three designs. The preliminary results with trypan blue dye suggested that Design II had superior performance compared to Designs

I and III in terms of linear relationship between absorbance and concentration and coherence with the control values. Due to poor absorbance and coherence results obtained for Design III microchip, it was not used for further testing. To improve the performance of Design I, it was re-designed to incorporate symmetry in the design and tested further with blue and yellow dyes. The results are discussed in the following paragraphs.

After the redesigning of microchip Design I, the tests were performed with aqueous colored solutions of yellow and blue food colors. Controls were performed in 96-well plates by pipetting color combinations in correct volumes that were the expected outcomes of the microchip wells. The absorbance spectrum of each well of the well-plate and microchips was recorded and plotted as OD versus wavelength in the range of 300-700 nm. The spectral signatures of control, microchip Design I (modified) and Design II are shown in Figure 4.6.

The corresponding plots of OD at peak wavelength verses concentration of blue dye for the control and the two microchips are shown in Figure 4.7. It represents OD or absorbance value of each well at 630 nm wavelength at varying blue concentrations (0 to 100%). Similar statistical tools were used to determine the accuracy of the mixtures produced by the microchips. The AUC and R^2 values are tabulated in Table 4.2.

Figure 4.6: Full spectra of blue and yellow color combinations for control and microchips.

The spectra were recorded from the wells of 96-well plates prepared manually, from microchips with Design I (modified) and Design II, respectively.

Figure 4.7: Absorbance vs. concentration of blue dye for control, Design I (modified) and Design II microchips.

The R^2 and AUC values are given in Table 4.2

Table 4.2: Comparison of area under curve (AUC) and regression coefficient of determination (R^2) for control, Design I (modified) and Design II microchips.

Experiment	AUC (curve) Sq. units	AUC (regression line) Sq. units	Absolute area difference (curve) Sq. units	Absolute area difference (line) Sq. units	R^2 value for regression line
Control	97.86	97.65	0.00	0.00	1.00
Design I (modified)	99.80	98.96	-1.94	-1.30	0.97
Design II	71.33	72.83	26.53	24.82	0.98

The AUC difference between control and the modified Design I was negligible (Table 4.2) as compared to the difference with Design II. This reflects the coherence of the microchip experimental results with those of the control. The performance of microchip Design I after incorporating symmetry had greatly improved, as seen from the significantly reduced AUC difference and increase in R^2 value, suggesting both coherence with the expected trend and linear increase in OD with increase in dye concentration. However, the R^2 value for Design II was higher than that of Design I, similar to the previous results with trypan blue dye, suggesting linear relationship between OD and dye concentration.

4.1.3 Microchip flow visualization studies

The flow visualization studies were performed on microchip Design I (modified) and Design II. Colored solutions dissolved in liquids of different viscosities were injected from both inlets of the microchips, yellow DI water being constant for all experiments. The frames were captured for the first second at intervals of 200 ms after the start of simultaneous pumping of fluid from both syringe pumps. Frames were also captured after 10 s and 1 min. The mixing efficiency was based on visual mixing of yellow and blue color fluids seen at 10 s after pumping, compared to the desired colors captured from control wells of 96-well plates. This was because pictures taken at a later time resulted in similar results for all flow rates. This was confirmed by visualizing the wells after 1 min, all of whose visual perceptions were satisfactorily comparable to the control (Table 4.3). The images of the wells of both microchips are tabulated in Appendix D (Tables D-1 to D-6).

From the tabulated pictures, it can be observed that after 1 min of injecting fluids into the microchip at all flow rates applied (2 to 16 mL/min), the fluids mixed completely (absence of any separate color vortices) and corresponded nearly to the expected OD values (see Table 4.3). Therefore, it can be suggested that at flow rates greater than 2 mL/min, the time required for the fluids to mix in the microchip wells is up to 1 minute. In the case of fluids with viscosities higher than that of water, higher flow rates may be applied. In the present study, maximum flow rate applied was 16 mL/min, which could bring about desired mixing in up to 10 s for all fluids used in the study. The desired mixing was visually compared to wells imaged after 1 min and control wells (of the 96-well plate) so that the OD of the uniformed mixture could be taken.

The Reynolds number was also calculated for the microchips, to establish flow properties in the microchannels. For water as the fluid with density 997 kg/m^3 at 20°C (ρ), dynamic viscosity $1 \text{ mPa}\cdot\text{s}$ (η), characteristic dimension of the microchannel $200 \text{ }\mu\text{m}$ (channel

width, l) and velocity of the fluid in the channels as 2.9-3 cm/s (u), the Re was calculated using the formula in Equation 2.1 (Chapter 2 – Literature Review),

$$\text{Re} = \rho \frac{ul}{\eta} \quad (2.1)$$

The Re calculated was in the range 0.0578-0.0599, that falls well in the range of creeping or laminar flow (Section 2.2.2.1 – Reynolds number and laminar flow). The fluid velocity in the microchannels was determined by videotaping the flow from the start of pumping to the time the fluid reached the wells.

Based on the results, across different flow rates and fluid viscosities used to determine mixing efficiency, higher flow rates of 16 min/mL (limited by the diameter of a 20 mL syringe) from both pumps was required for adequate mixing of fluids in the wells of the microchip. As per the design of the microchips, the two fluids entered from the inlets with pressure relating to the flow rate set on the programmable pump, and entered the respective wells from both sides into a resultant mixture. Fluids entering at higher flow rates and hence, higher pressure were observed to mix in 10 s or less post pumping. At all the flow rates used in the experiment, every well gave uniform mixture after 1 min of pumping, as seen from the tabulated results (Appendix D). In case of fluids with higher viscosity than water, setting a higher flow rate than water did not improve or enhance the process of mixing compared to 16-16 mL/min set on both syringe pumps.

On the other hand, based on the results of the statistical analysis of spectrometric recordings, it was concluded that the microchip with Design II based on symmetry was the preferred choice for further experiments of investigating combinatory drug effects on cells. This microchip enabled the integration of various steps resulting in reduced manpower, time and reagents required compared to conventional mixing techniques. All further experiments with cells and their cultures were performed with microchip Design II, unless mentioned.

Table 4.3: Images of yellow-blue combinations taken from the 96-well control experiment. The OD values at peak wavelength (nm) of the control are compared with those of microchip Design I and II.

Concentration (%) Yellow-Blue	Control Image	Control OD Peak wavelength (nm) / Absorbance	Design I OD Peak wavelength (nm) / Absorbance	Design II OD Peak wavelength (nm) / Absorbance
100 - 0		430 / 0.6610	430 / 0.7568	430 / 0.6502
80 - 20		430 / 0.6060	430 / 0.5552	430 / 0.6200
60 - 40		630 / 0.8090	630 / 0.6830	430 / 0.5034
50 - 50		630 / 0.9720	630 / 1.09124	-
40 - 60		630 / 1.1070	630 / 1.3174	630 / 0.8184
20 - 80		630 / 1.5470	630 / 1.5656	630 / 1.2642
0 - 100		630 / 1.9150	630 / 1.7430	630 / 1.5512

4.1.4 Initial tests on CDSS

Initial experiments carried out on CDSS were to examine long-term cell growth in the microchip wells and biocompatibility of the microchip material to sustain living cells. Cells from two broad categories were selected to demonstrate the system capabilities. Bacterial *E. coli* (MC1061) and *S. aureus* (ATCC 29213) were selected from the suspension cell category. Mammalian neuroblastoma and intestinal smooth muscle cells were selected from the adhesion cell category. The cells were inoculated in the wells of sterile microchips by injecting from the two inlets separately. Their growth was monitored over time.

For the bacteria, the OD was taken at various time intervals for 18-24 hours and related to standard microbiological colony counting by plating the dilutions on nutrient agar media. The results obtained were satisfactory in terms of biocompatibility and cell sustenance. Figure 4.8a shows the two growth curves for the *E. coli* binary fission behavior across the 18 hour long culture duration. These preliminary experiments for monitoring of cells produced growth curves very similar to those of standard bacterial growth curves obtained by sub-culturing bacteria into standard falcon tubes [211], suggesting a favorable environment for cell growth and successful monitoring on the system.

Similar growth curves were obtained for *S. aureus* 24 hours growth in nutrient media, injected into the well of the microchip and monitored in the same way as *E. coli*. The curves are shown in Figure 4.8b.

The results of the drug combination case studies that were extended for *E. coli* have been discussed in the following paragraphs of this section. The MIC, FIC and FICI for ampicillin and kanamycin were investigated and the averaged results are tabulated in Table 4.4. The FICI for the combination of the two antibiotics against *E. coli* fell in the

range for additivity [5] or positive interaction since it was less than unity. The combination and single antibiotics were also investigated on the CDSS.

Table 4.4: The MIC and FIC of the two antibiotics when used alone and in combination.

MIC when single drug was used ($\mu\text{g/mL}$)		MIC when drug used in combination ($\mu\text{g/mL}$)		FIC Index	Interpretation
Kanamycin	Ampicillin	Kanamycin	Ampicillin		
8	4	2	2	0.75	Additive

(a)

(b)

Figure 4.8: Bacterial growth curves obtained from the CDSS.

(a) Growth curves of *E. coli* and (b) growth curves of *S. aureus*. Graphs on left were obtained by counting viable CFU against time, and those on right were obtained by plotting optical density (OD) against time.

One of the prospective applications for the microchip was to perform rapid investigations of drug concoction on cell cultures. This was demonstrated with *E. coli* and antibiotics, ampicillin with DI water to produce increasing concentration of the drug in the wells. This was repeated with kanamycin with DI water and later with combination of ampicillin and kanamycin. The growth was monitored by recording OD and CFU/mL every 3 hours for

18 hours. The OD-time functions for the three sets of experiments mentioned above are given in Appendix E.

The CFU/mL versus time plots for ampicillin with DI water and kanamycin with DI water are shown in Figure 4.9. The arrow indicates the time at which injection of drug and DI water was carried out (i.e. 6 hours post inoculation). The plots provide growth curves of *E. coli* for each well (Figure 3.13, well 2 to 7, Chapter 3) over a period of 18 hours [212].

The time-kill plots were determined for the combination of 80% ampicillin and 20% kanamycin (v/v) (A-K-80-20%) that had OD value < 0.05 , comparable to the sterility control and 100% ampicillin or kanamycin concentrations as established from the OD results (Figure E-2, Appendix E). The time-kill plot for the combination in comparison with the individual antibiotic percentages is illustrated in Figure 4.10.

Images taken under the microscope at magnification 100, from the microchip wells while inside the incubation chamber of the CDSS are shown in Figure 4.11.

Figure 4.9: Plots of *E. coli* count vs. time for ampicillin and kanamycin experiments.

The percentages of the drugs, ampicillin (top) and kanamycin (bottom) are indicated on the respective graphs. The arrow along the abscissa indicates the time of injection of drug and DI water from the inlets of the microchip.

Figure 4.10: Time-kill plot for the 80-20% ampicillin-kanamycin combination on *E. coli*.
The plot compares the combination 80-20% (v/v) of ampicillin and kanamycin, with 80% ampicillin, 20% kanamycin and the growth control.

Figure 4.11: Pictures of the microchip wells taken directly under the microscope from the incubation chamber of the CDSS soon after inoculation.

(The images were taken at 400× magnification and scale bar at the bottom right of the images measures 50 μm)

The results of the mammalian cell culture in the wells of the microchip were compared to those of conventional culturing methods. Based on the visual microscopic comparison of the adhesion of the neuro-cells to the base of the culturing chamber, the results suggested satisfactory performance of the microchip material and culturing chamber, the wells. The

comparison of zero hour and 24 hour culture in the wells of the microchip and in the standard culture flasks are illustrated in Figure 4.12.

Figure 4.12: Mouse neuroblastoma cells (N2A) monitored in CDSS.

(a) Images of the neuro-cell cultures in the flask and microchip wells at '0' hour or immediately after inoculation of the two chambers. (b) Images of overnight cultures in the flasks and microchip wells taken after 24 hours of incubation. The cells in both culture chambers were adhered to the respective surfaces. (The images were taken at 400 $\times$ magnification and the scale bar at the bottom of the images measures 50 μm)

4.2 Control tests

The future experiments mainly focused on investigation of combination of drugs on *S. aureus* and *E. coli*. Baseline configuration to compare growth or killing of bacteria under external pressure of drugs was necessary for these experiments. The controls were setup with identical environmental conditions as those for the experimental tests and were repeated every time with an experiment for comparison. For the microchip related combination tests, the measured OD from the wells was interpreted as cell number and quantified into meaningful results. The general thumb rule that is widely accepted was applied for conversion; the relationship between cell number and OD is linear for OD values less than 0.4. The graphs in Figure 4.13 show the relationship between OD and Log_{10} of cell number for the three strains of bacteria used in the study. The measured OD values represent the ordinate axis (OD) and the corresponding cell number (CFU/mL) taken at the same time, indicated as Log_{10} conversion, represents the abscissa. Linear regression models were used to model a relation between the two for interpretation of OD values and cell number. The equations are displayed in the graph close to the respective regression line.

The time-kill control for comparisons of the assays performed on bacteria under influence of drug combinations, to determine bactericidal or inhibition activity, is shown in Figure 4.14 for the three bacterial strains.

Figure 4.13: OD versus Log₁₀ of cell number (CFU/mL) for *E. coli*, MSSA and MRSA.

The linear equation of the form of $y = mx + c$, represents the linear regression plot for each data set. The equation and coefficient of variance (R^2) of the predicted values are indicated in the respective color code.

Figure 4.14: The Time-kill control for *E. coli*, MSSA and MRSA.

4.3 Drug combination studies

The MIC, FIC and FICI values for ciprofloxacin, ofloxacin, phytochemicals and their combinations are given in Table 4.5 and 4.6, respectively.

Table 4.5: MIC, FIC and FICI of Ciprofloxacin (cipro) with quercetin (quer), PCA, rutin (rut) and EG.

Organism	MIC when single drug was used ($\mu\text{g/mL}$)		MIC when drug used in combination ($\mu\text{g/mL}$)		FICI
	Cipro	Phytochemical	Cipro	Phytochemical	
MSSA	1	Quer - 512	0.25	Quer - 256	0.75
	1	Quer - 512	0.5	Quer - 128	0.75
	1	PCA - 4096	0.25	PCA - 2048	0.75
	1	PCA - 4096	0.5	PCA - 1024	0.75
	1	Rut - 4096	0.5	Rut - 2048	1.00
	1	EG - 1024	0.25	EG - 512	0.75
MRSA	1	EG - 1024	0.5	EG - 256	0.75
	1	Quer - 512	0.25	Quer - 256	0.75
	1	Quer - 512	0.5	Quer - 128	0.75
	1	PCA - 4096	0.25	PCA - 2048	0.75
	1	Rut - 4096	0.5	Rut - 2048	1.00
	1	EG - 1024	0.25	EG - 512	0.75
	1	EG - 1024	0.5	EG - 256	0.75

The FIC indices of the two fluoroquinolones with the phytochemicals fell in the range of additivity and positive interaction ($\text{FICI} > 0.5$ and ≤ 1.0) and none of the combinations had shown antagonism in any of the repetitive microdilution assays. These studies were helpful in discerning qualitatively, the antibiotic-phytochemical combinations and their interactions.

The microchip experiments with the same combinations were performed using the MIC values from the tables as the initial concentration of the injecting drug; antibiotic from one inlet and phytochemical from the other. The OD-time functions over a period of 24 hours were obtained for each combination and for each strain, MSSA and MRSA. These are

given in Appendix F. The useful combinations based on averaged T_{24} results were determined and further tested for bactericidal or inhibitory activity against the two strains by performing the time-kill assays.

Table 4.6: MIC, FIC and FICI of Ofloxacin (oflox) with quercetin (quer), PCA, rutin (rut) and EG.

Organism	MIC when single drug was used ($\mu\text{g/mL}$)		MIC when drug used in combination ($\mu\text{g/mL}$)		FICI
	Oflox	Phytochemical	Oflox	Phytochemical	
MSSA	1	Quer - 512	0.5	Quer - 256	1.00
	1	Quer - 512	0.5	Quer - 128	0.75
	1	PCA - 4096	0.5	PCA - 2048	1.00
	1	Rut - 4096	0.5	Rut - 2048	1.00
	1	EG - 1024	0.5	EG - 512	1.00
MRSA	1	Quer - 512	0.25	Quer - 256	0.75
	1	Quer - 512	0.5	Quer - 128	0.75
	1	Quer - 512	0.5	Quer - 256	1.00
	1	PCA - 4096	0.5	PCA - 2048	1.00
	1	Rut - 4096	0.5	Rut - 2048	1.00
	1	EG - 1024	0.5	EG - 512	1.00
	1	EG - 1024	0.5	EG - 256	0.75

The time-kill plots of the combinations obtained from the microchip studies are given in the graphs in Figure 4.15 and 4.16 for ciprofloxacin and its combination with the phytochemicals against MSSA and MRSA, respectively. The time-kill plots for ofloxacin in combination with the phytochemicals are given in Figures 4.17 and 4.18, against MSSA and MRSA, respectively.

It can be observed from the time-kill plots in Figures 4.15 to 4.18, that for quercetin and EG, all four combinations (Ant20-Phy80, Ant40-Phy60, Ant60-Phy40 and Ant80-Phy20) showed bactericidal or inhibitory activity against both MSSA and MRSA. For PCA most selected combinations were bacteriostatic, with Cip/Of120-PCA80 - a combination of ciprofloxacin (20%) with PCA (80%) v/v, being bactericidal for both MSSA and MRSA. Rutin, on the other hand, was only bactericidal for MSSA in the Cip20-Rut80 combination

and bacteriostatic for Cip80-Rut20 combination. In combination with ofloxacin, even though the FICI determined was suggestive of additivity, the microchip studies did not yield any combinations to test further for antibacterial activity.

Figure 4.16: Time kill plots of ciprofloxacin with a phytochemicals in working combinations on MRSA.

From top left, ciprofloxacin with quercetin (Starting concentration = 1 and 512 $\mu\text{g/mL}$ for ciprofloxacin and quercetin, respectively), top right, ciprofloxacin with protocatechuic acid (4096 $\mu\text{g/mL}$), bottom left, ciprofloxacin with rutin (4096 $\mu\text{g/mL}$) and bottom right, ciprofloxacin with ethyl gallate (1024 $\mu\text{g/mL}$). The legends give the percentage of drug/phytochemical in combination or alone.

Figure 4.17: Time kill plots of ofloxacin with a phytochemicals in working combinations on MSSA.

Ofloxacin with quercetin (top) (Starting concentration = 1 and 512 $\mu\text{g/mL}$ for ofloxacin and quercetin, respectively), ofloxacin with protocatechuic acid (mid) (4096 $\mu\text{g/mL}$) and ofloxacin with ethyl gallate (bottom) (1024 $\mu\text{g/mL}$). The legends give the percentage of drug/phytochemical in combination or alone.

Figure 4.18: Time kill plots of ofloxacin with a phytochemicals in working combinations on MRSA.

Ofloxacin with quercetin (top) (Starting concentration = 1 and 512 $\mu\text{g/mL}$ for ofloxacin and quercetin, respectively), ofloxacin with protocatechuic acid (mid) (4096 $\mu\text{g/mL}$) and ofloxacin with ethyl gallate (bottom) (1024 $\mu\text{g/mL}$). The legends give the percentage of drug/phytochemical in combination or alone.

After careful scrutiny of the effective combinations of antibiotic ciprofloxacin with phytochemicals, MSSA was exposed to these combinations for 15 days (360 hours). The T₂₄ samples were tested for ciprofloxacin susceptibility by running a disk diffusion test on each sample in duplicates. The zone diameters were correlated with those provided by the manufacturer of the antibiotic disks, which classified the test strain into - susceptible, intermediate or resistant. The averaged zone diameters are given in Figure 4.19 over a period of 360 hours (15 days).

Based on the disk diffusion tests results, it can be observed from Figure 4.19, that *S. aureus* exposed to ciprofloxacin at MIC or sub-MIC levels develop resistance to the drug after four days of exposure (96 hours). For lower concentrations of 50% of MIC and less, the resistance was detected on the second day. On the other hand, ciprofloxacin in combination with phytochemicals in specific percentage mixtures produced by the microchip, show low level or no resistance. Resistance to ciprofloxacin is mainly observed at higher concentration of ciprofloxacin and lower concentration of phytochemicals (for example, Ant80-Phy20). With rutin combinations and Ant80-Phy20 as an exception to have developed resistance at the end of 15 days, the phytochemicals were able to potentiate ciprofloxacin activity and suppress the occurrence of resistance, based on the disk diffusion results.

Figure 4.19: Graphical representation of the disk-diffusion tests carried on MSSA, drug and drug combination exposed strains.

The red lines parallel to the x-axis divide the zone diameters to susceptible, intermediate and resistant regions for the ciprofloxacin disks (concentration = 5 μg). The names of the strains are indicated in the legend of each graph as Ant%-Phy% (% - percentage of the drug present in a mixture v/v).

The results of the disk diffusion were further verified by performing population analysis profiling of the test samples after every 72 hours, at T_{72} , T_{144} , T_{216} and T_{360} to determine heterogeneity in the population after continuous antibiotic pressure. The PAP results of some of the ciprofloxacin sub-MIC exposed strains and ciprofloxacin-phytochemical combinations exposed strains are shown in Figure 4.20. (For complete PAP results of ciprofloxacin MIC, sub-MIC and combinations exposed strains, refer to Appendix G). The PAP studies of the samples performed at every 72 hours revealed the presence of subpopulations capable of growing on plates with $\geq 1 \mu\text{g/mL}$ of ciprofloxacin ($>$ ciprofloxacin MIC). These subpopulations were not seen in the T_0 PAP and for all the samples exposed to ciprofloxacin at MIC and sub-MIC levels. Several samples from the ciprofloxacin combination exposed group, including the ciprofloxacin-rutin combination (Cip20-Rut80 and Cip80-Rut20), the ciprofloxacin-quercetin combination (Cip80-Quer20) and the ciprofloxacin-EG combination (Cip20-EG80 and Cip80-EG20) showed growth on plates with $1 \mu\text{g/mL}$ ciprofloxacin in the PAP results, but no growth was observed on $2 \mu\text{g/mL}$ plate at T_{360} .

The AUC of each of the ciprofloxacin and combination exposed strain were compared with those of the controls of the particular set. For example, for the PAP at T_{72} , the control for all the strains (ciprofloxacin and combination exposed) was the PAP of MSSA ATCC strain at T_{72} . This PAP-AUC criterion for PAP analysis has been adopted from Walsh *et. al.* and Wootton *et. al.* [201, 202]. It has been extensively used to establish hetero-resistance to vancomycin in *S. aureus*. Based on AUC comparison of test PAP and that of parent ATCC PAP, an estimate of hetero-resistance to ciprofloxacin in *S. aureus* exposed to drugs and drug combinations, could be established. The PAP-AUC ratios that fell in the range of 0-1.2 indicated homogeneous population compared to ratios greater than 1.2, with no mutants selection at higher ciprofloxacin concentrations (1 and $2 \mu\text{g/mL}$). These were later verified by series of tests, like re-determination of MIC of specifically selected

mutants, mutation frequency calculations and molecular analysis (discussed later). The PAP-AUC comparison is given in Table 4.7.

Table 4.7: PAP-AUC comparison of stains exposed to ciprofloxacin and ciprofloxacin-phytochemical combinations for 360 hours, with those of respective controls.

	Strain ^a	PAP-AUC ratio at T ₇₂	PAP-AUC ratio at T ₁₄₄	PAP-AUC ratio at T ₂₁₆	PAP-AUC ratio at T ₃₆₀
Control	MSSA (ATCC 29213)	1.00	1.00	1.00	1.00
Ciprofloxacin MIC and sub-MIC concentrations	Cip100	3.75	8.55	7.14	10.08
	Cip80	4.51	8.56	8.42	10.79
	Cip60	5.06	9.92	6.49	14.92
	Cip40	1.54	3.59	3.14	6.59
	Cip20	0.94	0.62	1.82	2.85
With PCA	Cip20-PCA80	0.77	1.36	1.41	1.00
	Cip40-PCA60	0.89	1.38	1.17	1.87
	Cip80-PCA20	3.17	0 ^b	0 ^b	3.12
With Rutin	Cip20-Rut80	1.01	1.19	6.86	7.78
	Cip80-Rut20	3.92	7.13	1.00b	8.01
With Quercetin	Cip20-Quer80	0 ^b	0 ^b	0 ^b	0 ^b
	Cip40-Quer60	0 ^b	0 ^b	0 ^b	4.38
	Cip60-Quer40	0 ^b	3.94	0 ^b	2.66
	Cip80-Quer20	3.52	7.02	6.16	8.79
With EG	Cip20-EG80	0.93	7.13	0 ^b	7.99
	Cip40-EG60	0.88	3.28	2.57	3.84
	Cip60-EG40	1.51	3.38	3.39	3.36
	Cip80-EG20	3.73	7.77	6.87	8.12
^a The strain names are represented as drug, to which it was exposed to for 15 days, followed by the percentage of its MIC. The ratios highlighted in red fall in the range of 0-1.2 indicating coherence with control AUC, hence more homogeneous population and no or less ciprofloxacin resistance. ^b The '0' ratio indicate that there was no bacteria in the sample retrieved at that particular time interval from the respective well and therefore, a PAP could not be performed.					

From Table 4.7, it can be observed that the T₃₆₀ samples for all strains had developed mutant populations and therefore the PAP-AUC ratio were all greater than 2, indicating that the strains had developed varied degree of hetro-resistance to ciprofloxacin at the end of day 15. The mutants isolated from the higher ciprofloxacin concentration plates (1 or 2 µg/mL) were passaged seven times on drug-free agar before their MICs were determined.

The mutants hence isolated and stored, were further allowed to undergo a series of tests, including mutation frequency calculations and further selection of mutants at 2 and 4 × their respective MICs. The MICs of these mutants were also determined and mutants with high MICs were selected for molecular analysis (results discussed in the next section).

Figure 4.20: PAP results of selected ciprofloxacin exposed and ciprofloxacin-phytochemical exposed strains.
 (Combinations indicated as Cip%-Phy%, % - percentage of MIC of respective drug v/v).

4.4 Detection of mutation in *S. aureus*

Following the isolation of mutants from the PAP studies, they were passaged on drug-free agar seven times to assess the stability of the hetro-resistance developed to ciprofloxacin, based on PAP and disk diffusion results. Generally, mutants are capable of undergoing further mutations even when the antibiotic pressure is removed. Therefore, the stability of the resistance was necessary to assess by repeated incubation on drug-free agar for 24 hour-periods each followed by MIC determination. The mutation frequencies for the isolated strains were also evaluated on ciprofloxacin-drugged agar plates having 2 and 4 × MIC of the respective strain at the end of 360 hours. These are tabulated in Table 4.8 with their respective MICs.

Based on the mutation frequency evaluation, the *S. aureus* strains obtained at the end of 15 days after ciprofloxacin exposure, showed higher selection of mutants compared to those exposed to ciprofloxacin-phytochemical combinations. Mutant selection was observed at four times MIC concentration for the ones exposed only to ciprofloxacin. The MICs of the final strains increased up to 16 folds compared to the MIC of the parent strain, and also compared to the T₃₆₀ sample of the control strain. The mutation prevention concentration or MPC window for the control strain was determined in the range of 1-4 µg/mL of ciprofloxacin. However, as seen from the table, there were mutants selected from significantly higher concentration of ciprofloxacin (32-64 µg/mL), indicating the development of ciprofloxacin-resistant *S. aureus*. For the T₃₆₀ samples exposed to the combinations, the mutation frequency remained lower than the corresponding ciprofloxacin-only exposed strains ($\leq 10^{-6}$). In some cases, there were no mutants selected at two and four times MIC. The mutation frequency was hence less than 10^{-9} . The MICs of these strains was also maintained at ≤ 2 µg/mL, with Cip80-Quer/EG20 being the exceptions, perhaps due to high ciprofloxacin concentration.

Table 4.8: Mutation frequency of T₃₆₀ samples of *S. aureus* exposed to ciprofloxacin alone and ciprofloxacin in combination.

	Strain ^a	MIC of Ciprofloxacin (µg/mL)	Mutation Frequency	
			2× MIC	4× MIC
Control	MSSA (ATCC 29213)	1	4×10 ⁻⁷	< 10 ⁻⁹
Ciprofloxacin only				
Ciprofloxacin MIC and sub-MIC concentrations	Cip100	16	10 ⁻¹	10 ⁻⁶
	Cip80	8	10 ⁻¹	9×10 ⁻⁷
	Cip60	8	10 ⁻¹	9×10 ⁻⁷
	Cip40	8	10 ⁻¹	3×10 ⁻⁷
	Cip20	4	10 ⁻¹	5×10 ⁻⁷
Ciprofloxacin in combination				
With PCA	Cip20-PCA80	1	< 10 ⁻⁹	< 10 ⁻⁹
	Cip40-PCA60	1	< 10 ⁻⁹	< 10 ⁻⁹
	Cip80-PCA20	2	1.5×10 ⁻⁶	< 10 ⁻⁹
With Rutin	Cip20-Rut80	2	1.6×10 ⁻⁶	< 10 ⁻⁹
	Cip80-Rut20	2	2×10 ⁻⁶	< 10 ⁻⁹
With Quercetin	Cip20-Quer80	1	< 10 ⁻⁹	< 10 ⁻⁹
	Cip40-Quer60	2	< 10 ⁻⁹	< 10 ⁻⁹
	Cip60-Quer40	2	1.6×10 ⁻⁶	< 10 ⁻⁹
	Cip80-Quer20	4	3×10 ⁻⁶	< 10 ⁻⁹
With EG	Cip20-EG80	2	2.3×10 ⁻⁶	< 10 ⁻⁹
	Cip40-EG60	2	1.5×10 ⁻⁶	< 10 ⁻⁹
	Cip60-EG40	2	< 10 ⁻⁹	< 10 ⁻⁹
	Cip80-EG20	4	3×10 ⁻⁶	< 10 ⁻⁹
^a The strain names are represented as drug, to which it was exposed to for 15 days, followed by the percentage of its MIC				

The MICs of the isolates obtained from plates with respective MIC or twice MIC of ciprofloxacin are summarized in Table 4.9. Strains exposed to ciprofloxacin at MIC and sub-MIC (strains - Cip100 to Cip20) showed resistance to ciprofloxacin with MIC ≥ 32 µg/mL (as per CLSI guidelines, MIC ≥ 4 µg/mL indicated ciprofloxacin resistance). This resistance was further confirmed by molecular analysis of selected strains. The isolates were passaged on drug free agar at least seven times before the molecular studies were carried out.

Table 4.9: MIC of ciprofloxacin for mutants selected from *S. aureus* drug exposed strains

Strain	× MIC	MIC (µg/mL) of selected mutant strains from <i>S. aureus</i>
Control (MSSA ATCC 29213)	1	2
Cip100	2	64
Cip80	2	32
Cip60	2	32
Cip40	1	32
Cip20	1	8
Cip20PCA80	1	2
Cip40PCA60	1	2
Cip80PCA20	2	4
Cip20Rut80	2	4
Cip80Rut20	2	16
Cip20Quer80	-	1
Cip40Quer60	2	2
Cip60Quer40	2	2
Cip80Quer20	1	8
Cip20EG80	2	2
Cip40EG60	2	2
Cip60EG40	1	2
Cip80EG20	2	4

For MIC ≥ 8 µg/mL, genetic evaluation of the QRD region and *norA* promoter sections of the bacterial genome were performed. The suspected mutants selected were, Cip100, Cip80, Cip60, Cip40, Cip20, Cip80Rut20 and Cip80Quer20. The parent MSSA ATCC 29213, served as the reference. The conserved sequences of DNA gyrase, DNA topoisomerase IV and *norA* genes of *S. aureus* were extracted from the public database (GenBank) as additional reference.

The QRD regions and *norA* gene sections of the suspected mutants were sequenced and aligned with the parent MSSA ATCC 29213 and *S. aureus* sequence from the published database. The alignment revealed that the QRDR and *norA* genes were indeed highly

conserved regions. The nucleotide and protein alignment showed unaltered *grlA*, *gyrA*, *gyrB* and *norA* genes. However, a single nucleotide change was found in the coding sequence of the *grlB* gene for all mutants exposed continuously to ciprofloxacin. Figure 4.21a shows the Adenine nucleic acid at sequence number 1415 of the coding sequence of GenBank and MSSA ATCC, was substituted by Thymine for the suspected mutants, except in Cip80Quer20 and Cip80Rut20, where no change was observed. As a result, at codon 470, Asparagine was substituted by Isoleucine (see Figure 4.21 b) [213]. This mutation was similar to the one found by Stutandar *et. al.*, where they had exposed the parent *S. aureus* strain (MSSA ATCC) to 50% MIC (0.5 µg/mL) of ciprofloxacin for seven days [193, 194]. Interestingly, there was no genetic variation found in isolates (MIC ≥ 8 µg/mL) exposed to ciprofloxacin combination with the phytochemicals (quercetin and rutin).

(a)

Figure 4.21: Multiple sequence alignment for mutants, parent strain MSSA ATCC and database derived sequence.

(a) Shows the nucleotide alignment for a region of *grlB* gene for mutants and reference coding sequences. A point substitution was observed in strains Cip100, Cip80, Cip60, Cip40 and Cip20 in the 1415 nucleic acid (A → T) [213]. (b) The corresponding protein sequence alignment for Gr1B protein, that showed mutation in the 470 codon, where Asparagine was substituted by Isoleucine for the mutants [213].

4.5 Maggot extracts combination studies

In the present study maggot-clarified extract, excretions/secretions (ES) and gaseous excreta/metabolites (gEM) using larvae of *L. cuprina* blowfly were investigated. Several methods were employed to extract the metabolites from late second/early third-instar maggots, some based on previously applied successful methods and others based on suggested antimicrobial and disinfection actions of maggots. These extracts were screened against *Staphylococcus aureus* (MSSA ATCC 29213) and *Escherichia coli* (MC 1061) bacterial strains. Based on the results, the ES and gEM demonstrated muted bacterial growth. Although the killing effects of these compounds may not be completely bactericidal as compared to synthetic antibiotics, an investigation to understand its effective dose and collection/extraction methods are important for further detailed *in-vitro* and *in-vivo* analysis.

The extracts obtained were also screened in combination with ciprofloxacin to study their effects on bacterial growth and inhibition. These studies were also performed on the CDSS. In combination with ciprofloxacin at sub-inhibitory concentrations, certain combinations revealed anti-staphylococcal activity, with bacterial reduction of up to 50% from the starting inoculums concentration. The results are discussed in the following sections.

4.5.1 Excretions/secretions/gaseous metabolites antibacterial activity

The control graphs for MSSA and *E. coli* were same as illustrated in Figure 4.13. The graphs represent OD against logarithm of cell number in a bacterial suspension (CFU/mL) in the respective broth media incubated at 37 °C for 24 hours. These control experiments were also used as reference for the tests performed with maggot ES and bacteria.

The GC-MS analysis of the maggot ES obtained by Method A, C, D and E showed the presence of phenylacetaldehyde only in the ES extracted by Method E. Quantitative analysis established an average of 84.5 mL of phenylacetaldehyde in one liter of ES extracted in PBS base.

The subsequent bacterial screening tests carried out for ES prepared by various methods are summarized in Table 4.10. However, according to the standard MIC test assays, the starting concentration of the drug, in this case the ES, must be diluted 2-folds making the original concentration lower. The tests performed in 96-well plates showed no observed antibacterial activity, as all the wells were turbid after overnight incubation (see Figure 4.22). This indicated that there was no significant antibacterial activity observed in the diluted ES forms for most of the methods. For the Methods B and B-modified, where live maggots were used in bacterial broth, the results showed increased bacterial growth after 24 hours. The starting bacterial inoculum concentration for MSSA was 2×10^7 CFU/mL and for *E. coli* it was 1.5×10^6 CFU/mL. The bacterial concentration increased more than

1,000,000-folds from the initial concentration with live maggots or its body fluid extracts. This supported the work reported by Cazander and co-workers, where they used live maggots in liquid media with bacterial suspension [209]. Similarly for Methods C and D with overnight incubation of maggots with or without nutrient for maggots, the bacterial concentration increased after ES addition compared to control [160]. These screening tests corroborated with the results of the GC-MS analysis of the maggot ES.

Table 4.10: Optical Densities and Bacterial concentration calculated after 24 hours of incubation of bacteria under ES influence.

Method No.	Optical Density after 24 hours (at 600 nm)		Bacterial concentration after 24 hours (per mL)	
	MSSA	<i>E. Coli</i>	MSSA	<i>E. Coli</i>
Control	0.315	0.23	2.69E+16	3.55E+12
A	0.104	0.309	1.27E+09	9.12E+14
B	0.453	0.409	1.66E+21 ^a	1.05E+18 ^a
B (modified)	0.178	0.32	4.68E+11	2.04E+15
C	0.102	0.112	1.07E+09	8.71E+08
D	0.099	0.25	8.51E+08	1.45E+13
E	0.065	0.115	5.62E+07	1.10E+09
^a The control graph for OD vs. Log of CFU/mL was valid for OD values upto 0.4, the actual viable cell count for MSSA was 1.8E+15 and 9.4E+9 for <i>E. coli</i> in method B.				

However, the original ES obtained by Method E was able to show bacterial reduction of approximately 30% from the starting bacterial concentration in case of MSSA (Figure 4.22). The percentage reduction was calculated as relative Log₁₀ reduction from the starting bacterial inoculum concentration. For this method the initial bacterial concentration of MSSA was 3×10¹⁰ and that for *E. coli* it was 1.5×10⁹ CFU/mL. The time-kill plot for MSSA revealed greater than 3 Log₁₀ decrease in bacterial number compared to the control (Figure 4.23), indicating significant bacterial growth inhibition. With *E. coli*, the results were consistent with the above methods, with no significant bacterial reduction.

Figure 4.22: MIC assay for maggot ES extracted by various methods.

The wells show no visible bacterial growth inhibition and hence MIC could not be determined with the concentrations obtained.

Figure 4.23: Time-kill plot for MSSA exposed to maggot ES and control (without ES).

The pH of the ES obtained by Method E had an average value of 8.7 and that with meat was 6.75. This clearly indicated that addition of meat made the pH of the ES in the acidic range but close to optimum for bacterial growth. In alkali pH value of 8.7, the bacterial growth would be stunted, which was also observed in the antibacterial screening results.

The pH of ES obtained by other methods (A, C and D) fell in the optimum range of close to 6.8-7.

The gEM collected as explained in Section 3.4.2.6 was tested using GC-MS analysis. The components detected in the gaseous mixture with their retention times are summarized in Table 4.11.

The agar plates streaked with gram-positive and gram-negative bacterial strains and directly exposed to gEM in its immediate environment clearly demonstrated stunted growth with 250 maggots in the maggot plate as illustrated in Figure 4.24 [160].

Table 4.11: Gas chromatography-mass spectrometry result of gaseous excreta/metabolites collected by incubating maggots for 24 hours [160].

Classification	Retention time (min)	Compound Detected
Aldehyde	8.95	Heptanal
	12.77	Octanal
	16.16	Nonanal
	19.25	Decanal
	22.11	Undecanal
	24.78	Dodecanal
	27.30	Tetradecanal
	31.95	Hexadecanal
Aliphatic Ester	34.09	Octadecanal
	14.40	2(3H)-Furanone, 5-ethylidihydro-
	23.45	2(3H)-Furanone, dihydro-5-pentyl-
	26.15	2(3H)-Furanone, 5-hexyldihydro-
	28.68	2(3H)-Furanone, 5-heptyldihydro-
	17.36	Pentanoic acid, 2-propenyl ester
Ether	33.60	Butyric acid, 4-pentadecyl ester
	29.69	Oxirane, tetradecyl-
	15.05	Tetrahydrofuran, 2-propyl- \$ 2-Pro
Ketone	29.07	Furan, 2-butyltetrahydro-
	15.73	2-Nonanone
	18.83	2-Decanone
	20.60	2h-pyran-2-one, tetrahydro-6-propyl
	26.91	2-Tridecanone
	21.69	2-Undecanone
	24.37	2-Dodecanone
	29.30	2-Tetradecanone
Phenol & Derivatives	30.97	4-Octen-3-one
	31.57	2-Pentadecanone
Siloxane	12.02	Phenol
	27.08	Butylated Hydroxytoluene
Alcohol	12.32	Cyclotetrasiloxane, octamethyl-
	21.26	2-Hexen-4-ol, 5-methyl-

Figure 4.24: MSSA (top) and *E. coli* (bottom) exposed to maggot gEM.

(a) MSSA/*E. coli* control for the gEM experiment. There were no maggots used in this experiment and the air was allowed to flow through the setup. Pictures b-d show plates exposed to 100, 200 and 250 maggots, respectively. There is stunted growth seen in plates in both cases when 250 maggots were placed in the maggot plate in the gas collection setup.

4.5.2 Combination of ES with ciprofloxacin

Similar combination studies were carried out with maggot ES extracted by Method E explained in Section 3.4.2.5, in combination with ciprofloxacin. Maggot ES extracted by Method E had shown maximum bacterial growth inhibition compared to other methods (see previous section). The microchip was inoculated with the maggot ES followed by ciprofloxacin addition, data on bacterial growth was recorded at 0, 8 and 24 hours after the bacterial suspension was exposed to drug and drug combinations.

Figure 4.25: OD readings directly taken from the microchip wells.

OD vs. time when only ciprofloxacin and PBS were injected from the two inlets of the micro-device (top). OD vs. time when ES with bacterial suspension was injected from both inlets, followed by ciprofloxacin and PBS from respective inlets (bottom).

The graphs in Figure 4.25 illustrate the results of OD versus time functions in different wells. From the graphs, it can be seen that with ciprofloxacin at MIC and sub-MIC concentrations, there is significant bacterial growth at all concentrations after 24 hours. The only exception was observed at 100% (= MIC) concentration, where bactericidal effect was observed. Whereas, with maggot ES and ciprofloxacin combinations, though no significant growth inhibition was observed in Well 7, (100% ES concentration), stunted growth was observed in ES with 80 and 60% ciprofloxacin. Comparing with 60% ciprofloxacin only, it can be deduced that the antimicrobial activity in the maggot ES partially potentiated the activity of ciprofloxacin and sub-MIC levels [160].

The microchip results were verified by plating the 24 hour sample from all the wells onto IS agar plates to determine the bacterial concentration (CFU/mL). The percentage reduction was calculated as relative Log_{10} reduction from the starting bacterial inoculum concentration, in this case, was 3×10^{10} (OD ~ 0.12). The percentage reduction of bacterial number was plotted for ciprofloxacin with and without maggot ES, obtained from the microchip (see Figure 4.26).

Stunted bacterial growth can be observed when maggot ES was used alone as compared to 100% ciprofloxacin (Figure 4.26), where bactericidal effect was observed. However, in combination with ciprofloxacin (at 80% and 60% MIC) the maggot ES showed significant reduction, up to 50% from the starting inoculum. When compared with corresponding ciprofloxacin concentrations, the reduction is less than 5% and in some cases there was no reduction observed.

Figure 4.26: Percentage reduction of bacterial count after 24 hours of exposure.

The reduction percentage was calculated relative to the initial bacterial concentration at '0 hour', after 24 hours of exposure of MSSA to ciprofloxacin at MIC, sub-MIC concentration and maggot ES in combination with ciprofloxacin at various concentrations.

5 DISCUSSION

The present investigation aimed to create a system for simultaneous drug-drug analysis on cell outcomes. The cell culturing platform coupled with microchannels for separating and mixing drugs was designed, calibrated and tested. The fabrication techniques to manufacture the microchip were optimized for rapid prototyping of the device using SU-8 master and PDMS mold. The initial trials on the system using adhesion and suspension cell types along with drug combination case-studies were useful to establish protocols for different cell types. The later part of the project was narrowed down to investigations and detailed analysis of drug-phytochemical combination effects on gram-positive *S. aureus*; suspension type bacterial cells. The selected phytochemicals were able to potentiate the selected antibiotic at different levels. In some cases, it even assisted in delaying the development of resistance, which otherwise was observed after 72 hours of antibiotic exposure at near MIC levels. The same antibiotic was investigated in combination with maggot extracts, which by itself had little antibacterial activity. It was also able to potentiate the activity of the antibiotic at near MIC levels. This chapter would encompass the explanation and rationalization of the results obtained from the investigations performed in this project, their deviation from expected results or controls (if any) and comparison with similar published work (if any).

5.1 Microchip experiments

The three microchips calibrated and tested using dye solutions were compared using statistical tools. The measurement of performance for each microchip was based on the concentration mixtures obtained in their respective wells. These mixtures were measured by recording their spectral signatures directly from the wells. Based on the specific concoction of the two input fluids in each well, the intensity and absorbance from the

spectrum was compared to those of the control mixtures obtained by manual pipetting and mixing. Based on the results (Section 4.1.2), microchip Design III was discarded for further tests due to poor resultant mixtures and issues of diffusion between wells. The initial Design I faced problems of unequal end volumes in the wells and bubble entrapment in the sharp turning (at 90° angles) microchannels and daughter channels. Whereas, Design II provided higher accuracy of serial dilution with higher coefficient of variance of linear regression model used to model concentration and absorbance, and greater coherence with control.

Design I was later modified to incorporate symmetry in its design similar to Design II and the sharp angled turns were replaced by curvaceous microchannels, keeping the basic design principle similar. This was due to erroneous results and experimental problems faced when the experiments were carried out with colored fluids for the testing and calibration of former Design I. The most frequently occurring problem was that of entrapment of air bubbles in the microchannels of the smaller sub-systems. This significantly depreciated the performance of the microchip. The entrapment of bubble(s) caused complete or partial blockage of the channel. As a result, the concentration in the following well in the sub-system was affected, which gave an erroneous value of absorbance during the spectrometric reading. This could have been partly due to the hydrophobicity of the channel material (PDMS) and was overcome by washing the channels with 70% ethanol frequently. A credible reason for bubble entrapment was also due to the presence of sharp corners at the turns of the microchannels (see Figure 5.1 and 3.2, Chapter 3). Studies show that sharp corners cause residual liquid or bubble entrapment in the microchannels and cause the flow to become almost stationary at that point. Chen and co-workers [214], in their study, deduced this phenomenon by using two different channel geometries, one with sharp corners and other with flat corners. They found that, though the sharp corners in the channels enhanced its mixing capabilities, the problem of bubble entrapment was unavoidable during the cleaning process. These

bubbles in turn obstructed the flow of liquid around the sharp corners and prevented continuous flow [214]. On the other hand, Rosaguti *et. al.* [215] showed that sharp corner geometry led to generation of recirculation regions at the corners that caused increased pressure drops at that region and beyond. In a similar study by Geyer *et. al.* in the following year [216], it was demonstrated using serpentine microchannel flow, that sharp turns led to flow separation in the inside corners of the bends that caused an undesirable effect, as at low Reynolds number the flow is expected to be of Hele-Shaw type with no flow separation regardless of the channel geometry.

Lee *et. al.* [217], suggested that the presence of bends that cause the undesirable effects are usually accepted and cannot be avoided in most of the microfluidic device designs due to space and technology constraints. In their study, they investigated that the flow rate and pressure drop through a microchannel with a single sharp turn at right angle changed significantly when compared with a smooth curved channel and a straight channel [217]. Therefore, to avoid bubble entrapment, flow separation and undesirable changes in pressure drop across the microchannels, Design I was modified to incorporate smooth bends (see Figure 5.1).

Figure 5.1: Comparison between the sharp corner geometry of Design I (left) and smooth curved channel geometry of the modified Design I (right).

The flow becomes even in the smooth geometry compared to flow via sharp corners.

The erroneous spectrometric absorbance readings from wells of former Design I were also attributed to the difference in the channel lengths of those connecting the inlet to the middle wells and those connecting the outer wells. This resulted in extra collection of fluid

at the middle three wells as compared to the outer wells, because the applied pressure at the inlet remained the same for the entire system and hence, the volume flow rate. Therefore, while the middle three wells filled up completely the outer wells were 3/4th filled. An increase in the volume and the length of the liquid column through which the incident light passed, changed the absorbance reading significantly. This is because the initial conditions no longer remained the same for all the wells. The importance of this condition is elucidated clearly by the Beer's law, given by,

$$A = ebc \quad (5.1)$$

where, A is absorbance (no units, since $A = \log_{10} P_0 / P$), P_0 is the radiant power of the entering beam of light into the solution, P is the radiant power of the transmitted beam, e is the molar absorptivity with units, $\text{L mol}^{-1} \text{cm}^{-1}$, b is the path length of the sample in cm and c is the concentration of the compound in solution, expressed in mol L^{-1} . e is kept constant since the concentration of the well remained the same for single absorbance reading. Hence, to obtain proportional relationship between absorbance and concentration of the dye, b must be kept constant, that is, the volume of fluid in each well and hence, the path length.

For Design I, the main channels that fed the sub-systems were different in length, and so the end volumes received by the outer wells was less than that received by the inner wells. For Design II, proportionality was governed by symmetry of the feed micro-channels. This was confirmed in the results when compared with the expected trend or the control line (Figure 4.5, Chapter 4). Finally, for Design III, the deviation in fluid volumes in the wells occurred due to variation in travelling distance for fluid as well as the diffusion between different wells with time. The latter occurred due to the large cross-section of the channels designed with aim to derive a simple microchip design. The lower absorbance value observed for the microchip with Design III (with a large AUC difference of over 98 sq. units, Table 4.1, Chapter 4) was mainly because of the small volumes involved in the

actual microchip than the volume used for the control experiment. For the control set, the total volume for each concentration mixture was 50 μL . While, the average volume in each well for Design I and II was 50 μL , that in wells of Design III was less than 35 μL . Again, according to Beer's law (see Equation 5.1), the absorbance (A) is directly proportional to the path length (b) keeping the other quantities constant. In the case of the microchips with Design III, the volumes were much lower and hence, there was an observed deviance from the reading of the control and from the expected trend. Design III was hence discarded for further tests and experiments.

The dye experiments were repeated with food color dyes (blue and yellow) on new batch of microchips with Design I (modified) and Design II. The results indicated more accurate mixing with high R^2 values for both designs. For Design I, the R^2 value was 0.97 and that for Design II it was 0.98. However, in this case, the AUC for Design II was greater than Design I, suggesting better coherence of the latter with the control. However, during the experiments, the problem of bubble entrapment still persisted in Design I (modified).

The microchip material, PDMS is naturally hydrophobic. To make the PDMS channels hydrophilic for longer periods, they must be washed with 70% ethanol after use and stored in ethanol bath when not in use. Generally, microchip with Design I had greater channel surface area in contact with incoming fluids due to more channels and hence, was susceptible to a higher probability of occurrence of bubbles. In microchip with Design II, there were comparatively less number of channels and low surface area in contact with the fluid and consequently, the occurrence of bubbles in the microchannels was less frequent. Bubbles encountered in channels of Design II were mainly in the sharp turns (Figure 5.2) which were seen at some areas of the microchip edges. But their instances were few and did not affect the performance of the microchip. It was overcome by washing the channels with 70% ethanol few times and storing the microchip in ethanol bath overnight from time to time.

In the modified Design I, however, entrapment of air bubbles in the microchannels of the sub-systems was observed frequently (Figure 5.3) and the channels were needed to be washed by ethanol more often to increase wettability of the microchannel network. This depreciated the performance of the microchip.

Figure 5.2: Sharp edges in microchip Design II.

(a) The picture of microchip Design II (close-up), the sharp edged are highlighted and enclosed in dotted box. (b) and (c) Entrapment of bubbles in the sharp edges of the microchannels. (The scale bar at bottom right of b and c measures 500 μm)

The entrapped bubbles completely (see Figure 5.3, top left) or partially (see Figure 5.3, top right) obstructed the flow of liquid in the channels of Design I. A bubble in any of the ten daughter channels of a sub-system affected the accuracy of the mixture obtained in the following well. In addition, the constant pressure applied by the programmable pumps forced more volumes of fluids into adjacent wells due to greater pressure from the blocked channel(s). Hence, the final volume of well with a preceding blocked channel(s) would

receive lower volume, depending on the number of channels blocked and the adjacent well would receive higher volume. This resulted in inconclusive data as there was insufficient amount of fluid mixing at the desired well. The bubbles were periodically removed by injecting 70% ethanol from the inlets followed by DI water to increase the microchannel wettability.

Figure 5.3: Bubbles entrapment in channels of microchip Design I (modified).

Note the complete and partial obstruction of channels with bubbles (top left and right, respectively), bubbles in the wells and large channels (bottom left and right, respectively). (The scale bars at bottom right of each image measures 500 μm)

The pressure drop and flow rates required to obtain maximum mixing efficiency were studied by using liquids of different viscosities and colored dyes. The investigations were carried out by flow visualization, achieved with the aid of microscopic digital camera imaging. The evaluative tool used for earlier experiments was the spectrometric data and absorbance value of the resultant mixed solution. Flow visualization results were based on observations of color, homogeneity and strength of the swirling movements in the experimental images after a stipulated time, post pumping. As flow visualization is a qualitative tool, it was necessary to set a fixed parameter in order to critically evaluate the mixing performance from the experimental raw data. The experiments involved mixing of colored dyes of yellow and blue to obtain a green hue. Since the mixing concentrations were not standardized, a control color spectrum was obtained to compare the final mixed

output of each well at a given flow rate. The resulting shade of green was then implicative of mixing performance. However, it was discovered under spectrometer testing, the wavelengths of the resulting green colors did not lie in the green spectrum range. Rather a decreasing blue peak and increasing yellow peak intensity was observed (Figure 5.4).

Figure 5.4: The use of blue gradient as quantitative analysis of color experiment results.

The top left graph shows the spectrum of each combination of blue and yellow in the control experiment. Increasing blue peak and corresponding decreasing yellow peak can be observed in the graph. The top right graph shows the blue and yellow gradient with respect to their respective percentage in the mixture. The bottom image shows the hue of green as a function of blue gradient in the control wells.

Therefore, in the event of comparison of color shades, the decreasing blue gradient was used to evaluate the experimental data with respect to the control. In Figure 5.4, the yellow peaks (top right graph, in black) were considerably small compared to the blue peaks (top right graph, in red). Additionally, the figure also highlights that the blue gradient was significantly steeper than the yellow gradient and was thus, useful to detect more accurately the variations across different concentrations. This explains why the blue gradient was selected over the yellow gradient for comparison of the green hue in the experimental wells (Figure 5.4, bottom image). All subsequent evaluations were made in comparison to the blue gradient.

Microscope images that were captured at high speed rates during the flow visualization studies have been tabulated in the previous chapter (Section 4.1.3, Chapter 4). The high speed camera was set to snap images every 200 ms for the first second that allowed the mixing action to be recorded. This was followed by snap shots of frames every 200 ms after 10 s of pumping and finally, one frame after 1 min. This was applied to all experiments for all wells and at all flow rates. Due to space constraints, only a few sets of these data were shown. The results can be explained by taking examples from the picture tables shown in Appendix D. One such example has been illustrated in Figure 5.5. Especially when flow rates were slow, for instance at 2 mL/min, the camera was able to produce an informative visual record of the mixing action. It was easy to track the arrival and subsequent mixing of the two fluids, with the microscope focused at the middle of a well.

Figure 5.5: Comparison of images taken during the flow visualization experiments.

Flow visualization experiments, five images taken successively within the first second (every 200 ms) after pumping at 2 mL/min from both inlet sides, depicting yellow water and blue ethanol/acetic acid mixing. (a) Design II - well 3 (60% yellow water-40% blue ethanol), (b) Design II - well 2 (80% yellow water-20% blue acetic acid) and (c) Design I - well 4 (40% yellow water-60% blue acetic acid).

Applying flow visualization to assess mixing efficiency, it is evident that the passive mixing strategy of utilizing high pumping pressure is capable of delivering high performance mixing in microchips Design I and Design II. There are numerous instances that can be seen from the tabulated images, at which the solution has mixed or settled

successfully into a homogeneous solution at high flow rates (16 mL/min). To corroborate this point, several images are highlighted in Figure 5.6 taken after 10 s of pumping fluids through both the inlets at 16 mL/min flow rate. The homogeneities of the solutions in those images demonstrate the high performance mixing that can be achieved at high flow rates.

Figure 5.6: Homogeneous solution obtained after 10 s of pumping.

(a) 10 s image of 80% Yellow Water – 20% Blue Ethanol at 16 mL/min, Microchip Design II (b) 10 s image of 50% Yellow Water – 50% Blue acetic acid at 16 mL/min, Microchip Design I (c) 10 s image of 80% Yellow Water – 20% Blue acetic acid at 16 mL/min, Microchip Design I and (d) 10 s image of 20% Yellow Water – 80% Blue acetic acid at 16 mL/min, Microchip Design II.

Referring to well no.1 of microchip Design I of water-ethanol combination (Table D-3, Appendix D), the strong swirling action in the images taken at one second of the pumping aids in the mixing process and the subsequent 10 s image show homogeneity of the mixture obtained. Images taken after one minute, as seen from the tables in Appendix D, show that most of the solutions had settled down homogeneously into the required shade. Hence, it can be interpreted from that, in most cases, one to nine seconds may not be an optimal mixing time frame; there may still be mixing occurring depending on the flow rates. Secondly, it appeared that the stabilizing time for all flow rates (low and high) was one minute, where all mixing seems to have been completed and all solutions were stable. Within one minute, all solutions would have achieved homogeneous status as seen in the images in Tables D-1 to D-6. Therefore, comparison of mixing performance across flow rates cannot be made on the one minute images, as all mixing had concluded and homogeneity was already achieved. As such, comparison was evaluated on the basis of the 10 s images.

In this passive mixing procedure, fluids must enter at high pressure to induce chaotic advection in the wells and due to its design, induce disturbances in the flow patterns, from microscale to macroscale. These abrupt disturbances in the flow path were able to boost mixing efficiency considerably as the increase in contact surface between two fluids would have resulted in rapid mixing [82]. On the other hand, if the fluids entered at low pressure, mixing would occur solely by diffusion and that would be a slow process. A diffusion-based mixing scenario would require more than 10 s to accomplish. According to *Poiseuille's Law* (Equation 2.6, Chapter 2, reiterated here) flow rates can be increased to attain higher pressure. The equation below shows the proportional relationship between pressure drop and flow rate.

$$Q = \frac{\pi R^4 \Delta p}{8 \eta l} \quad (5.2)$$

$$\rightarrow Q \propto \Delta p$$

where, Q is the volume rate of flow, Δp is the pressure drop over length l along a channel of radius R , and η being the fluid viscosity [57]. Therefore, higher flow rates result in greater pressure which in turn induces chaotic advection and greater turbulence to achieve optimal mixing performance. Consequently, a higher flow rate would be optimum for rapid mixing in the wells. An example from the results is quoted in the following paragraph.

As stated above, the comparison of mixing efficiency of each flow rate was evaluated on the basis of the 10 s images. Applying this, the 10 s row of images in Figure 5.7 is then able to corroborate the above observations by displaying swirling actions under 2 mL/min and a settled homogeneous solution under 8 and 16 mL/min.

Well No.		Flow Rate (ml/min)*			
		2 ml/min	8 ml/min	16 ml/min	
1 80W (yellow) – 20E (blue)	1s
	10s
	1min

Figure 5.7: Excerpt of Table D-3 (Appendix D) displaying mixing action across flow rates of 2 mL/min, 8 mL/min and 16 mL/min.

Since fluids of different viscosities were used to demonstrate the mixing efficiency, different flow rates were applied to different fluids. The standard viscosities of the fluids used in this experiment are tabulated in Table 5.1. The different viscosities of the selected fluids would mean that each fluid may arrive at the wells at separate timings. When this occurs, the fluids will be unable to mix as optimally as when both fluids make contact and mix upon impact. In response to this, different combinations of flow rates were employed during experimentation to determine if mixing could be improved. The two combinations used for the study were 5-10 mL/min and 10-16 mL/min. In these combination flow rates, lower flow rate was applied to the water (yellow) pump and higher flow rate was applied to the ethanol/acetic acid (blue) pump. However, experimental images suggested that using different combinations of flow rates may not have provided better mixing. In fact, the results of 16-16 mL/min appeared to be similar or better as compared to those of 5-10 mL/min and 10-16 mL/min (see Figure 5.8). One possible explanation could be that fluids like ethanol possesses higher viscosity but are also high in wettability and hence, able to travel through the microchannels faster than water even with higher viscosity.

Table 5.1: Viscosities of fluids used in the experiment

S. No.	Fluid	Viscosity (mPa-s)
1	DI water	1
2	Ethyl Alcohol	1.2
3	Acetic acid	1.23

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
1 80W (yellow) – 20E (blue)	1s
	10s
	1min

Figure 5.8: An example of water and ethanol mixing at a well. Excerpt of Table D-3 (Appendix D) displaying mixing action across flow rates of 16 mL/min, 5-10 mL/min and 10-16 mL/min

The miscibility of incoming fluids may have also played an important role in the rapid mixing process in the wells. Miscibility refers to a property of liquids to form a homogeneous solution in all proportions, in other words, to dissolve in each other. A higher miscibility or a greater dissolving ability would then translate to a direct improvement in mixing performance. In this project, ethanol and acetic acid were sufficiently miscible and mixed well with water. Both possess fine wettability properties with the polymer PDMS and thus, minimized the formation of bubbles in the microchannels, allowing mixing efficiency to be enhanced even at lower flow rates.

Figures 5.9 shows a series of 1s images of the water-ethanol, water-acetic acid and water-water combinations across low flow rate of 2 mL/min. It can be clearly seen that water with ethanol/acetic acid exhibits more effective mixing (stronger swirling patterns, Figure 5.9 a, b) due to higher miscibility as compared to the combination of water and water (weaker swirling patterns, Figure 5.9 c).

Figure 5.9: Comparison based on miscibility of mixing fluids.

First second images at flow rate of 2 mL/min of water with (a) ethanol, (b) acetic acid and (c) water. The miscibility of ethanol and acetic acid can be observed with its swirling action, it is faster compared to water-water mixture (seen after 1 s of pumping fluid).

5.2 Initial tests on the microchip and the CDSS

5.2.1 Biocompatibility and sustenance of cell growth

The preliminary studies performed with different cell types, tested the microchip along with the CDSS for successful growth, sustenance and monitoring of the inoculated cells. The microchip material, glass and PDMS, were adequately biocompatible with the cells chosen from the two categories. These materials have been previously used to fabricate microsystems for intended cell culturing and bioassays. The extensive use of the polymer PDMS in miniaturized devices that involve biological samples and assays have been reviewed extensively by Sia *et. al.* [218]. It covers the various advantages of the polymer over other materials, in being inexpensive, flexible, optically transparent, thermally and chemically stable. It is compatible with biological samples because of its permeability to gases, inert and nontoxic chemical structure, and impermeability to water. The critical advantage of this material over silicon and other polymers, is the ease by which it can be fabricated and incorporated to other systems and surfaces.

In the present study, the materials chosen for the fabrication of the microchip were based on its intended application of incorporating biological cells. Since biological samples were involved, PDMS was the preferred choice and glass was chosen as the base material. The cells that were tested on the microchip were bacterial (suspension type) and mammalian (adhesion type) cells. In a similar attempt to employ batch cultures of bacteria into a microchip, Chang *et. al.*, [121], successfully fabricated a hybrid PDMS/silicon device to sustain long-term cultures of bacteria in a single PDMS chamber (5.3 nL volume). Christen *et. al.* [120], had also reported a hybrid microchip for cell culture and incubation, with PDMS as the culturing platform (chamber volume, 12 μ L). Other microchips with multiple wells for cell culture have also been fabricated using PDMS, and have been discussed in Chapter 2, Section 2.2.4.

Although there are numerous other devices reported in literature for bacterial or mammalian *in vitro* cell culturing, the microchip fabricated in this project, was in addition, able to perform combinatory drug screening on the cells cultured in its wells. The microchip was not limited to a single culturing chamber, but several chambers connected by a microchannel network. It was capable of producing different drug or compound mixtures in its culturing wells. The volume of the chambers was 50 μ L each to sufficiently incorporate media and drugs for discernible detection of useful results in the form of drug combination effects. Moreover, due to its design, the microchip was capable of being fabricated by MEMS-based rapid prototyping and replica molding techniques, that uses single master to produce many PDMS replicas.

5.2.2 Case studies on *E. coli* with drug combinations

As case study, combination-drug investigations were performed on *E. coli* with a pair of β -lactam and aminoglycoside antibiotics, previously known to work in synergy (Section 2.1.2 – Antibacterial combination studies, Chapter 2). Beta lactams (e.g. ampicillin) kills bacteria by disrupting the cell wall while aminoglycosides (e.g. kanamycin) act through a

different mechanism inhibiting protein synthesis. As the bacterial cell wall was weakened by the action of ampicillin, kanamycin could easily permeate to cause inhibition of protein synthesis that subsequently led to cell death. The drugs chosen from the respective antibiotic families, ampicillin and kanamycin, were shown to have an FIC index of 0.75 and hence suggesting additivity and positive interaction. The combination was previously shown to have synergistic interaction due to their different targeted mechanism of actions, against both gram-positive and gram-negative bacteria [30, 31, 219-222]. The growing resistance to the penicillin type β -lactam antibiotics in the lactamase producing pathogens, led to the widespread use of this combination. However, in the recent past it has been advocated by many experts that the combination is more disadvantageous rather than advantageous. Paul *et. al.* [223], in their clinical findings for treatment of sepsis, emphasized that the combination of β -lactams and aminoglycosides should be avoided, as their results indicated no significant advantage over monotherapy with β -lactams alone. Their study was based on clinical trials on patients with septicemia, who were treated in different groups with combinations and with β -lactams alone. Later, Leibovici and Paul [224], also performed a study on patients with neutropenia and sepsis, with similar findings. Their additional findings emphasized increase in the rate of side effects by addition of aminoglycosides. It increased the risks of nephrotoxicity and other side effects relating to aminoglycosides.

In the case studies performed separately and in combinations, at various ampicillin and kanamycin concentrations on gram-negative *E. coli* on the CDSS, results had shown an optimal growth inhibition at a unique combination of the two drugs (80-20%, ampicillin and kanamycin, respectively). On the other hand, expected bacterial growth profiles were observed in the LB nutrient broth (illustrated in Section 4.1.4 and Figure 4.9) in the presence of varying concentrations of single drugs (from 0% to 100% of antibiotic MIC – wells 2 to 7). At higher drug concentrations (60% and 80%), *E. coli* growth was inhibited, indicated by the reduction of its density or OD (Appendix E) and CFU profiles (Figure 4.9,

Chapter 4) for the first few hours. But due to sub-MIC level, it was unable to inhibit the growth for long and a sharp increase in the bacterial density was observed after 24 hours. At lower drug concentrations ($\leq 20\%$), the inhibition of *E. coli* was not as noticeable as those with the higher drug concentrations in the early hours of incubation and the growth profile closely matched the growth control with no drug. For both the studies, the effect of the MIC level concentration for the two antibiotics (i.e., 100% ampicillin or kanamycin) was able to inhibit bacterial growth for 48 hours and closely followed the sterility controls, prepared separately with no bacterial inoculum. However, for the ampicillin graph at 40% drug concentration, an unexpected behavior of CFU profile was noted. A noticeable drop in CFU level was initially observed after the addition of the 40% ampicillin, which was later followed by a significant increase after the ninth hour before finally falling after 12 hours due to lack of nutrient supply. One possible likely explanation for this behavior would be that 40% ampicillin drug concentration would have resulted in the formation of mutant species resistant to the drug towards the ninth hour, which resulted in a resumption of the normal growth after 9 hours. Later, towards the twelfth hour and before the depletion of nutrient supply towards the eighteenth hour, a sharp decline in the *E. coli* growth, similar to higher drug concentration, was observed.

From the OD plots of ampicillin-kanamycin combination studies on *E. coli* (Appendix E) all the graphs were observed to be much lower than the control in general, with an exception of the 80-20% A-K, that represented 80% MIC of ampicillin in combination with 20% MIC of kanamycin (v/v). This combination, 80-20% A-K, was further investigated by performing the time-kill analysis and compared with single drugs at corresponding concentration and with the control. The time-kill plot achieved, showed greater than 3Log_{10} decrease from the most active drug component in the combination (in this case, was ampicillin) after 24 hours, but negligible decrease from the initial concentration. This suggested that the combination selected from the screening experiments performed on the CDSS had a bacteriostatic synergistic effect at 80-20% (v/v)

combination. The two antibiotics clearly inhibited bacterial growth compared to single antibiotics at the corresponding concentrations; 80% ampicillin only and 20% kanamycin only. The result, if extended to further trials, could be more beneficial than the earlier promoted additive and synergistic combinations of the two classes of antibiotics. These combinations were limited to concentration mixtures at 50-50% or 25-25% of the two drugs. Moreover, the combination selected here consisted of low concentration of aminoglycoside, kanamycin (20% MIC); the addition of which, was considered to increase the rate of side effects in the patients consuming the combination compared to those on monotherapy with β -lactams.

5.3 Drug-combination investigations on *S. aureus*

5.3.1 Drug-phytochemical combination investigations on CDSS

The FIC indices determined initially by the checkerboard method for ciprofloxacin and ofloxacin combinations with the four phytochemicals were in the range of 0.75 – 1.00, generally suggesting additivity. None of the combinations were antagonistic. The time-kill analysis of the combinations screened by the microchip experiments also showed bacteriostatic and bactericidal action on the growth of both MSSA and MRSA (see Figure 4.15-4.18, Chapter 4). The inhibitory and bactericidal combinations of ciprofloxacin were further made to undergo investigations on the CDSS for 360 hours on MSSA with replenishment of drugged media every 24 hours. The real time susceptibility of the parent strain, MSSA ATCC 29213, was tested by the disk diffusion test with 5 μ g ciprofloxacin disks every 24 hours. While every 72 hours the samples were made to undergo a PAP analytical test to check the heterogeneity of the population under drug influence.

As revealed from the disk diffusion tests (Figure 4.19, Chapter 4), most of the samples exposed only to ciprofloxacin at varying concentrations were found in the resistance zone of the susceptibility test. The number of exposed strains in the resistance category

increased with increasing time, and towards the end, at T_{360} , only the one exposed to 20% ciprofloxacin was found in the intermediate category; while all other strains showed resistance to the 5 μ g ciprofloxacin disks. On the other hand, in the case of ciprofloxacin-phytochemical combinations exposed strains, very few of them expressed resistance to ciprofloxacin disks (ciprofloxacin-rutin combinations). Ciprofloxacin with quercetin, PCA and EG exposed strains were either found in the intermediate range of susceptibility or in the susceptible range.

The PAP tests further validated the resistance development in drug exposed MSSA. The PAP of MSSA ATCC 29213 served as the reference and baseline for the comparison of the results. It demonstrated steep negative slopes and low AUC at all times during and after the experiments. The PAPs for strains exposed to ciprofloxacin and ciprofloxacin-phytochemical combinations were compared by finding the ratio of their AUC to AUC of the reference PAP at that time (see Table 4.7, Chapter 4). Wootton *et. al.* and Walsh *et. al.* [201, 202], carried out similar studies in 2001 to detect hetro-resistance to vancomycin in *S. aureus*. They formulated PAP-AUCs ratios and compared with previously prescribed standard ratios relating specifically to vancomycin resistance in *S. aureus*. In this case, however, the resistance profiles of *S. aureus* to ciprofloxacin were being established. Therefore, based on empirical data on PAP and the reference strain, a similar PAP-AUC method was adopted to hypothesize development of resistance. Ratios in the range of 0-1.2 were chosen as the range coherent with reference stain PAP and hence, susceptible to ciprofloxacin with near homogeneous populations. The ratios greater than 1.2 were considered incoherent with the reference strain, having broader negative slope and higher AUC values in the PAP graphs. Most of the strains exhibiting higher AUC-PAP ratio showed growth on 2 μ g/mL ciprofloxacin agar plates ($2\times$ MIC) which was the highest concentration used to determine the PAP. None of the ciprofloxacin-exposed strains possessed AUC ratio in the range coherent to the reference strain. On the other hand, many combination-exposed strains were coherent with the reference PAP. For example,

most of the ciprofloxacin-quercetin exposed strains had PAP-AUC ratio as zero, indicating no bacterial growth for most periods of the experiment. A very few strains were able to show T_{360} PAP-AUC ratio in the coherent range. Yet, interestingly, none of the combination-exposed strains showed growth at 2 $\mu\text{g/mL}$ ciprofloxacin plate in the PAP results (Appendix G, Figure G-2).

5.3.2 Detection of mutation in drug exposed *S. aureus*

Apart from the disk diffusion and the PAP tests, determination of MIC and mutation frequency of the T_{360} strains and the mutants selected during the PAP tests at $\times 2$ and $\times 4$ MIC, corroborated to the resistance profiles of the drug exposed strains. These tests had revealed a possibility that ciprofloxacin resistance had developed in the antibiotic exposed strains and in few stains that were exposed to the combinations. The MIC range of the ciprofloxacin-exposed strains was 8-64 $\mu\text{g/mL}$, which was much higher than the general susceptibility range of ciprofloxacin MIC for *S. aureus* ($1 \leq \text{MIC} \leq 4$) as per CLSI guidelines [197]. Some combination-exposed strains too were found to have MIC of 8 and 16 $\mu\text{g/mL}$. All these strains ($\text{MIC} \geq 8 \mu\text{g/mL}$) with phenotypic resistance to ciprofloxacin were made to undergo molecular studies to confirm the occurrence of genetic resistance.

The genetic confirmatory studies showed that mutation had indeed occurred on the mutants suspected with high ciprofloxacin MIC values ($\geq 8 \mu\text{g/mL}$). A point mutation in the *grlB* gene modified the 470th codon to replace Asparagine to Isoleucine in the GrlB subunit of topoisomerase IV enzyme. The MICs of the combination exposed strains were rather stable, and the ones suspected to have genetic mutation were interestingly the combinations with high levels of ciprofloxacin (80% or 0.8 g/mL), Cip80Rut20 and Cip80Quer20. The molecular analytical results however, confirmed no genetic variations in the two strains.

To understand the role of the mutation in the target enzyme coding regions in ciprofloxacin resistance, it is necessary to understand mechanism of action of the antibiotic. The antibiotic, ciprofloxacin belongs to the antibiotic class of fluoroquinolones that are commonly used for the treatment of *S. aureus* and related infections [225, 226]. Ciprofloxacin is a broad spectrum bactericidal antibiotic effective against both gram-positive and gram-negative bacteria, inhibiting bacterial enzyme, DNA type II topoisomerase important for DNA replication and cell division (for detailed mechanism of action refer Section 2.4.1, Chapter 2). However, due to its increased and over use in the past decade, many bacterial species including *S. aureus* have acquired ciprofloxacin resistance [227]. Ciprofloxacin resistance in *S. aureus* is mainly caused due to altered target or expulsion of drug from the cell (see Section 2.5.2.1 Resistance to ciprofloxacin, Chapter 2). In this case, as confirmed by the molecular analysis of the samples, the resistance was clearly due to altered target.

The most common single-step mutation found in quinolone resistant *S. aureus* was reported by Yamagishi *et. al.* [228], in the *grlA* gene. In this, Serine amino acid at codon 80 was substituted by Phenylalanine (also represented as S80F) and E84K, as a result of a single-step mutation in the *grlA* gene. Both mutations separately raised the ciprofloxacin MIC to 1.56 µg/mL. Similar mutations in the *grlA* gene were found by Ng *et. al.* [229] and Ferrero *et. al.* [178], as S80F, A116E/P and S80Y, E84K, respectively. The *grlA* mutations found by Ferrero *et. al.* were similar to those of Yamagishi *et. al.*, with an exception of the substitution protein at the 80th codon. While mutations in the *grlA* gene are more common; a mutation was reported by Fournier *et. al.* [230] in the *grlB* region as well, which increased the ciprofloxacin MIC to 2 µg/mL. This mutation resulted in substitution of Asparagine by Aspartate at codon 470 or N470D. Stutandar *et. al.* [193, 194], in their study, had also reported a point mutation in the *grlB* gene, where the parent strain, MSSA ATCC 29213 was continuously exposed to ciprofloxacin at half MIC for several days. Point mutations were observed in exposed strains isolated after 96 hours of

exposure of bacteria to the antibiotic. As suggested by Fournier and Hooper [231], mutation in GrlA subunit likely confers resistance by causing a reduction in the level of binding of quinolones to the enzyme-DNA complex and is predicted to have normal or near normal catalytic efficiency. This reduction leads to lower number of enzyme-DNA complexes in proper conformation for the drug to act and bind, to bring about its cleavage. This results in formation of fewer drug-enzyme-DNA complexes that would initiate events leading to cell death. The role of mutation in the *grlB* gene in ciprofloxacin resistance is not fully known, except that it alters the enzyme catalytic efficiency. The mutations found at the 470th codon in GrlB subunit at two instances stated above, is distant from the homologous putative sites of quinolone binding in GrlA and GyrA subunits, suggesting another mechanism of quinolone resistance and one possibly associated with altered enzyme catalytic efficiency [231]. Topoisomerase IV being the primary target for fluoroquinolones in *S. aureus*, mutations in *grlA* and *grlB* genes are more commonly found in case of quinolone resistance [229, 232].

Being a secondary target, single-step mutation conferring increased ciprofloxacin MIC has never been found to occur in GyrA and GyrB subunits of DNA gyrase. However, mutation in the *gyrA* is found only in highly resistant clinical strains of *S. aureus*, that also have mutation in *grlA* gene, as previously described. Alteration in *grlA* precedes that of *gyrA* in single step mutations, and any mutation in *gyrA* alone is silent in *S. aureus*.

5.3.3 Potentiating action of phytochemicals on ciprofloxacin activity

Antibiotic resistance in bacteria is a common consequence of antibiotic usage, due to the adaptable nature of bacterial pathogens (details of causes of antibiotic resistance are explained in Section 2.5, Antibiotic resistance, Chapter 2). Several pathogens are becoming resistant to several old and new antibiotics due to their use, overuse and abuse.

According to a report by World Health Organization (WHO) on Antimicrobial resistance [2], the consequences of the ‘wonder drug’ or antibiotic usage have become severe and leads to failed treatment of infectious diseases caused by resistant microbes. The report has also emphasized a possible beginning of a post-antibiotic era, due to the emergence of infectious diseases where resistance has developed for virtually all available drugs. In addition to antibiotic resistance, there are usually a number of side effects associated with antibiotics in general. Pertaining to quinolones alone, some of these side effects include seizures, respiratory failure, cardiac arrest and adverse interactions with caffeinated drinks [233]. Reductions of antibiotic dosages not only contribute to minimizing the side effects associated with them, but in some cases, play a role in delaying the development of resistance [234]. The reduction of antibiotic dose may be augmented by addition of alternative antimicrobial agents to give plausible synergistic outcomes. In the present study, phytochemicals were used in combination with the antibiotic with an aim to facilitate the reduction of synthetic drug dose. This would help to reduce antibiotic toxicity to some extent, adverse interactions and possibly delay in the development of adaptive resistance [51].

The aim of the 360 hour antibiotic and antibiotic combination investigations on *S. aureus* were to study the interactions of potentiating antibacterial phytochemicals on ciprofloxacin activity and their role in modulating antibiotic resistance. To make the results more diversified, the antibiotic was supplied in varying concentrations (100% = MIC to 20% of MIC) and their resistance profiles were determined by various methods mentioned in the previous sections. The resistance profiles of the ciprofloxacin exposed and combination exposed strains at the end of 360 hours can be explained by understanding the mechanism of action of the antibiotic and phytochemicals; on the basis of which the phytochemicals assist and potentiate the activity of ciprofloxacin in long term usage. These mechanisms are explained in the following paragraphs and correlated with that of ciprofloxacin.

Similar to the quinolones, quercetin and rutin (plant flavonoids) target type II DNA topoisomerases and induces DNA cleavage. These are therefore also referred to as topoisomerase poisons [235]. As seen from the results, the potentiating activity of quercetin with ciprofloxacin can be attributed to the high selectivity of quercetin for prokaryotic DNA gyrase, while ciprofloxacin primarily targeting topoisomerase IV [236, 237]. Together, it leads to cell lysis due to irreversible DNA damage upon replication and hence have a bactericidal effect. This was also revealed by the PAP tests results of the long term bacterial physiological analysis. The low mutation frequency and non-mutation selection for some combinations with quercetin are suggestive of delayed mutation. This delay can be attributed to the fact that both the drugs work on different and essential targets of the bacterial cell to bring about cell death. Quercetin in some studies has also shown to make the bacterial cell wall more permeable, allowing the drugs to enter through the cell wall [140]. As stated earlier, ciprofloxacin resistance in *S. aureus* is mainly due to altered target, usually mutation in genes coding the target enzyme and by multi-drug resistant or MDR efflux pumps [176, 237]. Also seen in the DNA evaluation of the ciprofloxacin-exposed strains, a genetic mutation was observed in one of the genes coding the topoisomerase II sub-units (*grlB*, encoding B subunit of topoisomerase IV) in the five exposed strains. In the case of ciprofloxacin and rutin combinations, the same potentiation was not observed even though rutin belongs to the same class of plant flavonoids. The MIC of one of the ciprofloxacin-rutin exposed strain was, in fact, 16 µg/mL. In this case however, both ciprofloxacin and rutin targets the bacterial topoisomerase IV, making it a competitive site [238]. Hence, even at high concentrations of rutin (3277 µg/mL or 80% MIC), potentiating activity was not observed.

The activity of ciprofloxacin with EG was comparable to that of ciprofloxacin with quercetin, in terms of bactericidal effects, potentiating activity and exhibiting lower mutation frequency. In general, alkyl gallates have an amphipathic molecular structure with a hydrophobic alkyl part (tail) and a hydrophilic pyrogallol moiety (head). They are

known to disrupt the fluidity of the lipid bilayer of the bacterial cell membrane. As explained by Kubo *et. al.*, the antibacterial activity of alkyl gallates does not depend on the alkyl chain length, but is responsible for its bactericidal activity [143]. Due to their molecular structure, they act as surfactants and inhibit the electron transport chain of the membrane, thereby inhibiting the respiratory system of the bacteria [143, 144]. Since, they do not have to enter the cell for their action, they are not usually affected by resistance causing mechanisms of bacteria. By further addition of ciprofloxacin against gram-positive *S. aureus*, the potentiation might have taken place due to disturbance in the fluid membrane by EG, allowing easy access to the antibiotic to its intracellular targets. The low mutation frequency and absence of phenotypic mutation in the T₃₆₀ sample also supports the potentiation of ciprofloxacin by EG. The mutation frequency and MIC of the selected mutants remained lower than corresponding ciprofloxacin exposed T₃₆₀ strains, except when the concentration of EG was low in combination with ciprofloxacin (e.g. Cip80-EG20).

The activity of ciprofloxacin with PCA also showed bactericidal effect in both short term and the long term studies. The MIC was same as the control strain throughout the 15 days and no mutants were selected at higher MIC from these samples. The molecular mechanism of action for PCA and related compounds is not completely understood, but their antibacterial properties have been demonstrated and reported by several groups [145, 146]. It is one of the major components isolated from Roselle calyx (*Hibiscus sabdariffa*) and barks of *Cananga odorata* medicinal plants; it has antibacterial action against most gram-positive and gram-negative bacteria. The absence of phenotypic mutation and low mutation frequencies observed in the results, suggests that PCA was able to potentiate ciprofloxacin activity and delay the occurrence of resistance, which was observed in the corresponding ciprofloxacin exposed strains.

In the present study, the role of phytochemicals in the delay of antibiotic resistance, suggest a possible incorporation of these ubiquitous elements of nature into treatments of

infections caused by resistant bacteria by common antibiotics. However, more research and *in-vivo* trials need to be conducted with these drug-phytochemical combinations. Even though plant-derived antimicrobials have been shown to be effective both *in-vitro* and *in-vivo*, and known for their resistance modulating capabilities, they are not yet available in mainstream medicine [7, 52]. Plant antibacterials tend to be concentrationally weak compared to most antibiotics and unable to be used alone for treatment of infections. As also seen in the current study, their MICs are 100 to 1000 folds higher than common antibiotics. For them to be used in a clinical scenario, the pharmacokinetic and pharmacodynamic (PK/PD) models of the drug must be taken into account. With such high MIC values *in-vitro*, the bioavailability of the drug *in-vivo* as effective dose may becomes difficult to reach. Many academic researchers have reviewed attractive potential of plant-derived antimicrobials, and have suggested that they may be useful when used alone [6, 7, 51, 52]. Plant antimicrobials with their narrow spectrum of action can target multiple sites in a bacterial cell, many of which are potentiating targets for a selected antibiotic. Their addition, phytochemicals as resistance modifying agents, could be beneficial when used in combination with strong antibiotics, which are known to encounter resistance during treatment. The bacterial cell acquires resistance against antibiotics within a few days to few weeks, but the addition of phytochemicals can make the treatment constructive for long term usage.

5.4 Maggot extracts and its combination with ciprofloxacin

The maggot extracts were collected by five methods and screened on gram-negative and gram-positive bacteria [160]. Based on the results, maggot ES extracted by Method E (Section 3.4.2.5, Chapter 3) demonstrated maximum bacterial growth inhibition in gram-positive *S. aureus*. It was also found to contain one of the antibacterial factors, phenylacetaldehyde, in discernable amounts. The ES extracted by this method was also

used in combination with ciprofloxacin to enhance the antibacterial activity of the ES and at the same time, help in reduction of antibiotic dose.

Maggot debridement therapy (MDT) has been in practice for many years for healing of chronic wounds. However, in the recent past, the interest has been in its disinfection and antiseptic activity on the wound. MDT is considered to be more advantageous than other conventional methods to treat infected wounds, especially by MRSA [157] [239]. Beasley and Hirst [157], have emphasized the role of maggot therapy in clearing multi-drug resistant (MDR) bacteria from infected chronic wounds where conventional methods have failed. The results were far superior compared to other methods for wound debridement, healing and disinfecting. MDT is particularly used for healing wounds infected by MDR MRSA. The disinfection action of maggots on the wound has been studied extensively over the past few years. There are three modes of action involved during the disinfection. These are summarized below,

- Movement and irrigation of the wound by ingestion of liquefied necrotic tissue by the maggots which promotes the formation of serous exudates,
- dilution of bacterial concentration caused by wound lavage due to irrigation
- secretion and/or excretion of antibacterial compounds by maggots, such as phenylacetaldehyde and phenylacetic acid formed by the larval gut symbiote *Proteus mirabilis*.

On the wound, the disinfection may be caused by one or a combination of two or more of these mechanisms. Some even suggested that the rise of pH of the wound milieu by the metabolic activity of the maggots inhibits bacterial growth [157]. They excrete compounds that lead to increased pH, such as ammonia and calcium carbonate. A comprehensive study of the disinfection action and wound healing in MDT has been done by Nigam *et. al.* [239].

Maggot ES have previously shown to have antibacterial activity against many bacterial pathogens. This was first demonstrated by Simmons and co-workers in 1935 followed by Pavillard and co-workers in late 1950s [153, 154]. The antibacterial factors were later studied by performing high-performance liquid chromatography (HPLC) and ultrafiltration to separate compounds in the ES. A factor possessing molecular weight of 6000 Da was purified by Friedman and coworkers [240]. Bexfield *et. al.* [158], later fractionated the ES into three fractions, based on their molecular mass, >10 kDa, 0.5-10 kDa and <500 Da. Significant antibacterial activity against *S. aureus* was observed in the < 500 Da and 0.5-10 kDa ultrafiltration fraction. They were found to be heat stable compounds, possessing non-proteinaceous and non-enzymatic structures. Several other groups demonstrated antibacterial activity in ES collected from maggots of *L. sericata*. Thomas *et. al.* [241], collected ES from aseptically raised maggots for the first time and detected antibacterial activity against *S. aureus*, *Pseudomonas aeruginosa*, *Streptococcus* group A and B and a clinical isolate of MRSA. Jaklia *et. al.* [159] studied the selective antibacterial activity of the maggot ES both *in vitro* and *in vivo* on the wound. They found that the ES is most active against gram-positive bacteria, such as *S. aureus*, abundant in wounds and less or no activity against gram-negative bacteria, especially *Proteus* spp. and *Pseudomonas* spp. strains. Huberman *et. al.* [55] had also isolated low molecular weight antibacterial compounds by HPLC and GC-MS from the maggot extracts of *L. sericata*. Similar studies on ES extracted from maggots of *L. sericata* were performed by Daeschlein *et. al.* [155] and Kerridge *et. al.* [56], showing antibacterial activity on selected bacterial pathogens.

Small molecular weight peptides, similar to those isolated from the maggot ES, are known to give insects their intrinsic ability to fight against pathogenic invaders. They are at some instances, even able to avoid emergence of resistance in bacteria [53, 242]. Maggots by itself have a wide range of defense mechanisms against microbes, and the presence of those in the ES, supports clinical findings and continued use of MDT in wound care.

These findings can help apply maggot-derived antibacterial factors to wider clinical applications, such as treatment of other external systemic infections. In addition, additives from natural resources, such as insect extracts, have advantages over many synthetic drugs in several ways. They are abundantly available in nature, having minimum or no side effects and many have shown to have intrinsic antibacterial properties due to their natural habitat. For example, defensin - a small, heat-stable, cationic antimicrobial peptide, is expressed by many Dipteran species in response to pathogen invasion and tissue damage [243, 244].

In most of the above findings, different methods were used for ES collection as well as for antibacterial screening. Bexfield *et. al.* [158] in their study, emphasized that the use of standard antibacterial assays such as, zone inhibition and microdilution assays, were ineffective when using maggot ES. This was also observed in the present study, when microdilution methods for determining MICs were employed using maggot ES.

It is also important to note that ES collection method is critical in order to detect direct or indirect antibacterial activity. Several methods were employed and demonstrated to extract or collect maggot ES (some methods were not included in this report). The antibacterial properties were identified by screening the collected extracts against *S. aureus* and *E. coli*, each representing bacteria from the gram-positive and gram-negative group, respectively. In addition, the methods used were based on different principles suggested in previous maggot-based studies.

Some of the screening results obtained in this study, coincided with previous work by Cazander *et. al.* [209]. They suggested that live maggots had no direct antimicrobial action on a bacterial suspension and rather the bacterial number increased after overnight incubation. But at the same time, it was suggested that ES collected by a similar method as Method E of this study, had no significant activity. The Method E was adopted from various maggot-based studies conducted previously to extract ES from maggots of *L.*

sericata (discussed in the previous paragraphs). Here, the original ES was collected in saline after 1 hour incubation in dark and used directly for screening, instead of breaking it into protein concentrations or fractions as done by Cazandar *et. al.* and Bexfield *et. al.* in their experiments [158, 209]. The original ES extracted also showed the presence of phenylacetaldehyde, which was not demonstrated in previous studies on ES. The extracts demonstrated antibacterial activity even after thermal sterilization, further supporting the results of Bexfeild *et. al.* [158].

It was also noted in the results that the ES collected by Method E did not have equal inhibitory activity on *E. coli* as for *S. aureus*. This result supported some previous studies with ES from other species of blowflies [159, 241], suggesting selective antibacterial activity of maggot ES. The ES was shown to be more effective against gram-positive bacteria compared to gram-negative. A 30% decrease of bacteria number from the starting inoculum concentration was observed with the ES extracted by Method E, while extracts collected by other methods did not have any direct antibacterial activity on both *S. aureus* and *E. coli*.

The gEM proposed and tested for maggots of *L. cuprina* for antibacterial activity was able to show partial growth inhibition in both bacterial species (when 250 maggots were used for gEM collection). This was further supported by the components detected by GC-MS analysis of the gEM. These included certain compounds that have potential antibacterial activity. For example, aromatic aliphatic esters of furanone and derivatives, and tetrahydrofuran esters have been previously shown to be antibacterial [245, 246]. Furanones alone have been used for antibacterial coating for biomaterials to reduce infection at the site of contact with the body [247, 248].

Complete bacterial inhibition was not observed with both ES and gEM collected from the maggots. This could be due to various reasons. The previous antibacterial screenings were done mostly using different blowfly specie, *L. sericata*. In this study, however, maggots of

L. cuprina blowfly were used for ES extraction. These are currently used in hospitals of Singapore for MDT on patients with chronic wounds. The results of the treatment have been positive with no traces of infection on the wounds [249]. The antibacterial factor(s) extracted in the ES, body fluids or gEM may be present in much diluted forms so as to only show stunted growth and inhibition. A combination with antibiotic was thus proposed and tested with the maggot ES, to enhance its antibacterial activity, and to possibly have a potentiating effect, similar to the phytochemicals, on the antibiotic activity.

The combination of ciprofloxacin with ES, as seen from the results, could help in potentiating ciprofloxacin activity at sub-MIC levels. However, with the density of maggots used in the present study (50 maggots per 100 μ l of PBS), total bactericidal effects could not be detected as shown in previous studies with *L. sericata* maggots [155, 158, 159]. Hypothetically, if the density of the ES preparation could be increased there would probably be improved bactericidal effects. Though, practical constraints to rear large number of maggots limited that possibility. Therefore, a combination therapy could be potentially useful and considered in appropriate situations. At this point, the resistance profile of *S. aureus* could not be determined under maggot ES influence similar to those determined under phytochemical influence, but the growth inhibition with ciprofloxacin with addition of maggot ES was significantly higher than ciprofloxacin alone. A likely implication of this result could be that the resistant sub-population had indeed taken over the susceptible ones at sub-MIC concentration of ciprofloxacin and the same was not true for the one with ES addition. Up to 50% reduction in bacterial number from the initial inoculum concentration was achieved with 80% and 60% MIC concentration of ciprofloxacin with maggot ES.

6 CONCLUSIONS

6.1 The experimental system: microchip and CDSS

6.1.1 Microchip

With an aim to expedite injection, separation, mixing and screening of drugs in combination on cells, three microfluidic devices were designed to be used as a platform to carry out these processes. Based on the quantitative spectrometric and qualitative flow visualization results, microchip with Design II presented superior performance among all the designs. The modified Design I also demonstrated accurate mixing performance, however, other issues such as, bubble entrapment and long term diffusion of fluid among wells of the sub-systems, limited its performance for long-term screening. Subsequently, all experiments were carried out using microchip Design II, unless otherwise stated.

The microchip material, PDMS and glass, were able to sustain cell cultures with prior treatment of the microchannels to impart hydrophilicity. It could be washed and reused after proper sterilization techniques, since, the microchip material was thermally and chemically stable.

6.1.2 CDSS

The Combinatory Drug Screening System or the CDSS was designed, fabricated and calibrated for sustenance of cell growth and to accommodate the microchip. The initial case studies on two categories of cells, adhesion and suspension types, successfully demonstrated the efficacy of the system for both short-term and long-term monitoring of both types of cells. It could be readily integrated to an inverted microscope for direct imaging of growing cells in the wells of the housed microchip. It could also be integrated

with a spectrometer setup for spectrophotometric analysis of the microchip wells during incubation. The limitations of the system were, however, the issues relating to evaporations of the fluids in the open wells of the microchip.

As a final concluding remark, the CDSS along with the microchip, could be used for long-term drug combination investigations, wherein, useful information of prolonged medications could be studied on selected cell systems.

6.2 Drug-phytochemical combinations

S. aureus, that is known to develop resistance to ciprofloxacin by adaptive mutation at near MIC levels, was stressed under drug-combination for 360 hours. The results demonstrated that most of the phytochemicals that were used in combination with ciprofloxacin were able to potentiate its activity. Consequently, most of the combinations studied were able to delay the occurrence of resistance in the test organism. The results were also compared with those of ciprofloxacin exposure only for the same period of time. The genetic mutations revealed in the ciprofloxacin exposed samples confirmed the CDSS findings. The conclusions on specific combinations are discussed below.

6.2.1 Ciprofloxacin-quercetin combination

The ciprofloxacin-quercetin combination was superior to all other combinations that were investigated, in terms of inhibiting bacterial growth for long-term (except combination Cip80Quer20). The disk-diffusion, PAP, mutation frequency and molecular analysis, revealed that quercetin successfully played a role in delaying the process of development of resistance to ciprofloxacin, which was apparent in the ciprofloxacin exposed strains. This can be attributed to the potentiating activity of the phytochemical on that of the antibiotic, by multi-target action on the bacterial cell, leading to bactericidal effects. The combinations Cip20Quer80, Cip40Quer60 and Cip60Quer40 - exposed strains maintained

the MIC value comparable to that of the parent strain (MSSA ATCC) even after 360 hours of drug exposure. This clearly suggested that the resistance to ciprofloxacin had been delayed by the addition of the phytochemical.

6.2.2 Ciprofloxacin-rutin combination

In the case of ciprofloxacin-rutin combinations that were selected by the CDSS tests, rutin was not as apparently involved in the process of delaying the resistance, as in case of quercetin. Rutin and quercetin both belong to the flavonoid class of phytochemicals, that disrupt the DNA replication of the bacterial cell. However, in combination with ciprofloxacin, the primary target of both ciprofloxacin and rutin was topoisomerase IV, which may have led it to become a competitive target site. Later, in the molecular analytical studies, even with high MIC value of the phenotypic strain exposed to Cip80Rut20 combination, no genetic mutation was observed in the final resistant strain. This also suggests partial potentiating activity of the phytochemical on antibiotic resistance.

6.2.3 Ciprofloxacin-protocatechuic acid combination

Even though the exact mechanism of action of PCA is not completely understood, yet the activity of the selected combinations of ciprofloxacin with PCA, Cip20PCA80 and Cip80PCA20, clearly suggested a delay in the development of ciprofloxacin resistance in the combination exposed strains. The experimental results were comparable to those of quercetin, also in terms of inhibition of bacterial growth for most of the intervals in the 360 hour period of exposure.

6.2.4 Ciprofloxacin-ethyl gallate combination

As described in the previous chapter, the mechanism of action of both ciprofloxacin and EG were entirely different, as one targeted the DNA replication mechanism and other

targeted the membrane fluidity. Together, this led to a bactericidal effect, shown by most of the combinations selected (except Cip80EG20). The mutation frequency and PAP tests, clearly suggested the delay in the development of ciprofloxacin resistance in the combination exposed strains, as compared to those exposed to ciprofloxacin alone.

6.2.5 Ofloxacin and phytochemical combinations

The combinations of ofloxacin with the four phytochemicals were studied for a period of 24 hours, unlike with ciprofloxacin. The combinations with rutin, however, showed no growth inhibition at T₂₄. With quercetin and ethyl gallate the results were similar to those with ciprofloxacin at T₂₄. With PCA, the combinations that showed bacterial growth inhibition were Cip20PCA80 and Cip40PCA60 only. In these combinations studies, however, the resistance profiles of the exposed strains could not be determined, unlike with ciprofloxacin, where exposure was for 360 hours. Further studies with long term exposures must be carried out that would reveal any potentiating action of the phytochemicals on the activity of ofloxacin.

6.3 Maggot ES and drug-maggot ES combinations

6.3.1 Maggot ES extraction

The maggot ES was collected by five methods, broadly classified based on their proposed antimicrobial action. Based on the findings, Method E had demonstrated superior antibacterial activity, compared to other methods. It revealed the presence of antibacterial compound, phenylacetaldehyde, in the GC-MS test results. This method has been used before by other groups in the past for extraction of ES from *L. sericata* maggots. In this study, the method was altered and screened for antibacterial action alone and in combination with ciprofloxacin. When used alone, it demonstrated partial inhibitory action on growth of gram-positive *S. aureus* with up to 30% growth reduction from the initial

inoculum concentration. No antibacterial activity was observed against gram-negative *E. coli*. The gaseous excreta/metabolites or gEM also demonstrated partial antibacterial activity against both gram-positive and gram-negative bacteria.

6.3.2 Ciprofloxacin and maggot ES combination

The combination of maggot ES with ciprofloxacin demonstrated up to 50% growth reduction from the initial inoculum concentration with 0.6 and 0.8 $\mu\text{g/mL}$ (60% and 80% MIC) of ciprofloxacin. This suggested partial enhancement of activity of the maggot ES by addition of sub-MIC of ciprofloxacin, and at the same time, reducing the total dosage of ciprofloxacin. The growth inhibition with ciprofloxacin and maggot ES addition was significantly higher than ciprofloxacin alone. For this study also the resistance profile of MSSA could not be determined due to 24 hour study only.

Hypothetically, if the density of the ES preparation could be increase there would be improved bactericidal effects, both alone and in combination. However, practical constraints to rear large number of maggots limited that possibility. Therefore, a combination therapy was proposed and studied that would be potentially useful and considered in appropriate situations.

7 FUTURE RECOMMENDATIONS

In this chapter, future work has been laid down to further improve the current system as well as to increase the scope of the work.

7.1 Microchip multiplexing

The microchip can be extended for investigations of combination by employing more than two drugs. Using the current microchip designs, this can be achieved by stacking a PDMS mold of one of the designs on top of another mold of the same design. A schematic of this kind of multiplexing is shown in Figure 7.1. The arrangement shown would generate mixture of four input fluids in each well.

Figure 7.1: Multiplexing of PDMS molds to employ multiple drug inputs.

The PDMS molds can be aligned and bonded to each other (left) and finally bonded to glass (right) with inlets on the sides. The schematic show four inlets at the sides of the PDMS molds.

7.2 Enhancement of the CDSS

The CDSS can be further improved by adding a humidity control system in the box incubator. In the current system, the evaporation of the low volume open wells was

overcome by placing moist pads in the box incubator, which greatly reduced the evaporation.

A programmable and automated injection and extraction control panel with reservoirs can be added to the system that would make the system capable of carrying out investigations with minimum manual interference. A simple schematic of the system is shown in Figure 7.2.

Figure 7.2: A simple schematic of the enhanced CDSS

The enhanced CDSS with humidity control unit and automated injection and extraction systems

7.3 Dynamic drug combination response

In combinations obtained in the current system, only a single combination can be investigated over time in a well. To introduce dynamic drug exposure on test cells, different input concentrations can be used, instead of $1 \times \text{MIC}$. Dynamic drug combination exposure can have diversified effects on the mutational behavior of impulsive bacterial

pathogens like *S. aureus*, that adapt to changing environment rapidly. This proposed phenomenon of dynamic drug combination exposure response or DDCR can be investigated using the CDSS by injecting input drugs at concentrations lower or higher than its MIC value. The resultant combinations in the wells would then be denominations of the input concentration.

7.4 Investigations with other cells and drugs

The present drug-phytochemical combination response can be studied on other bacteria of clinical importance pertaining to rapid development of resistance to antibiotics. These include, but not limited to, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Enterococcus faecium*, *Clostridium difficile* and *Streptococcus pyogenes*. Several species of these nosocomial and community acquired bacterial pathogens have been categorized world-wide as multi-drug resistant strains, isolated in different parts of the world [2].

Phytochemicals, other than the four used in this study, with known antimicrobial activity can be studied with related antibiotics in combinations, in which they are able to potentiate the antibiotic activity. Some of these promising phytochemicals include, epicatechin gallate, a major constituent of green tea; allicin, a major constituent of garlic; curcumin, a major constituent of turmeric; resveratrol, a constituent of red wine; ellagic acid, constituent of berries (raspberries, strawberries, cranberries and mulberries); tea tree oil; and capsaicin, a major constituent of chilli. All the above mentioned phytochemicals possess antimicrobial properties against both gram-positive and gram-negative bacteria. These can be combined with selected antibiotics with related or complementary mechanisms of actions and investigated on MDR bacterial species.

At the same time, cell systems other than bacterial and related drugs, can be incorporated into the system; such as, investigation of combination of several growth factors on plant cells. The work with adhesion cells can potentially be extended with a combination of

anti-cancerous drugs. The effects of different concentration mixtures of two drugs on the growth and multiplication of cancer cells can be studied *in vitro* for screening purposes.

7.5 Mechanism based drug interaction analysis

The current study can be extended by performing mechanism based drug interaction analysis. These kinds of studies involve drugs with similar mechanisms or targets in the same biochemical process of the test organism, such as folic acid biosynthesis in bacteria [250]. The appropriate concentration of drugs in combinations can be investigated based on their influence on the test organism. In case of bacterial pathogen as test organisms, the drugs may include antibiotics in combination of two or more or in combination with phytochemicals. Drug molecules can also be computationally designed using the 3D model of the target bacterial protein, belonging to the biochemical cycle. Two targets in an essential biochemical cycle would yield enhanced bactericidal outcomes. A similar study has been performed by Jayaraman *et. al.* [250], in which they investigated antifolate antibiotics in combination with phytochemicals that potentiate the activity of the antifolates against *P. aeruginosa*. They found that the phytochemicals block the function of dihydrofolate reductase (DHFR), an important enzyme in the folic acid biosynthesis pathway, by performing computational molecular docking of the phytochemicals in the active site of the enzyme. These interactions can provide significant leads into development of novel drug combinations with multi-targeting capabilities, thereby having an enhanced outcome.

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APPENDIX A

8-bit AVR® Microcontroller with 16 KBytes In-System Programmable Flash

Pin configuration of ATmega16

Block Diagram of ATmega16

General Features

1. High-performance, Low-power AVR® 8-bit Microcontroller
2. Advanced RISC Architecture
 - 131 Powerful Instructions – Most Single-clock Cycle Execution
 - 32 x 8 General Purpose Working Registers

- Fully Static Operation
- Up to 16 MIPS Throughput at 16 MHz
- On-chip 2-cycle Multiplier

3. Nonvolatile Program and Data Memories

- 16K Bytes of In-System Self-Programmable Flash
 - Endurance: 10,000 Write/Erase Cycles
- Optional Boot Code Section with Independent Lock Bits
 - In-System Programming by On-chip Boot Program
 - True Read-While-Write Operation
- 512 Bytes EEPROM
 - Endurance: 100,000 Write/Erase Cycles
- 1K Byte Internal SRAM
- Programming Lock for Software Security

4. JTAG (IEEE std. 1149.1 Compliant) Interface

- Boundary-scan Capabilities According to the JTAG Standard
- Extensive On-chip Debug Support
- Programming of Flash, EEPROM, Fuses, and Lock Bits through the JTAG Interface

5. Peripheral Features

- Two 8-bit Timer/Counters with Separate Prescalers and Compare Modes
- One 16-bit Timer/Counter with Separate Prescaler, Compare Mode, and Capture Mode
- Real Time Counter with Separate Oscillator
- Four PWM Channels

- 8-channel, 10-bit ADC
 - 8 Single-ended Channels
 - 7 Differential Channels in TQFP Package Only
 - 2 Differential Channels with Programmable Gain at 1x, 10x, or 200x
- Byte-oriented Two-wire Serial Interface
- Programmable Serial USART
- Master/Slave SPI Serial Interface
- Programmable Watchdog Timer with Separate On-chip Oscillator
- On-chip Analog Comparator

6. Special Microcontroller Features

- Power-on Reset and Programmable Brown-out Detection
- Internal Calibrated RC Oscillator
- External and Internal Interrupt Sources
- Six Sleep Modes: Idle, ADC Noise Reduction, Power-save, Power-down, Standby
- and Extended Standby

7. I/O and Packages

- 32 Programmable I/O Lines
- 40-pin PDIP, 44-lead TQFP, and 44-pad MLF

8. Operating Voltages

- 2.7 - 5.5V for ATmega16L
- 4.5 - 5.5V for ATmega16

9. Speed Grades

- 0 - 8 MHz for ATmega16L
- 0 - 16 MHz for ATmega16

10. Power Consumption @ 1 MHz, 3V, and 25 °C for ATmega16L

- Active: 1.1 mA
- Idle Mode: 0.35 mA
- Power-down Mode: < 1 μ A

Pin Descriptions

VCC Digital supply voltage.

GND Ground.

Port A (PA7...PA0) Port A serves as the analog inputs to the A/D Converter. Port A also serves as an 8-bit bi-directional I/O port, if the A/D Converter is not used. Port pins can provide internal pull-up resistors (selected for each bit). The Port A output buffers have symmetrical drive characteristics with both high sink and source capability. When pins PA0 to PA7 are used as inputs and are externally pulled low, they will source current if the internal pull-up resistors are activated. The Port A pins are tri-stated when a reset condition becomes active, even if the clock is not running.

Port B (PB7...PB0) Port B is an 8-bit bi-directional I/O port with internal pull-up resistors (selected for each bit). The Port B output buffers have symmetrical drive characteristics with both high sink and source capability. As inputs, Port B pins that are externally pulled low will source current if the pull-up resistors are activated. The Port B pins

are tri-stated when a reset condition becomes active, even if the clock is not running.

Port C (PC7...PC0) Port C is an 8-bit bi-directional I/O port with internal pull-up resistors (selected for each bit). The Port C output buffers have symmetrical drive characteristics with both high sink and source capability. As inputs, Port C pins that are externally pulled low will source current if the pull-up resistors are activated. The Port C pins are tri-stated when a reset condition becomes active, even if the clock is not running. If the JTAG interface is enabled, the pull-up resistors on pins PC5 (TDI), PC3 (TMS) and PC2(TCK) will be activated even if a reset occurs. Port C also serves the functions of the JTAG interface and other special features of the ATmega16.

Port D (PD7...PD0) Port D is an 8-bit bi-directional I/O port with internal pull-up resistors (selected for each bit). The Port D output buffers have symmetrical drive characteristics with both high sink and source capability. As inputs, Port D pins that are externally pulled low will source current if the pull-up resistors are activated. The Port D pins are tri-stated when a reset condition becomes active, even if the clock is not running.

RESET Reset Input. A low level on this pin for longer than the minimum pulse length will generate a reset, even if the clock is not running. Shorter pulses are not guaranteed to generate a reset.

XTAL1 Input to the inverting Oscillator amplifier and input to the internal clock operating circuit.

XTAL2 Output from the inverting Oscillator amplifier.

AVCC AVCC is the supply voltage pin for Port A and the A/D Converter. It should be externally connected to VCC, even if the ADC is not used. If the ADC is used, it should be connected to VCC through a low-pass filter.

AREF AREF is the analog reference pin for the A/D Converter.

APPENDIX B

Program Code

This program was produced by the CodeWizardAVR V1.25.3 Evaluation Automatic Program Generator

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<http://www.hpinfotech.com>

Project: Temperature & LCD

Version:

Date: 3/6/2007

Author: Jong Foo/Shuchi

Company: NTU

Comments:

Chip type: ATmega16

Program type: Application

Clock frequency: 3.686400 MHz

Memory model: Small

External SRAM size: 0

Data Stack size: 256

*****/

```
#include <mega16.h>
```

```
#include <lcd.h>
```

```
#include <delay.h>

#include <stdio.h>


// Alphanumeric LCD Module functions

#asm

    .equ __lcd_port=0x15; PORTC

#endasm


#define ADC_VREF_TYPE 0x00

#define sensor1 0x00 // Front

#define sensor2 0x01 // Rear


// Declare your global variables here

unsigned char lcd_buffer[33];

int chamber1, chamber2;

int setpt=37; // Set point of temperature usually 37 °C


// Read the AD conversion result

unsigned int read_adc(unsigned char adc_input)

{

    ADMUX=adc_input | (ADC_VREF_TYPE & 0xff);

    // Start the AD conversion

    ADCSRA|=0x40;

    // Wait for the AD conversion to complete

    while ((ADCSRA & 0x10)==0);

    ADCSRA|=0x10;

    return ADCW;

}
```

```
void main(void)
{
    // Declare your local variables here

    // Input/Output Ports initialization

    // Port A initialization

    // Func7=In Func6=In Func5=In Func4=In Func3=In Func2=In Func1=In Func0=In
    // State7=T State6=T State5=T State4=T State3=T State2=T State1=T State0=T

    PORTA=0x00;

    DDRA=0x00;


    // Port B initialization

    // Func7=In Func6=In Func5=In Func4=In Func3=In Func2=In Func1=In Func0=In
    // State7=T State6=T State5=T State4=T State3=T State2=T State1=T State0=T

    PORTB=0x00;

    DDRB=0xFF;


    // Port C initialization

    // Func7=In Func6=In Func5=In Func4=In Func3=In Func2=In Func1=In Func0=In
    // State7=T State6=T State5=T State4=T State3=T State2=T State1=T State0=T

    PORTC=0x00;

    DDRC=0x00;


    // Port D initialization

    // Func7=In Func6=In Func5=In Func4=In Func3=In Func2=In Func1=In Func0=In
    // State7=T State6=T State5=T State4=T State3=T State2=T State1=T State0=T

    PORTD=0x00;

    DDRD=0x00;
```

```
// Timer/Counter 0 initialization  
// Clock source: System Clock  
// Clock value: Timer 0 Stopped  
// Mode: Normal top=FFh  
// OC0 output: Disconnected  
TCCR0=0x00;  
TCNT0=0x00;  
OCR0=0x00;  
  
// Timer/Counter 1 initialization  
// Clock source: System Clock  
// Clock value: Timer 1 Stopped  
// Mode: Normal top=FFFFh  
// OC1A output: Discon.  
// OC1B output: Discon.  
// Noise Canceler: Off  
// Input Capture on Falling Edge  
// Timer 1 Overflow Interrupt: Off  
// Input Capture Interrupt: Off  
// Compare A Match Interrupt: Off  
// Compare B Match Interrupt: Off  
TCCR1A=0x00;  
TCCR1B=0x00;  
TCNT1H=0x00;  
TCNT1L=0x00;  
ICR1H=0x00;  
ICR1L=0x00;  
OCR1AH=0x00;  
OCR1AL=0x00;
```

OCR1BH=0x00;

OCR1BL=0x00;

// Timer/Counter 2 initialization

// Clock source: System Clock

// Clock value: Timer 2 Stopped

// Mode: Normal top=FFh

// OC2 output: Disconnected

ASSR=0x00;

TCCR2=0x00;

TCNT2=0x00;

OCR2=0x00;

// External Interrupt(s) initialization

// INT0: Off

// INT1: Off

// INT2: Off

MCUCR=0x00;

MCUCSR=0x00;

// Timer(s)/Counter(s) Interrupt(s) initialization

TIMSK=0x90;

// Analog Comparator initialization

// Analog Comparator: Off

// Analog Comparator Input Capture by Timer/Counter 1: Off

ACSR=0x80;

SFIOR=0x00;


```
// ADC initialization

// ADC Clock frequency: 57.600 kHz

// ADC Voltage Reference: AVCC pin

// ADC Auto Trigger Source: Free Running

ADMUX=ADC_VREF_TYPE & 0xff;

ADCSRA=0xA6;

SFIO&=0x1F;


// LCD module initialization

lcd_init(16);

lcd_gotoxy(0,0);

sprintf(lcd_buffer, "*** System ***");

lcd_puts(lcd_buffer);

lcd_gotoxy(0,1);

sprintf(lcd_buffer, "Initialization");

lcd_puts(lcd_buffer);

delay_ms(2000);


while (1)
{
chamber1=read_adc(sensor1)/2;
chamber2=read_adc(sensor2)/2;


lcd_clear();

lcd_gotoxy(0,0);

sprintf(lcd_buffer, "Front=%3ddegC",chamber1);

lcd_puts(lcd_buffer);
```

```
lcd_gotoxy(0,1);  
sprintf(lcd_buffer, "Rear =%3ddegC",chamber2);  
lcd_puts(lcd_buffer);  
  
if (chamber1<setpt) {PORTB.0=1;}  
else if (chamber1==setpt) {PORTB.0=~PORTB.0;}  
else PORTB.0=0;  
  
if (chamber2<setpt) {PORTB.2=1;}  
else if (chamber2==setpt) {PORTB.2=~PORTB.2;}  
else PORTB.2=0;  
  
if (chamber1<setpt-1|chamber2<setpt-1) {PORTB.1=1;}  
else PORTB.1=0;  
  
delay_ms(10000);  
}  
}
```

APPENDIX C

Layout transparencies of the microchips

(a)

(b)

(c)

Figure C-1: Layout transparencies for (a) Design I (b) Design I (modified), (c) Design II and (d) Design III.

The transparencies with clear field and dark features were for use with positive photoresist, where the UV-exposed areas of the photoresist dissolve in the developer. The transparencies with clear features and dark fields were for use with negative photoresist.

APPENDIX D

Microchip flow visualization studies

The flow visualization studies on the two designs of the microchip are summarized in the following tables. The tables show images captured after injection of different color and different viscosity fluids at specific time intervals (Section 3.1.1.3)

Table D-1: Flow visualization of wells of microchip Design I under the microscope at 100× magnification over varied flow rates, with water as fluid from both inlets.

Well No.		Flow Rate (ml/min)*		
		2 ml/min	8 ml/min	16 ml/min
1 80% (blue) – 20% (yellow)	1s
	10s
	1min
2 20% (blue) – 80% (yellow)	1s
	10s
	1min
Well No.		Flow Rate (ml/min)*		
		2 ml/min	8 ml/min	16 ml/min
3 60% (blue) – 40% (yellow)	1s
	10s
	1min
4 40% (blue) – 60% (yellow)	1s
	10s
	1min

Table D-1 cont...

Well No.		Flow Rate (ml/min)*		
		2 ml/min	8 ml/min	16 ml/min
5 & 6 50% (blue) – 50% (yellow)	1s
	10s
	1min
7 100% (yellow)
8 100% (blue)		Scale:

*The flow rates mentioned in the table were same for both syringe pumps

Table D-2: Flow visualization of wells of microchip Design II under the microscope at 100× magnification over varied flow rates, with water as fluid from both inlets.

Well No.		Flow Rate (ml/min)*		
		2 ml/min	8 ml/min	16 ml/min
1 100 (yellow)
2 80 (yellow) – 20 (blue)	1s
	10s
	1min
3 60 (yellow) – 40 (blue)	1s
	10s
	1min
4 40 (yellow) – 60 (blue)	1s
	10s
	1min
5 20 (yellow) – 80 (blue)	1s
	10s
	1min
6 100 (blue)		<div> Scale: 0.5 mm </div>		

*The flow rates mentioned in the table were same for both syringe pumps

Table D-3: Flow visualization of wells of microchip Design I under the microscope at 100× magnification over varied flow rates, with water from one inlet and ethanol from the other.

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
1 80W (yellow) – 20E (blue)	1s
	10s
	1min
2 20W (yellow) – 80E (blue)	1s
	10s
	1min

Table D-3 cont...

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
3 60W (yellow) – 40E (blue)	1s
	10s
	1min
4 40W (yellow) - 60E (blue)	1s
	10s
	1min

Table D-3 cont...

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
5 & 6 50W (yellow) - 50E (blue)	1s
	10s
	1min
7 100W (yellow)
8 100E (blue)

Scale: 0.5 mm

W-water, E-ethanol

*The flow rates mentioned in the table were same for both syringe pumps, except for in last two columns. The higher value of flow rate in both columns referred to flow rate applied to ethanol syringe pump.

Table D-4: Flow visualization of wells of microchip Design II under the microscope at 100× magnification over varied flow rates, with water from one inlet and ethanol from the other.

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
1 100W (yellow)
2 80W (yellow) – 20E (blue)	1s
	10s
	1min
3 60W (yellow) – 40E (blue)	1s
	10s
	1min

Table D-4 cont...

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
4 40W (yellow) – 60E (blue)	1s
	10s
	1min
5 20W (yellow) – 80E (blue)	1s
	10s
	1min
6 100E (blue)		Scale: 0.5 mm				

W-water, E-ethanol. *The flow rates mentioned in the table were same for both syringe pumps, except for in last two columns. The higher value of flow rate in both columns referred to flow rate applied to ethanol syringe pump.

Table D-5: Flow visualization of wells of microchip Design I under the microscope at 100× magnification over varied flow rates, with water from one inlet and acetic acid from the other.

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10ml/min	10-16 ml/min
1 80W (yellow) – 20V (blue)	1s
	10s
	1min
2 20W (yellow) – 80V (blue)	1s
	10s
	1min

Table D-5 cont...

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
3 60W (yellow) – 40V (blue)	1s
	10s
	1min
4 40W (yellow) - 60V (blue)	1s
	10s
	1min

Table D-5 cont...

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
5 & 6 50W (yellow) - 50V (blue)	1s
	10s
	1min
7 100W (yellow)
8 100V (blue)

Scale: 0.5 mm

W-water, V-acetic acid

*The flow rates mentioned in the table were same for both syringe pumps, except for in last two columns. The higher value of flow rate in both columns referred to flow rate applied to acetic acid syringe pump.

Table D-6: Flow visualization of wells of microchip Design II under the microscope at 100× magnification over varied flow rates, with water from one inlet and acetic acid from the other.

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
1
2 80W (yellow) – 20V (blue)	1s
	10s
	1min
3 60W (yellow) – 40V (blue)	1s
	10s
	1min

Table D-6 cont...

Well No.		Flow Rate (ml/min)*				
		2 ml/min	8 ml/min	16 ml/min	5-10 ml/min	10-16 ml/min
4 40W (yellow) – 60V (blue)	1s
	10s
	1min
5 20W (yellow) – 80V (blue)	1s
	10s
	1min
6 100V (blue)		Scale: 0.5 mm				

W-water, V-acetic acid. *The flow rates mentioned in the table were same for both syringe pumps, except for in last two columns. The higher value of flow rate in both columns referred to flow rate applied to acetic acid syringe pump.

APPENDIX E

Kanamycin, Ampicillin and its dilutions on *E. coli*

The OD versus time functions of ampicillin and kanamycin dilutions on *E. coli* performed in the microchip (refer Chapter 3, Section 3.2.1) are summarized in the graphs below. The plot shows the recorded OD of *E. coli* in each well (wells 2 to 7) marked in Figure 3.12 (Chapter 3) under the influence of antibiotics for 18 hours. The graph legend indicates the percentage of drug with corresponding percentage of DI water in v/v mixture. The 100% drug indicates the concentration of the drug equal to its MIC and no DI water dilution, followed by 80% which indicates 80% of the MIC with 20% DI water (v/v) and so on.

Figure E-1: The plots of Optical Density of the wells at 600 nm wavelength vs. time for ampicillin and its dilution (left), and for kanamycin and its dilution (right)

Ampicillin-Kanamycin combinations on *E. coli*

The OD versus time function for ampicillin and kanamycin combination case studies is illustrated in the plot below.

Figure E-2. The plot of Optical Density of the wells at 600 nm wavelength vs. time for the combination of ampicillin and kanamycin.

The combinations are represented as, A-K-80-20%, represents 80% of MIC of ampicillin with 20% of MIC of kanamycin (v/v) and so on.

APPENDIX F

Combination of ciprofloxacin and phytochemicals on MSSA and MRSA

The OD versus time functions of ciprofloxacin and the four phytochemicals on MSSA and MRSA performed in the microchip (refer Chapter 3, Section 3.3) are summarized in the graphs below (Figure F-1, F-2). The plot shows the recorded OD of MSSA or MRSA in each well (wells 2 to 7) marked in Figure 3.12 (Chapter 3) under the influence of antibacterial combination for 24 hours. The graph legend indicates the percentage of drug with corresponding percentage of DI water or phytochemical in v/v mixtures. The 100% drug indicates the concentration of the drug equal to its MIC. Similarly, the OD-time function for ofloxacin and its combination with phytochemicals is given in Figure F-3 and F-4.

OD-Time function for Ciprofloxacin at MIC and sub-MIC (MSSA) **OD-Time. Cip1-Quer512 (MSSA)** **OD-Time. Cip1-PCA4096 (MSSA)**

Figure F-1: The plots of Optical Density of the wells at 600 nm wavelength vs. time for ciprofloxacin and its dilution (top) followed by ciprofloxacin with phytochemical combinations against MSSA.

Figure F-2: The plots of Optical Density of the wells at 600 nm wavelength vs. time for ciprofloxacin and its dilution (top) followed by ciprofloxacin with phytochemical combinations against MRSA.

OD-Time function for Ofloxacin at MIC and sub-MIC (MSSA) **OD-Time. Of11-Quer512 (MSSA)** **OD-Time. Of11-PCA4096 (MSSA)**

Figure F-3: The plots of Optical Density of the wells at 600 nm wavelength vs. time for ofloxacin and its dilution (top) followed by ofloxacin with phytochemical combinations against MSSA.

Figure F-4: The plots of Optical Density of the wells at 600 nm wavelength vs. time for ofloxacin and its dilution (top) followed by ofloxacin with phytochemical combinations against MRSA.

APPENDIX G

Disk Diffusion Test

The averaged zone diameters for the disk diffusion tests carried out every 24 hours on each of the ciprofloxacin and its combinations exposed strains are given in Tables G-1 to G-5.

Table G-1: Averaged zone diameters of MSSA ATCC 29213 exposed to ciprofloxacin at MIC and sub-MIC levels for 360 hours*.

Time (hours)	Control MSSA ATCC 29213	Cip100	Cip80	Cip60	Cip50	Cip40	Cip20
0	25	25	25	25	25	25	25
24	21	BDL	30	25	15	15	17
48	21	BDL	15	17	15	16	17
72	21	23	16	17	14	15	18
96	21	13	14	15	15	15	16
144	21	11	13	12	13	12	15
168	21	10	13	12	13	14	15
192	21	11	13	12	13	16	16
216	23	10	13	11	13	16	15
240	23	10	12	12	13	15	13
264	23	10	12	12	14	13	14
312	21	9	11	10	11	14	16
336	21	9	13	11	15	13	16
360	21	9	12	11	10	13	14

Table G-2: Averaged zone diameters of MSSA exposed to ciprofloxacin (cip) in combination with quercetin (quer)*.

Time (hours)	Cip20-Quer80	Cip40-Quer60	Cip60-Quer40	Cip80-Quer20
0	25	25	25	25
24	BDL	BDL	BDL	BDL
48	30	BDL	20	15
72	30	BDL	BDL	17
96	BDL	30	30	15
144	BDL	20	BDL	16
168	BDL	BDL	25	17
192	BDL	30	BDL	15
216	BDL	BDL	BDL	16
240	BDL	25	BDL	15
264	BDL	22	22	17
312	BDL	22	20	15
336	BDL	BDL	20	18
360	BDL	30	16	16

Table G-3: Averaged zone diameters of MSSA exposed to ciprofloxacin (cip) in combination with rutin (rut)*.

Time (hours)	Cip20-PCA80	Cip40-PCA60	Cip60-PCA40	Cip80-PCA20
0	25	25	25	25
24	23	22	23	29
48	BDL	22	23	28
72	BDL	23	23	BDL
96	BDL	23	25	BDL
144	25	27	23	23
168	23	24	23	25
192	24	23	24	20
216	23	23	23	BDL
240	23	23	23	30
264	23	23	19	25
312	BDL	28	18	17
336	23	22	18	17
360	23	22	18	17

Table G-4: Averaged zone diameters of MSSA exposed to ciprofloxacin (cip) in combination with rutin (rut)*.

Time (hours)	Cip20-Rut80	Cip40-Rut60	Cip60-Rut40	Cip80-Rut20
0	25	25	25	25
24	19	20	20	15
48	19	21	20	15
72	19	20	21	15
96	18	20	21	15
144	25	19	20	15
168	BDL	19	21	15
192	13	19	19	14
216	11	17	18	11
240	15	17	19	15
264	15	17	19	14
312	BDL	17	19	16
336	30	17	20	15
360	14	17	19	10

Table G-5: Averaged zone diameters of MSSA exposed to ciprofloxacin (cip) in combination with EG*.

Time (hours)	Cip20-EG80	Cip40-EG60	Cip60-EG40	Cip80-EG20
0	25	25	25	25
24	23	26	30	28
48	25	25	17	BDL
72	30	24	18	15
96	21	20	18	16
144	19	22	21	16
168	21	18	17	15
192	15	17	21	16
216	BDL	21	17	15
240	18	18	16	15
264	21	18	19	15
312	23	21	18	16
336	25	21	17	15
360	25	21	17	15

***Legend for Tables H-1, H-2, H-3, H-4 and H-5:**

	more than equal to 21 mm- Susceptible
	Between 16 – 20mm - Intermediate
	less than 15 mm - Resistant

Population analysis profiling (PAP)

The PAP was carried out for the combinations for which, bactericidal or bacteriostatic outcomes were established in the time-kill assays. The PAPs for those ciprofloxacin-MIC, sub-MIC and combinations exposed strains are shown in Figure G-1 and G-2, respectively.

Figure G-1: PAP for MSSA ATCC parent strain exposed to 100%, 80%, 60%, 40% and 20% MIC of ciprofloxacin for the parent strain.

Figure G-2: PAP for MSSA ATCC parent strain exposed to Ciprofloxacin-phytochemical combinations (combinations indicated as Cip%-Phy%, % - percentage of MIC of respective drug v/v)

APPENDIX H

Experimental Setup

Figure H-1: Experimental Setup

The setup shown here is based on the block diagram in Figure 3.9, Chapter 3.

GLOSSARY

2D	Two Dimensional
3D	Three Dimensional
A/D	Analog/Digital
Ant	Antibiotic
ATCC	American Type Culture Collection
BHK	Baby-Hamster Kidney
BioMEMS	Biological Micro-Electro-Mechanical System
BSAC	British Society for Antimicrobial Chemotherapy
CDSS	Combinatory Drug Screening System
CFU	Colony Forming Unit
Cip or Cipro	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute (formerly, NCCLS)
CoNS	Coagulase-Negative Staphylococci
CSC	Cancer Stem Cells
DI	De-Ionized
DNA	De-oxyrbo Nucleic Acid
DRIE	Deep-Reactive Ion Etching
EG	Ethyl Gallate
ES	Excretion/Secretion
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
GC-MS	Gas Chromatography-Mass Spectrometry
HMDS	Hexamethyldisilazane
ICP	Inductively Coupled Plasma

IPA	Iso Propyl Alcohol
MDR	Multi-Drug Resistant
MEMS	Micro-Electro-Mechanical System
MIC	Minimum Inhibitory Concentration
MPC	Mutation Prevention Concentration
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin Sensitive <i>Staphylococcus aureus</i>
N2A	Mouse Neuroblastoma cell lines
NSC	Normal Neural Stem Cells
OD	Optical Density
Ofi or Oflox	Ofloxacin
PBP	Penicillin Binding Protein
PBS	Phosphate Buffer Saline
PCA	Protocatechuic Acid
PCR	Polymerase Chain Reaction
PDMS	Poly-dimethylsiloxane
Phy	Phytochemical
PTFE	Poly-tetrafluoroethylene
QRDR	Quinolone Resistance Determining Regions
Quer	Quercetin
Re	Reynolds Number
RIE	Reactive Ion Etching
RNA, rRNA, tRNA	Ribonucleic Acid, ribosomal RNA, Transfer RNA
Rut	Rutin
SCC	Staphylococcal Cassette Chromosome

STS	Surface Technology System
T _#	After "#" hours or at "#" hours
UV	Ultra Violet
VRSA	Vancomycin Resistant <i>Staphylococcus aureus</i>
μPIV	Micro Particle Image Velocimetry
μTAS	Micro Total Analysis System