ANTIBIOTIC SUSCEPTIBILITY OF CHEMICALLY-DISPERSED *PSEUDOMONAS AERUGINOSA* BIOFILM CELLS

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A Master dissertation for the study programme Master in Drug Development

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SAMENVATTING

*P. aeruginosa* is een multidrug resistente opportunistische bacterie die vooral in immuungecompromitteerde patiënten aanleiding geeft tot infecties van de luchtwegen en urinewegen. Door de multidrug resistentie faalt standaard antimicrobiële therapie vaak in het doden van alle cellen in de biofilm. Daarom wordt meer aandacht besteed aan het zoeken naar alternatieve behandelingen voor de multidrug resistente species.

Dit onderzoek bestudeert de antibiotica gevoeligheid van gedispergeerde *P. aeruginosa* biofilm cellen aan tobramycine, meropenem en ciprofloxacine. De biofilm werd gedurende vier dagen in een flow-cell opgegroeid met een minimaal medium dat glutamaat (1,8 mM) bevat als enige koolstofbron. De glutamaat concentratie werd vertienvoudigd (18 mM) om dispersie te诱导eren. Deze verhoging van de glutamaat concentratie resulteerde in een viervoudige verhoging in gedispergeerde cellen in vergelijking met een controle biofilm (1,8 mM Glu) na 15 min behandeling met 18 mM glutamaat. De effluent cellen van de glutamaat geïnduceerde en de controle biofilm (1.8 mM Glu) werden behandeld met 10 µg/ml tobramycine, 10 µg/ml meropenem of 1 µg/ml ciprofloxacine gedurende 1 tot 5 u. Planktonische cellen afkomstig van een overnacht cultuur werden gebruikt als controle. De controle en de glutamaat geïnduceerd gedispergeerde biofilm werden behandeld met 10 µg/ml tobramycine, 10 µg/ml meropenem of 1 µg/ml ciprofloxacine voor 24 u.

Er werd aangetoond dat geïnduceerde gedispergeerde cellen (3,4-log reductie) significant minder gevoelig zijn voor tobramycine dan spontaan gedispergeerde cellen (4,6-log reductie) en dan planktonische cellen (5,6-log reductie) na 5 u. Bovendien werd aangetoond dat geïnduceerde gedispergeerde cellen (3,5-log reductie) minder gevoelig zijn aan ciprofloxacine dan planktonische cellen (3,9-log reductie) met een significant verschil in afdoding na 3 u behandelen. Bij behandeling met meropenem werd geen significant verschil in afdoding gevonden. De biofilm behandeld met tobramycine, meropenem of ciprofloxacine, toonde geen significant verschil in afdoding tussen de geïnduceerd gedispergeerde en de controle biofilm.
**SUMMARY**

*P. aeruginosa* is a multidrug resistant opportunistic bacterium which causes infections of the urinary tract and the respiratory tract, especially in immune compromised patients. Due to the multidrug resistance, standard antimicrobial therapies nearly always fail to kill all the cells in the biofilm. Therefore, more attention is being paid to searching alternative treatments for the multidrug resistant species.

This research studies the susceptibility of dispersed *P. aeruginosa* biofilm cells to antibiotics such as tobramycin, meropenem and ciprofloxacin. The biofilm was grown in a flow-cell for 4 days with a minimal medium containing glutamate (1.8 mM) as sole carbon source. This glutamate concentration was then increased ten-fold (18 mM) to induce dispersion. The increase in the glutamate concentration resulted in a four-fold increase in dispersed cells compared to the control biofilm containing only 1.8 mM glutamate minimal medium. The four-fold increase in dispersed cells was observed after 15 minutes of 18 mM Glu treatment. The effluent cells from the glutamate induced and the control biofilm (1.8 mM Glu) were treated with 10 µg/ml tobramycin, 10 µg/ml meropenem or 1 µg/ml ciprofloxacin for 1 to 5 hours. Planktonic cells from an overnight culture were used as control. The control and glutamate induced dispersed biofilms were treated with 10 µg/ml tobramycin, 10 µg/ml meropenem or 1 µg/ml ciprofloxacin for 24 hours.

It was shown that induced dispersed cells (3.4 log reduction) are significantly less susceptible to tobramycin than spontaneously dispersed (4.6 log reduction) and planktonic cells (5.6 log reduction) after 5 hours of treatment. Moreover, it was shown that induced dispersed cells (log reduction 3.5) are significantly less susceptible to ciprofloxacin than planktonic cells (log reduction 3.9) after 3 hours of treatment. No significant difference in killing was observed after treatment with meropenem. The biofilm treated with tobramycin, meropenem or ciprofloxacin showed no significant difference in killing between the induced dispersed and the control biofilm.
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7. LITERATURE LIST
Rpm: Revolutions per minute
RT: Room temperature
SDS: Sodium dodecyl sulphate
SNP: Sodium nitroprusside
Tobra: Tobramycin
tRNA: Transfer ribonucleic acid
TSA: Tryptone soy agar
WHO: World health organisation
µg: Microgram
µl: Microliter
µM: Micromolar
1. INTRODUCTION

Recently, the WHO issued a list of resistant bacteria for which novel antibiotics are immediately required as these bacteria are resistant to the current available treatments and pose a great risk to the public health (1). The listed critical bacteria include *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and members of the Enterobacteriaceae. Bacteria have two modes of living, in which they either appear as planktonic or sessile cells. Planktonic bacteria are cells which have a free-living existence whereas sessile cells are attached to a surface. Biofilms are defined as attached bacterial cells embedded in a self-produced protective barrier called the matrix. The matrix is composed of extracellular proteins, extracellular polysaccharides, extracellular DNA as well as cell lysis products and material that bacteria encounter in their environment (2) (3). The matrix components are used by the bacteria as interconnecting products and hold the bacterial cells together. In addition, there are water channels present in the biofilm to allow the transport of water, nutrients and other substances to the cells embedded in the matrix. Biofilms come in all sizes and shapes such as mushroom, pillar-like structures or microcolonies and can be composed of a single bacterial species or multiple species (4). The biofilm life cycle consists of three different steps: initiation, maturation and dispersion as illustrated in figure 1.1.

Because the bacterial cells in the biofilm are closely packed together, the surrounding matrix acts as a protective barrier against various forms of stress including nutrient deprivation, toxic agents, such as antibiotics, pH changes but also the host immune system (5). This barrier increases the complexity of destroying the biofilm as opposed to planktonic cells, which are not surrounded by a protective barrier. This is the main reason these biofilms often lead to persistent chronic infections of the skin and mucus layers, as the barrier provides resistance towards most common antibiotics and explains the urgent need for new antibiotics as stated by the WHO (1). Bacterial species often involved in these biofilm infections are *P. aeruginosa*, *Escherichia coli*, *Staphylococcus spp.*, and *Enterococcus spp.* (2).
Figure 1.1.: Schematic presentation of the biofilm lifecycle consisting of the three steps: initiation, maturation and dispersion. This figure was adopted and modified from Petrova et al. 2016 (5).

1.1. PSEUDOMONAS AERUGINOSA
Approximately ten percent of the hospital acquired infections involve P. aeruginosa and are mostly located in either the urinary or the respiratory tract (6). P. aeruginosa is an opportunistic pathogen meaning only immune compromised patients might be infected, resulting in endocarditis, pneumonia, bacteremia, otitis externa, eye infections, skeletal infections or skin infections (7). Since this species shows high
resistance towards most antibiotics and disinfectants, it was listed as critical by the
WHO in the urgent search for novel treatment strategies (1).

*P. aeruginosa* is a Gram negative (G⁻) bacterium which is surrounded by an outer
membrane. *P. aeruginosa* belongs to the class of the Gammaproteobacteria in the
genus *Pseudomonas* (8). *P. aeruginosa* cells are rod-shaped with polar flagella and
contain a single circular chromosome (9). The bacteria have a facultative anaerobic
respiratory metabolism and can use oxygen as a terminal electron acceptor (8).
Nevertheless, they can also use other components as a terminal electron acceptor,
including NO₃ (10).

1.1.1. Antibiotic resistance
Since *P. aeruginosa* has an alarmingly high multidrug resistance, it was enlisted by
the WHO as critical (1). The species possesses two mechanism to acquire resistance
during treatment with antibiotics, either through mutations that alter the expression
and function of the encoded mechanisms or through acquiring resistance genes via
horizontal gene transfer (11).

The bacterial cells also possess several trump cards to protect themselves from
harmful substances. The major difference between Gram-positive and -negative
bacteria is that Gram-negative bacteria possess an outer membrane which protects
them from harmful substances including antibiotics (12). The outer membrane
contains porins which are channels connecting the periplasm to the extracellular
environment. They only allow small molecules to pass through and do not allow entry
of larger molecules such as peptides. This limit decreases the permeability of the
outer membrane and protects the bacteria from the intracellular accumulation of
antibiotics resulting in an increased survival (12). Additionally, *P. aeruginosa* has
developed multidrug resistance through the presence of multidrug efflux pumps,
which include MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN (13). For
example, MexXY-OprM extrudes aminoglycosides antibiotics, while others are known
to extrude tetracyclines, fluoroquinolones, macrolides, β-lactams and other types of antibiotics.

Resistance against several classes of antibiotics is known. First, aminoglycosides antibiotics such as tobramycin or gentamicin, which are commonly used in the treatment of *P. aeruginosa* infected cystic fibrosis (CF) patients, work by inhibiting translation (14). Four known resistance mechanisms exist against aminoglycosides (15). Firstly, resistance occurs through enzymatic modification by enzymes that either acetylate, phosphorylate or adenylate the aminoglycosides (15). This modification in turn inactivates the aminoglycosides. In addition, resistance against penicillin antibiotics and carbapenems antibiotics is known. Resistance against penicillin is achieved through the production of β-lactamases which disrupt the β-lactam ring of the antibiotics (11). Resistance against carbapenems antibiotics is due to an increase in the production of carbapenem hydrolysing enzymes. Secondly, there is resistance through the impermeability of the outer membrane (15). Thirdly, resistance exists through efflux pumps, which pump the antibiotic out of the cell. The last mechanism is through adaptive resistance, which is due to an aminoglycoside-induced efflux system (15).

1.2. INITIATION OF BIOFILM FORMATION

Free living planktonic *P. aeruginosa* cells possess flagella allowing them to move and encounter new biotic and abiotic surfaces (4) (16). The planktonic cells can then attach to a surface to form a biofilm. This transition from a free-living state to a sessile state completes the first step of the biofilm life-cycle: initiation (figure 1.1.).

However, when attaching to a surface, repulsive forces need to be conquered (17) (18). These exist between the negative surface charges of the cells and the negative charges on the desired surface. Van der Waals forces, flagella and fimbriae are used to overcome these inhibiting repulsive forces allowing the bacteria to attach to a surface (17). Flagella allow active motility to the surface which is important for the movement of the cell and the transport to the infection site since it allows the cells to
swim (19) (20). Flagella also possess adhesion properties but those properties are not a necessity for the attachment. Passive motility to a surface is due to Brownian motion or fluid flow and also contributes to the attachment (2).

Elsewise type IV pili are also necessary for attachment (2). Once the cells have formed a monolayer they move over the surface by a twitching motility which is achieved by a repeated extension and retraction of type IV pili (21). Twitching motility contributes to the virulence of the species since it allows the bacteria to enter and exit epithelial cells (22). Type IV pili are also necessary for the formation of a mature biofilm. However, extensive motility is known to inhibit the formation of microcolonies in biofilms. Host immune systems might promote this extensive motility to prevent biofilm formation (19). Human lactoferrin, an iron chelator, is such an immune response. Another form of movement is called swarming which is accomplished by flagella and type IV pili (23). Swarming motility is promoted when amino acids are the only nitrogen sources present (23). _P. aeruginosa_ is one of the rare species that possess three ways of movement: swimming, swarming and twitching motility (23).

Initially, the attachment is reversible meaning that bacterial cells can return to the bulk phase. This process is also known as desorption (5). However, under certain conditions, this attachment becomes irreversible. Several factors are known to occur during the transition from a reversible to an irreversible attachment including the reorientation from apical to longitudinal attachment, the formation of cell clusters and _lasB_ activation (20). The _lasB_ gene encodes an elastase, and is required for the swarming motility which contributes to the virulence of _P. aeruginosa_ (24) (25).

Attachment leads to surface-induced gene expression and synthesis of numerous proteins is found to be affected (26). For example, alginate, which is a part of the biofilm matrix, synthesis requires the alternative sigma factor AlgT (27). Once the bacteria are attached to a surface this sigma factor down regulates genes involved in motility, by example flagella (4). Conversely, other genes such as _algC_, involved in alginate synthesis, are upregulated (4). However, alginate is not necessary for biofilm formation (28). The surface induced gene expression prepares the bacterial cells to
enter the next phase of the biofilm lifecycle: maturation, as illustrated in figure 1.1. (4).

1.3. MATURATION OF BIOFILMS
The second step is maturation (figure 1.1.) which is characterised by an increased production of the matrix components and cellular division. In addition, biofilms will form a mushroom or pillar-like structure (3).

The matrix functions as a diffusion barrier which causes different nutrient gradients in the biofilm. Consequently, the biofilm is divided into two parts. First, the cells near the surface of the biofilm are characterized by a higher metabolic activity because these are near the nutrient source (2). Last, the part closest to the attached surface is characterized by cells that have a lower metabolic state, which is caused by an oxygen and nutrient deficiency.

1.3.1. Matrix production
The matrix is constituted out of extracellular polysaccharides, extracellular proteins and extracellular DNA. In addition, the matrix consists of cell lysis products and material originated from the surface surroundings (3).

Three extracellular polysaccharides have been described in P. aeruginosa biofilms: Pel, Psl and alginate (17). Psl is polysaccharide composed of D-mannose, D-glucose and L-rhamnose, Pel is a glucose rich exopolysaccharide (2) (29). Pel and Psl are matrix substances essential for the biofilm formation, regulated by the pel and psl gene respectively (17). Alginate, which is not required for primary biofilm development, provides strength and flexibility to the matrix and is essential for the retention of water and nutrients in the biofilm (30) (31). It also plays an important role in acquired antibiotic tolerance. The AlgC bifunctional enzyme plays a role in alginate production and lipopolysaccharide production (LPS) (17). Nevertheless, AlgC is also
important in the Pel and Psl synthesis and is essential for the matrix production (17). Other extracellular polysaccharides, such as levan and cellulose, have been described in other *Pseudomonas* species (32).

In addition to polysaccharides, proteins play an important role in the matrix composition. Proteins such as amyloid and CdrA have been described in *P. aeruginosa* biofilms. Amyloids are insoluble extracellular proteins and contribute to the strength of the biofilm. It also allows the bacterial cells to aggregate and aids in biofilm formation (33). CdrA has been described to bind to Psl which might facilitate polysaccharide-cell binding. As described earlier, flagella and type IV pili play an important role in the attachment to a surface (32).

The last matrix component is extracellular DNA (eDNA) and functions as a cell-to-cell interconnecting factor, which might lead to a more robust biofilm (30) (34). However, eDNA is also involved in the stalk formation of the biofilm, since it is more abundant in the inner part of the biofilm stalk.

1.4. DISPERSION OF BIOFILMS

There are several processes by which cells can leave the biofilm and these represent the last step of the biofilm lifecycle as seen in figure 1.1. Dispersion refers to the release of cells from the biofilm. This can be divided into different types depending on the factors that initiate the dispersion: spontaneous, induced and physical disruption (5). Dispersion allows the cells to escape the biofilm to colonize new surfaces and contributes to the spreading of the bacterial infection (10).

Dispersed cells show a transcriptomic difference from planktonic and biofilm cells (35). Numerous known and unknown genes are upregulated or down regulated in dispersed cells compared to biofilm and planktonic cells. For example, dispersed cells have a higher expression level of virulence genes such as *secB* and a lower expression level of genes involved in iron uptake such as *pvdS* compared to biofilm and planktonic cells (35).
1.4.1. Spontaneous dispersion
If the microcolony diameter of a biofilm reaches a critical 80 µm, the cells in the biofilm will disperse spontaneously (36). The multicellular structure and the barrier function of the matrix create a lack of nutrients and oxygen together with an accumulation of waste products resulting in stress. Initially, this stress will result in a lower metabolic state as described earlier. However, with a continuously present stress, the cells will eventually disperse from the biofilm. Spontaneous dispersion requires self-produced enzymes which destroy the matrix. This is in contrast with induced and physical dispersion in which external factors are required (5). When the cells leave the biofilm by spontaneous dispersion the biofilm structure is primarily characterised by central voids or cavities (36).

Enzymes which play a role in matrix-decomposition and are produced by the bacterial cells, include alginate lyase and glycosyl hydrolase PslG (5) (37). In addition, rhamnolipids also play a role in matrix decomposition (32). Alginate lyase degrades alginate, while PslG degrades the extrapolsaccharide matrix component PsL (38). Rhamnolipids are surface-active glycolipid biosurfactants and can aid in dispersion (32). It is also possible for the cells to produce extracellular enzymes that will decompose the substrate to which the biofilm is attached (39).

1.4.2. Induced dispersion
Dispersion might also be induced by signals originating from the environment resulting in so called induced dispersion. Theoretically, every environmental change might induce dispersion. However, several components have been described as dispersion inducers.

One of these is sodium nitroprusside (SNP) (10). SNP is a nitric oxide releasing compound which will induce dispersion when a biofilm is treated with low concentrations (around 500 nM) of SNP (10). In contrast, higher concentrations (in the millimolar range) have been shown to promote biofilm formation. P. aeruginosa can produce NO during anaerobic respiration from NO₃⁻ or NO₂⁻ as the terminal
electron acceptor. Low NO concentrations promote the transition to planktonic cells by induction of the phosphodiesterase DipA. This leads to lower concentrations of the secondary messenger, c-di-GMP and dispersion (40).

Another way of inducing dispersion is by a sudden increase of the carbon source (41). The carbon sources that have been described to induce dispersion include glucose, glutamate, succinate and citrate with succinate being the most effective (41). The sudden increase leads to a reduction in pilA-dependent β-galactosidase activity, as well as a reduction in pilA expression which are both important for the surface attachment and increased gene expression of flagella (41). In addition, other components such as detergents and iron chelators have been described to induce dispersion (42).

1.4.3. Physical disruption
Physical disruption consists of desorption and detachment (5). First, during desorption, as described during biofilm initiation, reversible attached cells will leave the surface.

Secondly, when a change in shear force occurs, for example due to the formation of air bubbles, detachment can be observed. Detachment can further be divided into several categories depending on the underlying machinery: grazing, abrasion, erosion and sloughing (5). Abrasion occurs due to the collision with particles residing in the bulk liquid (5). Grazing is when the biofilm is consumed by other organisms including eukaryotes such as protozoa (5) (43). As illustrated in figure 1.1, erosion is the constant release of bacterial cells during the biofilm lifecycle caused by fluid shear (39). This shear might also result in the release of biofilm clumps which is called sloughing (5).

1.5. BIOFILM FORMATION INVOLVES THE SECONDARY MESSENGER C-DI-GMP
The transition between the planktonic lifestyle and the sessile lifestyle is regulated by
the secondary messenger bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (44).

Biofilm formation is characterized by an increase of c-di-GMP while the planktonic lifestyle is marked by low c-di-GMP concentrations as illustrated in figure 1.2. (45). The concentration of c-di-GMP is regulated by enzymes such as phosphodiesterase (PDE) which degrade the second messenger to 5'-phosphoguananylyl-(3'-5')-guanosine (pGpG), and diguanylate cyclase (DGC) which forms c-di-GMP starting from two GTP molecules (46). DGCs contain a GGDEF active site motif whereas PDEs contain an EAL or HD-GYP domain (47).

![Figure 1.2](image-url)

Figure 1.2: a) c-di-GMP influence on sessile and free-living cells. b) c-di-GMP concentrations in the biofilm life-cycle. This figure was adopted and modified from Römling et al. 2006 (44).
Increased c-di-GMP concentrations result in the production of matrix components such as extracellular polysaccharides and adhesins, which aid in the attachment and prevent multiple forms of movement found in planktonic and dispersed cells. Accordingly, high c-di-GMP concentrations contribute to the decreased susceptibility of biofilm cells (47). In contrast, dispersed cells show a lower concentration of c-di-GMP (figure 1.2.b) and a higher concentration of the enzymes that destroy the matrix. When bacterial cells activate PDE’s, they manage to induce dispersion because it lowers the concentration of c-di-GMP leading to the upregulation of proteins involved in the motility (46).

One of the methods to regulate c-di-GMP concentrations is by communication via quorum sensing (QS). QS allows bacteria to measure cell density and plays a role in the secretion of extracellular DNA, which is a part of the matrix (17) (48). QS molecules play a critical role in the biofilm life-cycle. Such molecules in G- bacteria are called N-acyl homoserine lactones (AHL) and belong to the LuxI/LuxR-type quorum sensing system (49). \textit{P. aeruginosa} synthesises two such molecules: N-(3-oxododecanoyl)-L-homoserine lactone and N-butyryl-L-homoserine lactone (50). N-(3-oxododecanoyl)-L-homoserine lactone is produced by an enzyme that is encoded in the \textit{lasl} gene (4). \textit{lasl} knockout mutants fail to build multicellular biofilms (4).

The molecules are also referred to as autoinducers, can diffuse through the cell membrane and are important in regulating gene expression. With an increased cellular density, the concentration of autoinducers is increased, resulting in the expression or repression of the regulated genes (4) (51). One of these genes result in the induced expression of TpbA (52). TpbA is part of a regulatory system, which will eventually lead to the breakdown of c-di-GMP. This c-di-GMP decrease will induce dispersion and therefore reduces the number of sessile cells (52). Interfering with the cell-to-cell signalling, can disrupt biofilm formation and can be applied as a therapy. This can be achieved by degrading the signalling molecules enzymatically, blockage of the signal receptor or blockage of the signal generation (53).
1.6. ROLE OF BIOFILMS IN HUMANS

Biofilms play an important and advantageous role in the development of the human immune system and the digestion (2). They are found on the epithelial lining of the gastrointestinal tract and skin. Nevertheless, they are also present on artificial hearts, catheters, contact lenses and other medical devices (54). When the equilibrium between the host and its microbiota is disturbed, pathogenic bacteria such as *P. aeruginosa* can cause an infection. This is especially the case in immunocompromised patients, patients with cancer or those who suffer from CF. Several diseases are caused by biofilm infections such as cystic fibrosis pneumonia, native valve endocarditis and otitis media (43).

Biofilms show a high antibiotic tolerance due to numerous mechanisms (55) (13). Because of this, standard antimicrobial therapies nearly always fail to kill all the cells in the biofilm leading to persistent chronic infections. This results in an increased hospital residence time, an increase in the cost of healthcare, treatment failure and eventually a higher mortality rate (56). Therefore, it is crucial to find new ways to treat these chronic biofilm infections but also to prevent them from occurring.

1.7. INFLUENCE OF ANTIBIOTICS ON BIOFILMS

Several factors are responsible for the increased resistance in biofilms when compared to planktonic cultures.

1.7.1. The matrix as a barrier

The matrix acts as a barrier in two ways. By acting as a diffusion barrier and by the binding of harmful substances to matrix components (57).

As explained earlier, the matrix possesses a diffusion barrier for nutrients. Likewise, this diffusion barrier accounts also for antibiotics and other harmful substances. This leads to lower nonlethal antibiotic concentrations inside the biofilm. The presence of biofilm nutrient and oxygen gradients allows the biofilm to be divided into two subparts, where the top part is characterised by fast dividing cells and the bottom by
slow dividing cells (2) (57). Depending on the mechanism of action of the antibiotics used, they will either work on the top part or the bottom part of the biofilm structure. The slow penetration of antibiotics due to the matrix diffusion barrier leads to an increased adaptation time for the biofilm cells which allows the bacteria to transform into a less susceptible state before the required antibiotic concentrations inside are achieved (58). Transforming to a less susceptible state by decreasing metabolic activity, leads to slower dividing cells. As most antibiotics work by acting on fast dividing cells, they will therefore be unable or work less efficiently on these adapted cells. The cells that are metabolically inactive are referred to as persister cells as the antibiotic treatment has no effect on them, although they have not undergone any genetic changes (59). Therefore, they are not resistant but tolerant to antibiotics.

For example, ciprofloxacin works by inhibiting DNA gyrase and will therefore only affect the fast dividing cells as these need DNA gyrase for replication (60). So, the cells close to the attached surface will survive the treatment whereas the top cells will not. Tetracycline’s mechanism of action is by inhibiting the attachment of aminoacyl-tRNA (transfer ribonucleic acid) to the ribosomal acceptor (A) site, which is necessary for translation (61). Slow growth or low metabolic activity decreases the antimicrobial susceptibility to tetracyclines (62). Therefore, the deeper cells survive while only the top cells are killed. The same effect is again true for tobramycin, which also inhibits translation.

The matrix does not only prevent diffusion of harmful substances but it can also protect the bacterial cells by binding with these harmful substances. For example, tobramycin works by inhibiting translation and is a positively charged molecule (63). The ionic interaction between tobramycin and matrix components such as alginate is responsible for the limited penetration (63). This mechanism is applicable to aminoglycosides in general (63).
1.7.2. Efflux pumps

Efflux pumps are not only present in the outer membrane of planktonic cells but also in the sessile cells of a biofilm (64). A recent study has shown that the MDR efflux pumps of the planktonic cultures do not have a higher expression in biofilms and do not account for the higher resistance (65). However, a new efflux pump has been discovered, that has a significant higher expression in biofilm cells and is coded by the PA1874-1877 operon (66) (35). The pump belongs to the ABC family and is the first pump of the ABC transporter family detected in the species, which accounts for the increased resistance observed in biofilm cells (66).

1.7.3. Biofilm gradients

Other antibiotics, including peptides such as colistin, will disrupt the membrane of the bacterial cells causing them to leak, resulting in cell death (67). When biofilms are treated with colistin, only bottom cells are killed. The rapidly dividing top cells could adapt to colistin which led to an increased tolerance (2). There are two tolerance mechanisms. Firstly, there is tolerance through the MexAB-OprM efflux pump. Secondly, there is tolerance due to the polymyxin resistance LPS modification mechanism or pmr (68). In contrast, the slow dividing bottom cells could not adapt, resulting in cell death. Keeping this in mind, a combined treatment with colistin and for example ciprofloxacin could kill both parts of the biofilm cells and was found to nearly kill the entire biofilm (2).

*P. aeruginosa* has an alarmingly high multidrug resistance (1). The species is resistant to a great number of antibiotics including carbapenems and aminoglycosides. The multidrug resistance can be tackled by developing new antibiotics. In addition, it can be tackled by increasing antibiotic diffusion or by inhibiting the formation of the biofilms. Inhibiting the biofilm formation can be achieved by targeting the attachment phase or inducing dispersion. During this thesis, we tried to treat the biofilm by inducing dispersion and to determine the vulnerability of the dispersed cells and the remaining biofilm.
2. OBJECTIVES

*P. aeruginosa* is an opportunistic pathogen with resistance towards most antibiotics including meropenem and aminoglycosides and accounts for 10 % of the acquired hospital infections. *P. aeruginosa* can form biofilms on biotic and abiotic surfaces including medical devices (54). Several factors are responsible for the increased resistance in biofilms such as the matrix barrier, matrix gradients and efflux pumps. More attention has been given to finding new therapies to treat these infections. One of these may be inducing dispersion, followed by antibiotic treatment.

The first main objective of this thesis was to induce dispersion by a 10-fold increase in the concentration of glutamate, the sole carbon source. Biofilms were grown in a flow-cell for 4 days and dispersion was induced using a ten-fold increase in the glutamate concentration. Evidence for glutamate-induced dispersion was shown by plating, microscopy and flow cytometry. In addition, cell viability was determined via flow cytometry and microscopy in combination with a Live/Dead staining.

The second objective was to determine the antibiotic susceptibility of both the dispersed cells and the remaining biofilm. This was tested on a 4-day old biofilm, in which glutamate-induced dispersion was induced. Then the susceptibility to tobramycin (10 µg/ml), meropenem (10 µg/ml) and ciprofloxacin (1µg/ml) was tested.
3. MATERIALS AND METHOD

3.1. BACTERIAL STRAIN

The bacterial strain used was *P. aeruginosa* PAO1. Pure cultures were made on TSA-agar (Lab M. Limited, UK) and incubated at 37°C. These were used to make the overnight cultures in 1.8 mM glutamate minimal medium.

3.1.1. 1.8 mM glutamate minimal medium

The glutamate minimal medium, similar to Sauer et al. 2004, consisted of 2.56 g/L of Na$_2$HPO$_4$ (Sigma-Aldrich, MO, USA), 2.08 g/L of KH$_2$PO$_4$ (Sigma-Aldrich, MO, USA), 1.0 g/L of NH$_4$Cl (Fisher Scientific, UK.), 0.04 g/L of CaCl$_2$$\times$2H$_2$O (Sigma-Aldrich, MO, USA), 0.05 g/L of MgSO$_4$$\times$7H$_2$O (Fluka Chemika), 0.064 mg/L of CuSO$_4$, 0.16 mg/L of ZnSO$_4$$\times$7H$_2$O (Sigma-Aldrich, MO, USA), 0.1 mg/L of FeSO$_4$$\times$7H$_2$O (Fluka Chemika), 0.004 mg/L of MnCl$_2$$\times$4H$_2$O (Sigma-Aldrich, MO, USA), and 1.8 mM of glutamic acid (Sigma-Aldrich, MO, USA) (41). The pH was set at 7.2 (HI 2210 pH meter, Hanna Instruments) and the medium was sterilised via filtration (Stericup, EMD Millipore Corporation).

3.1.2. 18 mM glutamate medium

To 1 L of 1.8 mM glutamate minimal medium, 2.74 g of glutamic acid was added. The pH was set at 7.2 and the medium was filter-sterilised.

3.2. GROWTH CURVE

Two times 5 ml of overnight culture PAO1 OD$_{600}$ = 0.05 was centrifuged at 4500 g for 5 minutes. The medium was discarded and the pellet was resuspended in 5 ml 1.8 mM or 5 ml 18 mM glutamate minimal medium. 100 µl of each was added to a 96 well plate (SPL Life Sciences, Korea). LB (Lab M. Limited, UK) was used as a blank and as growth control in a 1/50 dilution. This was incubated at 37°C while the absorbance at 590 nm was measured (Envision 2104 multilaber reader, PerkinElmer) every half an hour for 24 hours.
3.3. FLOW-CELL SETUP AND BIOFILM FORMATION

A microscope slide (Marienfeld) and a 24x60 mm coverslip (Normax) were mounted onto the flow-cell (Biosurface technologies). The flow-cell was connected with bubble traps (Biosurface technologies) and flow breaks (Biosurface technologies) via platinum tubing 3.2x6.4 (Novolab) as illustrated in figure 3.1.

![Figure 3.1. Flow-cell set-up. A general overview of the flow-cell setup is given, indicating the position of the different instruments.](image)

These were autoclaved for 20 minutes at 121°C. The flow-cell was connected to a 520 S peristaltic pump (Watson Marlow) and primed with 1.8 mM glutamate minimal medium. Then the flow-cell was inoculated with 5 ml *P. aeruginosa* PAO1, *OD*$_{600}$ = 0.05 and incubated under static conditions at room temperature (RT) for one hour. Finally, a flow was created of 0.2 ml/min at RT for four days.
3.4. INDUCTION OF DISPERSION

3.4.1. Determination of dispersion via plating
A four-day old biofilm was treated with 18 mM glutamate medium for 15 minutes. Then 100 µl effluent was collected in a 96 well culture plate every 15 minutes for 2.5 hours. Cell numbers were determined using plate counts in TSA. To determine biofilm cell numbers after treatment, the flow-cell was dismantled and the biofilm on the coverslip was scraped off using a cell scraper (Falcon) and 1 ml physiological saline. This was then used to determine cell numbers via plate counts in TSA.

3.4.2. Microscopy
A four-day old biofilm, grown in the flow-cell, was treated with 18 mM glutamate medium for 2 hours. The flow-cell was disconnected from the tubing to perform microscopy on it. The flow-cell was imaged (Olympus BX41, Olympus) with 40 times magnification using transmission light.

For Live/Dead fluorescence imaging, a four-day old biofilm was treated with 18 mM glutamate medium for 2 hours. The flow-cell was then stained using 3 ml L7012 Live/Dead BacLight solution (Fisher Scientific) containing 10.02 µM SYTO9 and 60 µM Propidium iodide (PI). This was then incubated in the dark at RT for 15 minutes. The coverslip and the microscope slide were collected from the flow-cell and fixed to a clean microscope slide and coverslip accordingly. Finally, these were imaged (Evos FL auto, Thermo Fisher Scientific) using 470 nm led light to visualize the live cells and 531 nm led light to visualize the dead cells. The used magnification was 40 times. The number of cells was then determined via surface coverage.

3.4.3 Flow cytometry
A four-day old biofilm was treated with 18 mM glutamate medium. The effluent (100 µl) was harvested in a 96 well plate every 15 minutes for 2.5 hours. Next, the effluent was centrifuged (Eppendorf 5804 R) at 2464 g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 100 µl Live/Dead solution and incubated in dark at RT for 15 minutes. Next, samples were centrifuged at 2464 g for
5 minutes. The staining solution was discarded and the pellet was resuspended in 100 µl physiological saline. Cell numbers were then determined using cell viability flow cytometry (Attune NxT, ThermoFisher Scientific).

3.5. MIC TEST

3.5.1. Antibiotic stock solutions
For ciprofloxacin (Sigma-Aldrich, MO, USA), meropenem (Sigma-Aldrich, MO, USA) and tobramycin (Tokyo Chemical Industry) a 10 ml stock solution of 1 mg/ml, 2 mg/ml and 2 mg/ml respectively was made. All stock solutions were made using millipore water, the solutions were sterilized using a 0.2 µm filter (Merck Millipore). To the stock solution of ciprofloxacin 1 ml 1 M HCL was added to dissolve the antibiotic.

3.5.2. Antibiotic solutions
The stock concentration of tobramycin was diluted to a 20 µg/ml solution using 1.8 mM glutamate minimal medium, 18 mM glutamate medium or MQ (Millipore Corporation). This was done similarly for meropenem (4 µg/ml) and ciprofloxacin (2 µg/ml).

3.5.3. Inoculum
The optical density (OD) of the overnight culture was set to $\text{OD}_{600} = 0.05$ using 1.8 mM glutamate minimal medium, 18 mM glutamate minimal medium or double concentrated MHB (Lab M. Limited, UK). The inoculum in double concentrated MHB was then diluted one over 100 using double concentrated MHB.

3.5.4. Plate preparation
A 1:2 dilution series of the antibiotic was made in a 96 well plate (SPL life sciences) using 1.8 mM glutamate minimal medium, 18 mM glutamate minimal medium or MQ. 100 µl sterile medium and MQ was used for the growth control and the sterility control. 100 µl of diluted overnight culture was added to each well, except the sterility control. To the sterility control, another 100 µl of medium was added. After incubation
at 37°C for 24 hours, the absorbance at 590 nm (Envision 2104 multilaber reader, PerkinElmer, Waltham MA, USA) was measured.

3.6. ANTIBIOTIC TREATMENT OF THE EFFLUENT AND REMAINING BIOFILM
The four-day old biofilm was treated with 18 mM Glu medium for 30 minutes. Next, effluent of both control and treatment were collected in a 15 ml tube for 15 minutes. The effluents were then divided over 5 wells in a 96 well plate, 100 µl per well. To these wells, 100 µl tobramycin (20 µg/ml) in 1.8 mM or 18 mM Glu was added and incubated at room temperature for 1 to 3 and 5 hours. 100 µl of untreated effluent was plated as a reference of the original bacterial load. An overnight culture (OD₆₀₀ = 0.05) in 1.8 mM or 18 mM Glu minimal medium was treated similarly. Untreated overnight culture was also plated to determine the start concentration of bacteria. Cell numbers were determined via plate count.

To the biofilm, 5 ml of 1.8 or 18 mM glutamate was added. To determine the biofilm cell numbers the flow-cell was dismantled and the biofilm on the coverslip was scraped off, using a cell scraper and 1 ml physiological saline. This was then used to determine cell numbers via plate counts on TSA. To the other biofilm, 5ml of 10 µg/ml tobramycin in 1.8 mM or 18 mM Glu minimal medium was added. These were incubated under static conditions at RT for 24 hours. Finally, these biofilms were also collected and plated as described above.

Likewise, the effluent and the biofilm were treated similarly with (1 µg/ml final concentration) ciprofloxacin or meropenem (10 µg/ml final concentration).
4. RESULTS

4.1. GROWTH CURVE

To assess the influence of a 10-fold increase in concentration of the carbon source, a growth curve was made. During the growth curve experiment, 1.8 mM Glu minimal medium was compared to 18 mM Glu minimal medium and LB-broth (positive control). As illustrated in figure 4.1, the generation time of *P. aeruginosa* in 1.8 mM Glu minimal medium was 9.7 hours per division. Whereas the generation time in 18 mM Glu minimal medium was 3.7 hours per division.

![Figure 4.1. Growth curve of *P. aeruginosa* in LB (growth control), 1.8 mM minimal glutamate medium and 18 mM glutamate medium. The absorbance at 590 nm was measured every half an hour for 24 hours at 37°C.](image)

4.2. EVIDENCE OF DISPERSION

4.2.1. Determination of dispersion via plating

To determine whether a tenfold increase of the sole carbon source could lead to dispersion, the effluent of a 4-day old biofilm was plated. Next, the fold change of the effluents, treatment effluent (18 mM Glu) divided by control effluent (1.8 mM Glu), was determined. This showed a significant increase (*p* < 0.05) in cell numbers from 0:00 h until 1:15 h (figure 4.2.).
As illustrated in figure 4.2.A, the highest fold change (4.38) was noticed after 15 minutes. The remaining biofilm of both the treated and untreated sample was plated after 2.5 hours. The cell numbers were compared with each other, but no significant difference ($p > 0.05$) was observed (figure 4.2.B).
4.2.2. Microscopy

To investigate if dispersion was induced using a ten-fold increase in the glutamate concentration, mature biofilms were grown for four days with 1.8 mM glutamate as sole carbon source (control) and compared to induced dispersed biofilms (treatment). Dispersion was induced with 18 mM glutamate. The microscopic pictures in figure 4.3 illustrate the biofilm before (Fig. 4.3.A) and after induced dispersion with 18 mM glutamate medium (Fig. 4.3.B).

![Microscopic images of biofilms](image)

**Figure 4.3.** Comparison of control and induced dispersed biofilms. Biofilms were grown in a flow-cell for 4 days with minimal medium containing 1.8 mM glutamate as sole carbon source. Induced dispersion by a 10-fold increase in glutamate concentration for 2 h. A) Control: 1.8 mM B) Induced dispersion: 18 mM C) Average diameter of cell clusters in a 4-day old biofilm before (1.8 mM Glu) and after induced dispersion with 18 mM Glu, n = 3. The scale bar indicates 100 µm. * = p < 0.05.

Dispersion will lead to a loss of cells in the biofilm and the formation of voids. Large cell clusters of 43 µm diameter are observed in Fig. 4.3.A and smaller cell clusters of 24 µm diameter with voids in Fig. 4.3.B. The difference in cell cluster diameter between the control (1.8 mM Glu) and the treated biofilm (18 mM Glu) was significant.
(p < 0.05). This indicates a decrease in cell cluster diameter after induced dispersion with 18 mM Glu.

To determine if there were clumps, which are cells with matrix, present in the effluent from the flow-cell of the control biofilm and the biofilm treated with 18 mM glutamate medium, microscopy was performed on the effluent of a four-day old biofilm after 15 minutes of treatment with 18 mM glutamate (figure 4.4.).

As illustrated in figure 4.4. there are no clumps present in the effluent of the control biofilm (1.8 mM). However, there are clumps present in the effluent of the induced dispersed biofilm (18 mM). In addition, more cells are seen in figure 4.4.B compared to figure 4.4.A.

To examine if there were more dead cells due to a ten-fold increase in the glutamate concentration, the biofilms were stained with 3 ml Live/Dead (Baclight, Fisher Scientific). Figure 4.5. illustrates the biofilm before (Fig. 4.5.A and C) and after a 2-hour treatment with 18 mM glutamate medium (Fig. 4.5.B and D).
Green cells are live cells and red cells are dead cells. The number of red cells was determined via surface coverage. There is no significant ($p > 0.05$) difference in the surface coverage of red cells in Fig. 4.5.D compared to Fig. 4.5.C, showing dispersion with 18 mM Glu does not kill the biofilm cells. Also, cell viability flow cytometry was used to examine if there were more dead cells due to induced dispersion with 18 mM Glu.

Figure 4.5. Life/dead fluorescence at 470 nm and 531 nm, and using transmission light at magnification 40. Biofilms were grown in a flow-cell for 4 days with minimal medium containing 1.8 mM glutamate as sole carbon source. Induced dispersion by a 10-fold increase in glutamate concentration. A) Control: 1.8 mM phase contrast image B) Induced dispersion, phase contrast image C) Control: 1.8 mM, Live/Dead fluorescence D) Induced dispersion, life/dead fluorescence E) Average surface coverage ($\mu m^2$) of red cells of the control biofilm (1.8 mM) compared to the treated biofilm (18 mM), $n = 3$. 
**4.2.3. Flow cytometry**

To examine if induced dispersion using 18 mM glutamate killed the biofilm cells, flow cytometry on the effluent of the flow-cell was performed after a Life/Dead staining. In the live cells, a similar trend to the plate counts was obtained, while this was not observed in the dead cells as shown in figure 4.6. This might indicate that the treatment (18 mM) did not kill the cells from the biofilm. Due to high variance, all time points were not significantly different from 1.

![Figure 4.6](image_url)

**Figure 4.6.** The fold change in the number of cells in the effluent of the flow-cell during treatment over time. A four-day old biofilm, grown in 1.8 mM Glu minimal medium was treated with a 10-fold Glu increase. The effluent was collected every 15 min for 2.5 h and cells were stained with Live/Dead followed by flow cytometry. The fold change, the treatment (18 mM) divided by control (1.8 mM) in effluent cells, is illustrated. A) The fold change in the number of live cells. B) The fold change in the number of dead cells. Error bars indicate standard error, (n = 3).

**4.3. MIC**

To have an idea about the antibiotic concentrations that should be used and whether these were medium dependant, MIC’s were determined (table 4.1.).

**Table 4.1.** MIC values (µg/ml) determined in 1.8 mM glutamate minimal medium, 18 mM glutamate medium and MHB for ciprofloxacin meropenem and tobramycin.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>1.8 mM Glu (µg/ml)</th>
<th>18 mM Glu (µg/ml)</th>
<th>MHB (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ciprofloxacin</td>
<td>0.0625</td>
<td>0.0625</td>
<td>0.156</td>
</tr>
<tr>
<td>meropenem</td>
<td>0.500</td>
<td>0.625</td>
<td>2.50</td>
</tr>
<tr>
<td>tobramycin</td>
<td>0.625</td>
<td>0.625</td>
<td>0.625</td>
</tr>
</tbody>
</table>
Based on these results and on literature, the following treatment concentrations were chosen: 10 µg/ml tobramycin, 10 µg/ml meropenem and 1 µg/ml ciprofloxacin.

4.4. ANTIBIOTIC TREATMENT OF DISPERSED CELLS AND REMAINING BIOFILMS
To investigate the effect of antibiotics including tobramycin, meropenem and ciprofloxacin on dispersed biofilm cells, 4-day old biofilms were dispersed using 18 mM glutamate for 15 minutes. The effluent of the flow-cell was treated with antibiotics for 1, 2, 3 or 5 hours, and plated. Overnight cultures were treated similarly. In addition, the remaining biofilm after dispersion was treated with antibiotics for 24 hours and plated.

4.4.1. Tobramycin
The effluent of the flow-cell and overnight cultures were treated with tobramycin (10 µg/ml). The effluent from the flow-cell after induced dispersion with 18 mM glutamate had a significantly less killing (p < 0.05) of cells after 1 to 3 and 5 hours of treatment with tobramycin than the effluent from the flow-cell in 1.8 mM glutamate minimal medium (figure 4.7.).

![Figure 4.7. Remaining CFU in the effluent of the flow-cell and overnight culture after a 1,2,3 and 5 h treatment with tobramycin (10 µg/ml). 4-day old biofilms were grown in a flow-cell with 1.8 mM glutamate minimal medium as only carbon source. Induced dispersion by a 10-fold increase in glutamate concentration. Overnight cultures were set to OD\textsubscript{600} = 0.05 using 1.8 and 18 mM of glutamate. Error bars indicate standard error (n = 3), * = p < 0.05.](image)
In addition, the effluent from the flow-cell after induced dispersion with 18 mM glutamate had a significantly less killing (p < 0.05) of cells after 1 to 3 and 5 hours of treatment with tobramycin than the planktonic culture in 18 mM Glu. The spontaneous dispersed cells showed a 4.6 log reduction whereas the glutamate-induced dispersed cells showed a 3.4 log reduction after 5 hours treatment. This indicates that glutamate-induced dispersed cells are more tolerant to tobramycin than spontaneously dispersed cells. Compared with the planktonic culture that showed a 5.6 log reduction after 5 hours treatment, indicating that glutamate-induced dispersed cells are more tolerant to tobramycin than planktonic cells. Spontaneously dispersed cells showed no significant difference with the planktonic culture after a 5-hour tobramycin treatment.

The remaining biofilm was treated with 10 µg/ml tobramycin. However, no significant difference was detected before and after treatment with tobramycin, nor between the control and the glutamate induced dispersed biofilm (figure 4.8.).

Figure 4.8. Remaining CFU of the biofilm after 24 h treatment with tobramycin (10 µg/ml). 4-day old biofilms were grown in a flow-cell with 1.8 mM glutamate minimal medium as only carbon source. Induced dispersion by a 10-fold increase in glutamate concentration with 18 mM glutamate medium. Error bars indicate standard error (n = 3).
4.4.2. Meropenem

Effluent from the flow-cell and overnight cultures were treated with meropenem (10 µg/ml). The glutamate induced dispersed cells showed a 2.4 log reduction after 5 hours treatment whereas the spontaneous dispersed cells showed a 1.8 log reduction. The overnight culture showed a 1.4 log reduction after 5 hours of meropenem treatment. The observed differences were not significantly different (p > 0.05) (figure 4.9.).

![Graph showing the influence of meropenem on dispersed and planktonic cells over time.](image)

**Figure 4.9.** The influence of meropenem (10 µg/ml) on dispersed and planktonic cells over time. Biofilms were grown in a flow-cell for 4 days with 1.8 mM glutamate minimal medium as sole carbon source. Dispersion was induced by a 10-fold increase in glutamate concentration. Overnight cultures were set to OD₆₀₀ = 0.05 using 1.8 and 18 mM of glutamate. Error bars indicate standard error (n = 3).

The remaining biofilm was treated with meropenem (10 µg/ml). When looking at the remaining biofilms, no significant difference was observed between the control and
the treated biofilms as illustrated in figure 4.10. Also, no significant difference was observed between the induced dispersed biofilm and the control biofilm.

Figure 4.10. Remaining biofilm after 24 h meropenem (10 µg/ml) treatment. Biofilms were grown in a flow-cell for 4 days with 1.8 mM glutamate minimal medium as sole carbon source. Dispersion was induced by a 10-fold increased glutamate concentration. Biofilms were treated with 10 µg/ml meropenem in medium. Error bars indicate standard error (n = 3).

4.4.3. Ciprofloxacin
As illustrated in figure 4.11, overnight cultures and the effluent from the flow-cell were treated with ciprofloxacin (1 µg/ml) for 1 to 3 and 5 hours. Spontaneous dispersed cells have a log reduction of 3.9 in comparison with induced dispersed cells with a log reduction of 3.5 after 5-hours of treatment with ciprofloxacin. The overnight culture in 1.8 mM glutamate showed a log reduction of 3.5 whereas the overnight culture in 18 mM glutamate medium showed a log reduction of 3.9. There was significant less killing (p < 0.05) in the induced dispersed cells compared to the overnight culture in 18 mM glutamate medium after 3 hours of treatment with
Ciprofloxacin. This indicates that induced dispersed cells are more tolerant to ciprofloxacin than planktonic cells (figure 4.1).

The remaining biofilms were treated with ciprofloxacin (1 µg/ml). As illustrated in figure 4.12, no significant difference was observed between the control and the treated biofilms. In addition, no significant difference was observed between the glutamate induced dispersed and the control biofilm grown in 1.8 mM glutamate minimal medium.
Figure 4.12. Remaining biofilm after treatment with ciprofloxacin (1 µg/ml) for 24h. Biofilms were grown in a flow-cell for 4 days with 1.8 mM glutamate minimal medium as sole carbon source. Dispersion was induced by a 10-fold increase in glutamate concentration with 18 mM glutamate medium. Biofilms were treated with 1 µg/ml ciprofloxacin in 1.8 or 18 mM glutamate medium. Error bars indicate standard error, (n = 3).
5. DISCUSSION

5.1. EVIDENCE OF BIOFILM DISPERSION

Dispersion, the process by which biofilm cells leave the biofilm, can be induced by environmental changes including an increase in the carbon source concentration (10) (41) (42). The dispersion method used in this thesis, a tenfold increase in the glutamate concentration as the sole carbon source, was similar to an earlier study (41).

The obtained plating data showed a significant (p < 0.05) 4.38-fold change in the number of effluent cells after 15 minutes. This was in agreement with the turbidity data from an earlier study (40), indicating that dispersion is induced early after the environmental change. To confirm that the increase in effluent cells was not due to growth, a growth curve was made. Although the 18 mM glutamate minimal medium showed an increase in growth speed, the generation time (3.7 h) was higher than the time needed to induce dispersion. Similar results were also observed by Sauer et al 2004 (41). Most studies determine dispersion via surface coverage of an untreated and treated biofilm (40) (34) (41). From the microscopic images a decrease in cell cluster diameter after induced dispersion with 18 mM Glu was also noticed.

To have an idea whether the dispersion was an active mode of dispersion, cell viability flow cytometry was performed. In active dispersion, cells release matrix degrading components. Whereas in passive dispersion, the matrix decomposition is due to a dispersal agent such as sodium dodecyl sulphate (SDS) (39). Glutamate induced dispersion is a form of active dispersion. During an active mode of dispersion, only an increase in live cells would be expected. In contrast to passive dispersion, where an increase in both live and dead cells may be expected. Despite the insignificant difference of the fold change in live cells from 1, a similar trend was noticed between the fold change in live cells determined via flow cytometry and plating. The high variance observed at 15 minutes in the fold change of live cells with flow cytometry, might be due to an outlier. The experiment should be repeated to determine if there was an outlier present. Flow cytometry results showed no
significant difference in the fold change of dead cells from 1, indicating that glutamate induced dispersion did not kill the biofilm cells.

In addition, fluorescence microscopy on a Live/Dead stained biofilm before and after treatment with 18 mM glutamate did not show more dead cells in the treated biofilm compared to the control, as determined via surface coverage. This indicated that the treatment with 18 mM glutamate did not kill the biofilm cells.

All the data mentioned above suggest that cells actively escaped the biofilm, leading to a decrease in the number of cells in the biofilm after a 10-fold increase in the glutamate concentration as sole carbon source. The biofilm mode of growth is only nutritionally advantageous when low nutrient concentrations such as glutamate are present (41). This could explain why a ten-fold increase in the glutamate concentration induced dispersion.

5.2. ANTIBIOTIC TREATMENT OF DISPERSED CELLS AND REMAINING BIOFILMS

The results obtained show that glutamate induced dispersed cells are more tolerant to tobramycin (10 µg/ml) than spontaneously dispersed or planktonic cells. In addition, induced dispersed cells were observed to be more tolerant to ciprofloxacin (10 µg/ml) compared to planktonic cells.

Experiments with a 5-hour treatment of induced dispersed, spontaneously dispersed and planktonic cells with tobramycin (10 µg/ml), showed a significant difference in the number of induced dispersed cells compared to spontaneously dispersed cells or planktonic cells. This indicates that glutamate dispersed cells are less susceptible to tobramycin than spontaneously dispersed or planktonic cells. The mechanism of action of tobramycin is inhibition of the movement of peptidyl-tRNA from the ribosome A-site to the P-site (2). A lower expression of genes involved in translation could possibly account for the reduced susceptibility of induced dispersed cells compared to planktonic cells. Nonetheless, transcriptomic data from Chua et al. 2014 observed
a higher expression of genes involved in translation in SNP induced dispersed cells compared to planktonic cells (35). However, there is no transcriptomic data available for glutamate induced dispersed cells. The reason for the decreased susceptibility to tobramycin of induced dispersed cells compared to planktonic cells remains unknown.

Microscopy performed on the effluent from the control and the induced dispersed biofilm showed the presence of clumps in the effluent from the induced dispersed biofilm. Tobramycin is positively charged and can bind to the matrix due to an ionic interaction with alginate or eDNA, both matrix components (63) (69). This reduces the effectiveness of tobramycin. Since the presence of clumps was only observed in the effluent from the induced dispersed biofilm, the binding of tobramycin to alginate or eDNA could be a possible explanation for the increased tolerance of induced dispersed cells compared to spontaneously dispersed cells. Further experiments are required to provide concrete evidence of matrix presence in the effluent of an induced dispersed biofilm and the absence of matrix in effluent from the control biofilm to support this hypothesis.

In addition, it was observed that induced dispersed cells are more tolerant to ciprofloxacin than planktonic cells after 3 hours of treatment. Ciprofloxacin works by inhibiting DNA gyrase and will therefore only affect the fast diving cells as these need DNA gyrase for replication (60). A possible explanation for the decreased susceptibility to ciprofloxacin of induced dispersed cells, could be that induced dispersed cells have a lower expression of genes involved in DNA replication. Transcriptomic data from Chua et al 2014 showed that SNP induced dispersed cells have a higher expression of genes involved in DNA replication (35). The reason for the increased tolerance of glutamate induced dispersed cells observed in our experiments is still not known.

No significant difference in the number of cells after 5 hours of treatment with meropenem (10 µg/ml) was observed between induced dispersed, spontaneously
dispersed, or planktonic cells. In addition, no significant difference was noticed in the number of cells between the biofilm before and after treatment with tobramycin, meropenem or ciprofloxacin for 24 hours. Due to the matrix gradients, cells deeper in the biofilm possess a lower metabolic activity than cells closer to the surface (55) (62). In addition, the gradients lead to decreased oxygen levels deeper inside the biofilm. The decreased oxygen concentration and lower metabolic activity lead to an increased tolerance of biofilm cells to antibiotics, including ciprofloxacin and tobramycin, as these work on fast dividing cells (2) (62). This could be the reason that no significant difference was observed between the biofilm before and after a 24 hour antibiotic treatment with tobramycin or ciprofloxacin.

In the future, it would be interesting to treat glutamate induced dispersed biofilms with tobramycin or ciprofloxacin and an iron chelator, as dispersed cells are more sensitive to iron stress than planktonic cells (35). This is similar to Chua et al (35). In addition, it would be interesting to compare the susceptibility of other antibiotics such as colistin or detergents of induced dispersed, spontaneously dispersed and planktonic cells.
6. CONCLUSION

In conclusion, chemical dispersion of *P. aeruginosa* PAO1 can be induced using a 10-fold increase in the glutamate concentration as sole carbon source. Based on antibiotic treatment using tobramycin, meropenem and ciprofloxacin before and after glutamate induced dispersion of the *P. aeruginosa* biofilm, it could be concluded that glutamate induced dispersed cells from a *P. aeruginosa* biofilm are more tolerant to tobramycin than spontaneous dispersed cells or planktonic cells. In addition, glutamate induced dispersed cells are more tolerant to ciprofloxacin than planktonic cells.

Experiments with the fold change, the number of cells from the effluent of the induced dispersed biofilm divided by the number of cells in the effluent from the control biofilm, showed the highest fold change after 15 minutes. In addition, microscopic images of the biofilm before and after 2 hours of glutamate induced dispersion showed smaller cell clusters and more voids in the induced dispersed biofilm than in the control biofilm. Microscopy on a Live/Dead stained biofilm before and after glutamate induced dispersion and flow cytometry on the effluent of the biofilm during induced dispersion showed that glutamate induced dispersion did not kill the biofilm cells. All the above led to the conclusion that cells were actively dispersed after a ten-fold increase in the glutamate concentration.

Based on antibiotic treatment on induced dispersed, spontaneous dispersed, planktonic and biofilm cells, it was shown that glutamate induced dispersed cells are more tolerant to tobramycin than spontaneous dispersed cells or planktonic cells. In addition, induced dispersed cells were shown to be more tolerant to ciprofloxacin than planktonic cells. In contrast to the results obtained for meropenem, which showed no significant difference between induced dispersed, spontaneously dispersed or planktonic cells. No difference was observed between the glutamate induced dispersed biofilm and the control biofilm after treatment with tobramycin, meropenem or ciprofloxacin.
Future experiments could evaluate the susceptibility of other antibiotics such as colistin, disinfectants or a combination after glutamate induced dispersion.
7. LITERATURE LIST


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INTERNATIONALIZATION AT HOME

1. Prof. Sandra Van Dulmen: Therapietrouw en communicatie; van woorden naar daden

Compliance refers to the amount of a prescribed dose of the drug taken by the patient. A good compliance is above 80% and is important to ensure the effect of the treatment and prevent complications. Compliance concerns a wide range of medicines. There are several reasons why patients show a low compliance and it can be divided into two subcategories: intentional and non-intentional noncompliance. The biggest reason for noncompliance is non-intentional by forgetting to take the medicine. It is a tenacious problem because so many factors are involved. The highest effect in improving the compliance is reached through working on the psychosocial factors. This all depends on good communication and thus contact with the caretaker. For example, when the doctor prescribes the drug, he should ask the patient to repeat the information to prevent noncompliance. On the other hand, the problem should also be addressed appropriately, by using non-open and non-directing questions.

2. Prof. Claus-Michael Lehr: Nanomedicine-Opportunities for non-invasive drug delivery and combatting infectious diseases

Drug delivery is difficult to achieve because the body possesses numerous barriers. There is a dual strategy to tackle these barriers. First, is the use of in vitro models of barriers. Second, is the use of carriers and technology. The gastro intestinal tract is characterised by the presence of oral and intestinal mucosa. Nanomedicines will lead to an increase in solubility, which will lead to a decrease in dissolution velocity due to the larger surface area. In IBD, nanomedicines can be used as a carrier because the inflamed mucosa will possess a higher permeability, allowing the particles to pass through. When looking at the skin, carriers have an improved effect and immune response and can be utilised in the form of an ointment for people with needle phobia. The lungs possess alveoli with a large surface area. A first in vitro case study for infectives has been performed for *P. aeruginosa* biofilms. Here, nanocarriers can be dispersed in water to create an
aerosol that can penetrate the lungs so that the drug is incorporated in the nanoparticles.

3. Prof. Bryony D. Franklin: Designing and evaluating interventions to reduce medication errors: challenges and opportunities
When administering drugs, you want to avoid side effects, medication errors and optimise the use by the patient. 1 to 2% of patients are harmed by medication errors. One way of avoiding such errors is by implementing an electronic prescribing system instead of using paper charts. However, stamping or printing the name on the medication order is also a solution. Another way of avoiding medication errors, is by preventing errors from reoccurring. This can be achieved by improving the pharmacists feedback to doctors when an error has occurred. Nonetheless, this led to little difference in prescribing error because the reasons for these medication errors vary widely and are complex. Other solutions are the usage of a checklist when prescribing, simply referred to as ‘check and correct’, or the use of Dr-CARD.

4. Mr. Pedro Facon: Reform royal decree nr. 78 and implications for health care workers
Belgium is well situated regarding expenditure in healthcare compared to other EU member states. The share of social security is still higher than other member states. Belgium has an 80 billion euros social security budget, which is not exaggerated in comparison to others.
Because Belgium has such a high expenditure for health care, it creates the illusion that it has the best health care sector of the world, which is far from correct. If we look at the expenditure Belgium scores less than expected in survival chances, which is a tool for measuring the quality of health care. Belgium scores high for accessibility. However, a low score is obtained for urgent assistance. Therefore, the goal is to achieve more value in the health care policy. There are several running reforming routes including the reformation of hospitals, plan eHealth, and plan incorporated care to increase the value. Another way to achieve this goal is by knowing more about the costs and having new treatments, medicines and technologies.