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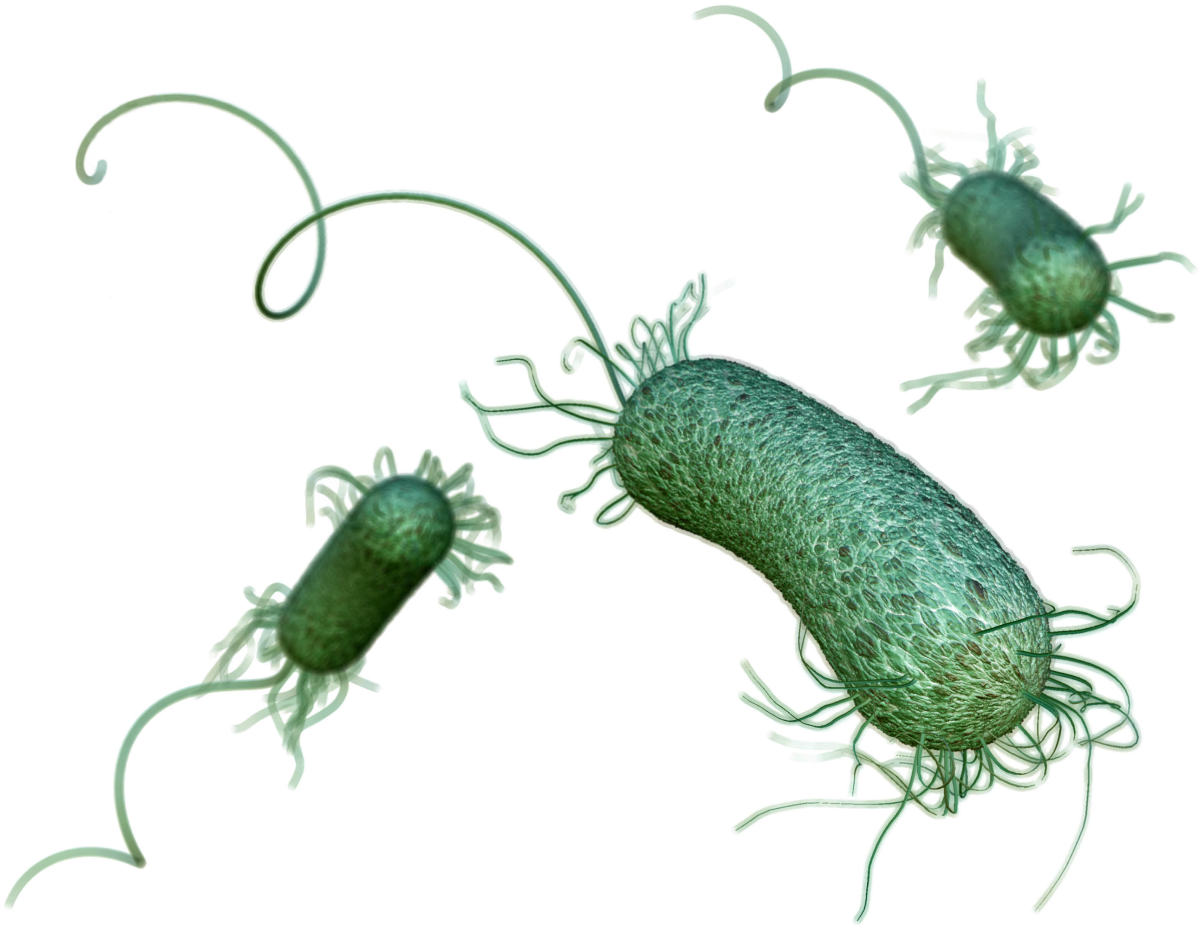

# Winning the Race:
## Bacterial Growth versus Dynamic Dosage of Antimicrobial Compounds

Master's thesis in Biomedical Engineering

DANIEL MAHR

Department of Physics
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2017

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Supervisor: Aida Bibic, Mölnlycke Health Care AB
Examiner: Julie Gold, Department of Physics

This Master's thesis was performed in the Preclinical Laboratory of Mönlycke Health Care AB (Gothenburg, Sweden). Certain data obtained during the Master's thesis work have been withheld from this public edition of the Master's thesis report due to proprietary reasons. These data have been made available to the Master's thesis examiner in order to carry out the examination of the Master's thesis.

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Cover: Three-dimensional computer-generated image of three multidrug-resistant *Pseudomonas aeruginosa* bacteria based upon scanning electron microscopic imagery. (CDC, 2013 [1]) More information about *Pseudomonas aeruginosa* can be found in section 2.3.1.

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# Abstract

Great pain and suffering to patients as well as high financial burden to health care systems are caused by chronic wounds. The interrupted wound healing mechanism is often due to increased bacterial load, whereof conventional treatment via antibiotics is affected by a recent increase in developing multiple antibiotic resistances. This suggests alternative treatments like wound dressings that release antimicrobial substances. A model for dynamic dosage of the antiseptic compound polyhexamethylene biguanide (PHMB) was introduced to investigate the time-kill behaviour of Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus*. This was hypothesised to allow findings about dependences on pharmacodynamic and pharmacokinetic parameters. Such knowledge might grant conclusions about necessary release kinetics of PHMB in wound dressings.
Four different concentration profiles of PHMB were used, starting at 0 µM and then reaching the respective minimum inhibitory concentration (MIC) against bacteria after 3, 6, 9, and 24 h by linear increase. Bacteria were cultured in these concentration profiles for 48 h and regular viable count was performed. Reaching the MIC of PHMB after 3 h resulted in killing *Pseudomonas aeruginosa*, reaching it after 6 h showed inhibited growth and reaching it after 9 h or later showed no effect. *Staphylococcus aureus* still were killed by reaching the MIC of PHMB after 6 h, whereas reaching it after 9 h showed inhibited growth only. Reaching it later did not affect bacterial growth. After 48 h of culturing, all populations recovered to stationary growth phase.
PHMB seemed to be more potent against Gram-positive *Staphylococcus aureus*. Conclusions about pharmacodynamic and pharacokinetic parameters of the effect of PHMB on bacteria were impeded as only a series of increasing, but no decreasing concentration gradients was tested. However, a greater effect during higher initial concentrations suggests dependence on the maximum concentration and on the time until the MIC is reached. Despite the necessity of further research that includes for instance a later decrease in concentration of PHMB, these early results suggest PHMB in wound dressings to be released as fast as possible and in a determined bactericidal dosage after application to the wound in order to act with highest efficacy of bacteria toxicity.

Keywords: polyhexamethylene biguanide, dose-response, time-kill, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, antimicrobial, pharmacodynamics, pharmacokinetics, wound healing, wound dressing.

# Acknowledgements

While my name might be unaccompanied on the front cover of this thesis, I am not by any means its sole contributor. This piece of research looks very different because of input, influence and expert knowledge originating from a whole lot of people. Therefore, prima facie, I would like to thank my supervisor Aida Bibic from Mölnlycke Health Care AB, whose selfless time and care were sometimes all that kept me going and who kept a sense of humour when I had lost mine. Enthusiasm, guidance, and unrelenting support have always been her principles - principles that also have to be credited to my committed and especially inspiring deputy supervisor Erik Gerner. They have regularly gone beyond their duties to fire fight my worries, concerns, and anxieties, and have worked to instill great confidence in both myself and my work.

I would also like to thank my manager Eric Wellner, whose maddening will to research and explore new things finally drove me to adore science even more. The door to his office has always been open whenever I ran into a trouble spot or had a question about my research or writing. He consistently allowed this paper to be my own work, but steered me into the right direction whenever I or he thought I needed it.

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At this point, I take the opportunity to express further gratitude to Gabriel Kaszonyi and his entire team for their help and support.

I place on record, my sincere thank you to my examiner Julie Gold, Associate Professor at the Division of Biological Physics of the Department of Physics at Chalmers University of Technology, and her team (especially Antonius Armanious, who supplied me with materials when the supplier could not supply) for the continuous, generous, and determined assistance.

Lastly, I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement not only through the process of researching and writing this thesis, but also throughout all my years of study. This accomplishment would not have been possible without them. Thank you.

Daniel Mahr, Gothenburg, June 2017

# Contents

# List of Figures

# List of Tables

# Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AUC | Area under the concentration-time curve |
| CFU | Colony-forming unit |
| $C_{max}$ | Maximum (peak) serum concentration |
| DNA | Deoxyribonucleic acid |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EPUAP | European Pressure Ulcer Advisory Panel |
| FBGC | Foreign body giant cell |
| GAG | Glycosaminoglycan |
| GPIb | Glycoprotein Ib (receptor for collagen and von Willebrand factor) |
| GPIIb/IIIa | Glycoprotein IIb/IIIa (receptor for fibrinogen and von Willebrand factor) |
| HMWK | High molecular weight kininogen |
| MBC | Minimum bactericidal concentration |
| MEC | Minimum effective concentration (i. e. MIC or MBC) |
| MHC | Mölnlycke Health Care AB (Gothenburg, Sweden) |
| MIC | Minimum inhibitory concentration |
| MRSA | Methicillin-resistant Staphylococcus aureus |
| NHS | National Health Service |
| P. a. | *Pseudomonas aeruginosa* (*P. aeruginosa*) |
| PHMB | Polyhexamethylene biguanide (Polyhexanide) |
| S. a. | *Staphylococcus aureus* (*S. aureus*) |
| SWF | Simulated wound fluid |
| T>MBC | Time above minimum bactericidal concentration |
| T>MEC | Time above minimum effective concentration |
| T>MIC | Time above minimum inhibitory concentration |
| TGF-$\beta$ | Transforming growth factor beta |
| TSB | Tryptic soy broth |
| TTC | Triphenyl tetrazolium chloride |
| VEGF | Vascular endothelial growth factor |
| vWF | Von Willebrand factor |

# 1
# Introduction

In the course of the evolution, the human body has developed a cascade of several remarkable coordinated sequences of events, which enables a healthy organism to heal a potential wound. A detailed description of this process can be found in section 2.2, however, a rather complex mechanism like this can be vulnerable to factors that are related to the patient, the wound, comorbidity, biophysiology, or health care. This might result in delayed or even prevented wound healing, often referred to as *chronic wound* or *hard to heal wound*, if a wound does not heal within three months. [2] Most common types of chronic wounds are leg ulcers, classified into venous, arterial, and diabetic leg ulcers, and decubitus ulcers (*pressure sores*), classified into degree of severity I to IV after EPUAP 2005. [3]

Leg ulcers mean a loss of skin tissue, including the epidermis and even parts of the dermis. These wounds are usually deep and weeping and in the area of the lower leg. [4] Main causes are chronic venous insufficiency after phlebothrombosis or severe varicosis (venous leg ulcer) [5], closure of a leg artery after peripheral arterial disease (arterial leg ulcer) [6] and diabetes mellitus (diabetic leg ulcer) [7], respectively. These kinds of wounds are correlated with great pain and suffering to patients, therefore significantly decreasing their quality of life physically as well as psychologically. [8–12]

On a microbiological view, the bacterial load of such wounds is the decisive factor, which interrupts wound healing. When a bacterial population in a wound proliferates to a level that is not manageable by its host anymore, the wound healing mechanism will be interrupted. [13] This disturbance often goes hand in hand with prolonged inflammatory response, changes in the molecular and cellular environment of the wound bed, as well as breakdown of granulation tissue. [8,14] Moreover, an increased burden not only for patients, but also for society and economy is the long-term consequence. [15,16]

The National Health Service (NHS) in the United Kingdom was assumed to be charged with 2.5–3.1 billion GBP per annum for chronic wound management in 2009. [17] Thereby, decubitus ulcers held by far the biggest proportion with over 90% of all chronic wounds. [18] More recent studies in the United Kingdom from 2015 and 2016 additionally took acute wounds into account and estimated the total load to 4.5–5.1 billion GBP. [16,19] Another important market in health care are the United States (US). The combined wound care burdened the country with around 50 billion USD per annum in 2010, which is together with the burden of the chronic wounds' treatment of 25 billion USD per annum in 2009 not less than 5% of the entire expenses on Medicare and Medicaid system in the US. [20,21] The US hold 6.5 million patients, who are already enduring a chronic wound. [22,23] In total,

the amount of the population within developed countries which will suffer from a chronic wound during its lifetime has been estimated to 1–2% in 2004. [24] A study about costs for health care of chronic wounds in Scandinavia came to the result that it accounts for 2–4% of the total health care expenses. [25]
An ageing population together with commonly predicted rises in healing potential affecting diseases like diabetes and obesity make the pervasiveness and occurrences of wounds and in particular chronic wounds very likely to be increased as well. [21, 26, 27] Therefore, more effective and efficient wound care to accelerate wound healing is crucial in order to not only relieve the financial burden of health care systems, but also to curtail healing times and levels of wound recurrence as well as to enhance outcomes. Injuries, in particular wounds of the skin, can lead to serious imbalances of several homeostasis systems of the human body, depending on their severity.
Usual attempts to face this increasing problem are trying to reduce the bacterial burden of a wound via antibiotics, which, however, is drastically affected by a recent increase of bacterial development of antibiotic resistance. [28] Therefore, methods of treatment are trending towards cutaneous antimicrobial substance loaded wound dressings as an alternative instead nowadays. [29–34] This could not only constitute a method of treatment of chronic wounds, but also an approach to prevent an acute wound from becoming chronic.
Polyhexamethylene biguanide (PHMB) is an antimicrobial agent, that has been used successfully in the field of wound dressings. [35,36] Little is known about connections between efficacy and potency and release kinetics, however, it has been compared to more commonly used antimicrobial compounds in wound dressings such as silver and is considered to be an adequate equivalent in its role as an antimicrobial agent. [37–39] Hence, PHMB appears to be an appropriate candidate as an antimicrobial agent for further investigations in research regarding its antimicrobial and bactericidal effect.
By reason of those uncertainties and of the previously mentioned burdens for patients, society and health care, the ultimate goal in this field is to improve available wound dressings that release antimicrobial substances to a level, which enables the release of antimicrobial substances in specific release profiles that kill bacteria with highest possible efficacy and potency. In doing so, costs for the medical device and health care sector could be decreased and simultaneously the patients' healing process improved. Furthermore, the known optimal parameters would allow the prevention of possible overdoses by overloaded or too rapid releasing wound dressings.

## 1.1 Aim and Hypothesis

The aim of this project is to investigate the effect of dynamic dosage of an exemplary antimicrobial substance, in specific PHMB, on the efficacy and potency of the antimicrobial effect on Gram-positive as well as Gram-negative bacteria. With a visualisation of time-killing as an objective, a model shall be introduced, which allows the simulation of the conditions of a treated chronic wound by an adjustment of the respective parameters, including but not limited to a specific concentration gradient of the antimicrobial substance.

*Staphylococcus aureus* is a Gram-positive bacterium and *Pseudomonas aeruginosa* is a Gram-negative bacterium; Those two pathogenic bacterial species shall serve as model bacteria. A dependence on pharmacokinetic and pharmacodynamic parameters such as the area under the concentration-time curve (AUC), the maximum (peak) serum concentration ($C_{max}$) or the time above the minimum bactericidal concentration (T>MBC) / time above the minimum inhibitory concentration (T>MIC) of PHMB is assumed. In addition, the time point of attack, meaning during the bacteria's lag phase, exponential phase (log phase) or stationary phase could play a larger role. Combinations of multiple parameters might be conceivable, too.

# 2

# Theory

This chapter shall include an overview of the theory of the field that is necessary for this project. The given framework is not limited to commonly accepted and established background knowledge, but also includes a survey of the area. Recent studies and new findings which partly have the potential to revolutionise traditional expertise may give the possibility to see the theory from a different angle.

## 2.1 Skin

Skin is the largest and simultaneously also the most versatile organ of the human body. Its surface is around $2\,m^2$, its weight is between 3 and 4 kg; Including the subcutaneous fat tissue, the weight of the skin is even about 16% of the body weight. The thickness of the skin varies between 1 and 2 mm, depending on the region of the body. [40] It serves as a separation between the body's inside and outside, protects against environmental influences, has representative and communicative power and ensures homeostasis. These comprehensive roles include most importantly the following functions: [41, 42]

1. Regulation of body temperature
2. Storage of blood
3. Protection against desiccation
4. Protection against external threads
5. Protection of genetic information against ultraviolet A (UVA) and partly ultraviolet B (UVB) radiation
6. Production of cholecalciferol (vitamin D3) by absorption of UVB radiation
7. Production of sebum
8. Reproduction of skin cells
9. Sensation of temperature, contact, pressure and pain
10. Barrier against diffusion of nutrients and ions

The skin can be divided into two main layers, which are the upper epidermis and the lower dermis. They are connected via a basement membrane and can be divided further into several sub-layers, which can be seen in Figure 2.1.

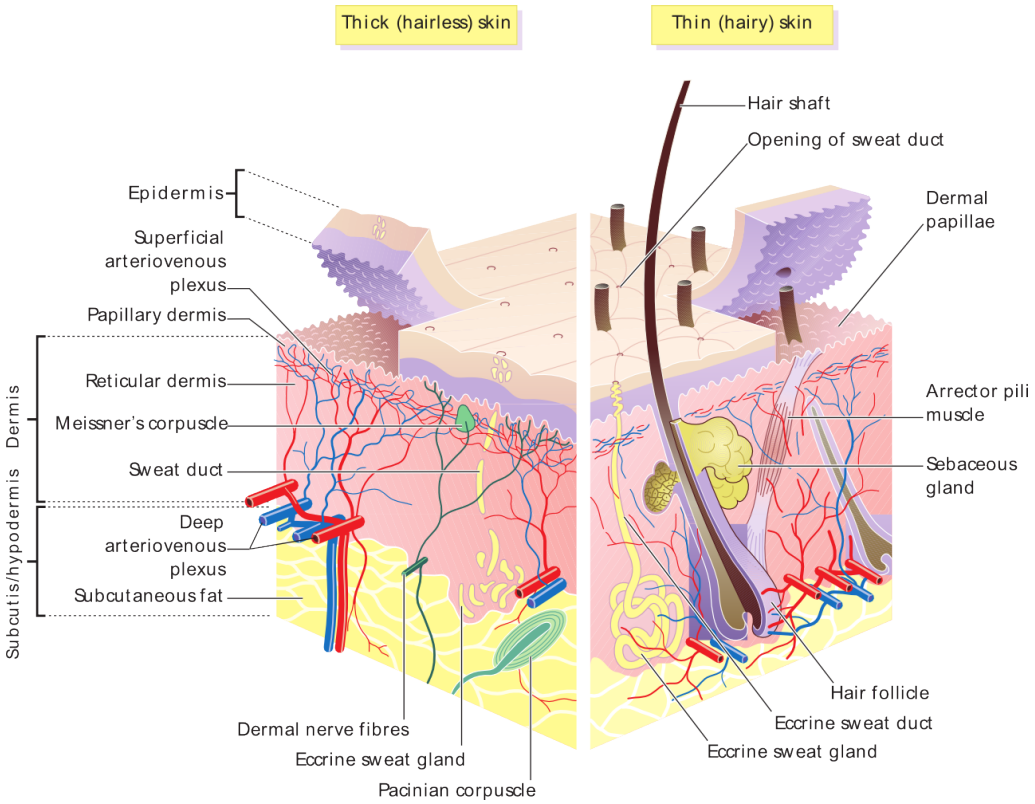


**Figure 2.1:** Different layers of skin, including epidermis, dermis, subcutis and skin appendages. Dermis consists of the papillary dermis (Stratum papillare) and reticular dermis (Stratum reticulare). (Wikimedia Commons, 2011 [43]) A detailed view of the epidermis is given in Figure 2.2.

### 2.1.1 Epidermis

The epidermis is only 50–100 µm thin, but can be thicker on palms and soles. [44] Its sub-layers are

- Stratum corneum
- Stratum lucidum (only on palms and soles)
- Stratum granulosum
- Stratum spinosum
- Stratum basale

and are characterised by multilayer, horny squamous epithelium, no blood vessels and numerous receptors like free nerve endings and Merkel cells. [40] The different layers are visualised in Figure 2.2.

The main cell type of the epidermis are keratinocytes. In the *Stratum corneum*, these cells are horny and have neither a nucleus nor organelles anymore, therefore considered as dead, yet no waste product but the differentiation's final purpose. They are 0.5 µm thin and 30 µm wide and piled in layers of about 25 (up to 100 on palms and soles). By *desquamation*, keratinocytes are shed individually after proteolytic dissolution of the desmosomes, which are holding them together. [44]

The lower, only on palms and soles occurring, and compared to the other layers rather thin *Stratum lucidum* contains transitional stages between non-horny and

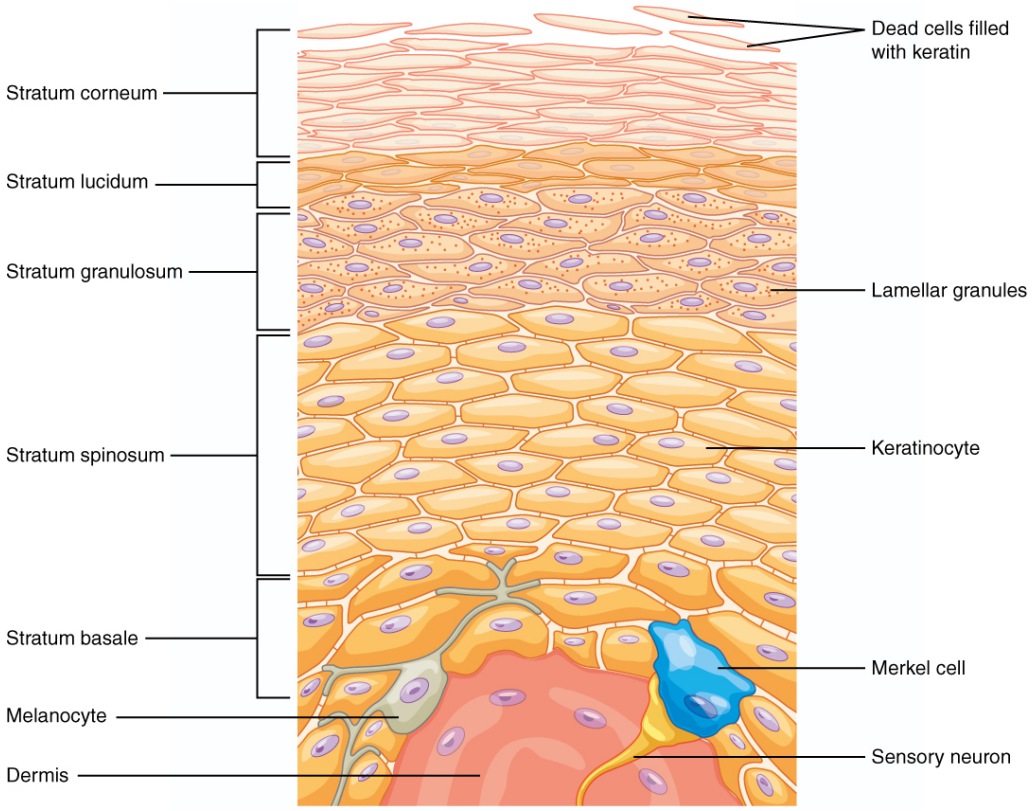


**Figure 2.2:** Detailed view of the epidermis of thick skin, which consists of five layers: Stratum basale, Stratum spinosum, Stratum granulosum, Stratum lucidum, and Stratum corneum. (OpenStax College, 2012 [45])

horny keratinocytes. [44]

Even lower, the *Stratum granulosum* can be found. Here, the three-layered keratinocytes still contain keratohyalin granules. Those do not secrete anything, but are aggregates of cytokeratin filaments and proteins, that are important for the later hornification. [44]

Below the Stratum granulosum, the *Stratum spinosum* is situated. In this stratum, keratinocytes are morphologically formed as polygons and piled in 2–5 layers. [44]

The lowest layer of the epidermis is the *Stratum basale*. It is made of single-layered prismatic cells, which are directly sitting on the basement membrane and is responsible for the continuous supplies of cells. The presence of stem cells and progenitor cells allow mitosis only in this stratum, whereas in the higher layers differentiation, maturation and hornification occur. The duration for a cell going through all of these strata is about 4 weeks. [44]

Other important cell types within the epidermis are melanocytes, Langerhans cells and Merkel cells. Melanocytes are only located in the Stratum basale and responsible for the pigmentation of the skin. These cells are able to synthesise the pigment melanin in the melanosome organelles and transfer these organelles to keratinocytes within the Stratum spinosum. One melanocyte supplies around 36 keratinocytes. Main purpose of melanin is the protection of the mitotic cells of the Stratum basale against genome-damaging UV radiation. [44]

Langerhans cells can mainly be found in the Stratum spinosum. They are dendritic cells of the epidermis and therefore antigen-presenting cells. Langerhans cells sit in regular distances between the keratinocytes and are responsible for the immune response of the skin by controlling their domain with long foothills. Antigens can be intercepted gaplessly this way. [44]
Merkel cells are the only institution of surface sensibility which is based on an actual cell instead of free nerve endings. Merkel cells are sitting in the Stratum basale, but also in the outer root sheath of hair follicles. They are counting to neuroendocrine cells; Basal, an axon ending fits closely. [44]

### 2.1.2 Dermis

The below the epidermis located dermis occupies most of the skin's thickness. Its sub-layers are

- Stratum papillare
- Stratum reticulare

and are characterised by collagenous, elastic connective tissue, good vascularisation and innervation and numerous receptors like free nerve endings, Meissner's corpuscles, Ruffini corpuscles and Pacinian corpuscles. [40]
The upper and thinner *Stratum papillare* consists of loose connective tissue. It contains mainly type III, but also type I collagen fibres, elastic fibres and various cells, primarily immune cells. Each papilla contains a capillary loop and a lot of free nerve endings, sensing pain and itching. On palms and soles, also Meissner's corpuscles can be found within the papillae, which convey touch sensation. These corpuscles are ovally formed piles of Schwann cells with collagen fibrils in between. The collagen fibrils are anchored to the basement membrane. Axon endings meander through the Schwann cells' pile. With this setup, each movement of the basement membrane moves the Schwann cells and can be recognised. [44]
The lower and thicker *Stratum reticulare* consists of taut, woven connective tissue. It gives the skin its mechanical resistance. The main components of the extracellular matrix (ECM) in the Stratum reticulare are the fibrous proteins type I collagen and elastin, both assembled as fibres. The collagen fibres run corrugated and in a latticed manner, which allows the tissue to be stretchable under tension. When stretched to a parallel alignment, the tear-resistant properties of collagen grant high tensile strength. By removing the tension, elastic fibres re-establish the original condition. Besides, Ruffini corpuscles can be found within this stratum and allow elongation sensation by a branched axon ending, which is attached to collagen fibrils and therefore recognises movements of these. [44]
Vibration sensation is given by Pacinian corpuscles, which are located in the *subcutis* below the Stratum reticulare. [44] The subcutis, as well as skin appendages, are not covered here.

## 2.2 Wound Healing

Wound healing is a natural biologic process, starting only few minutes after the injury has occurred. Blood platelets arrive at the injured location and try to seal

it. [46]
There is no way of an actual healing acceleration, however, wound pain, any complication or infection, a delay and a possibly affected cosmetic result can be treated and perhaps alleviated or prevented. That is, in order to achieve a complete recovery both in function and cosmetics (*restitutio ad integrum*), although barely attainable. Tetanus and sepsis are exemplary diseases as a consequence of a wound, which might be preventable by antiseptic treatment (disinfection) and removal of foreign bodies of the wound. [46]
There are several phases of wound healing, which occur temporally overlapping and successively. The based on light microscopical investigations classification is neither uniform nor unchallenged. Nevertheless, the phases this section is based on are shown in Table 2.1. Most important phases shall be covered in the following.

**Table 2.1:** Normal wound healing phases of vascularised connective tissue; occurring chronologically successively and partly overlapping following an injury

| Sequence | Respective phase | Section |
|---|---|---|
| I | Blood Coagulation | 2.2.1 |
| II | Inflammatory Phase | 2.2.2 |
| III | Proliferative Phase | 2.2.3.1 |
| IV | Repair Phase | 2.2.3.2 |
| IV.1 | ECM Production | |
| IV.2 | Angiogenesis | |
| IV.3 | Granulation Tissue | |
| V | Remodelling Phase | 2.2.3.3 |
| VI | Scar Formation | 2.2.3.4 |

### 2.2.1 Blood Coagulation

A complex set of reactions between platelets and coagulation proteins, which are dependent on each other, happen to occur after an injury in order to maintain hemostasis. This hemostatic mechanism opposes several activation and inhibition systems, leading to blood coagulation as an early important phase in wound healing. Blood platelets derive from megakaryocytes, which differentiated like erythrocytes and unlike lymphatic leukocytes from myeloid stem cells of differentiated pluripotent hematopoetic stem cells. Platelets are actually fragments of megakaryocytes' cytoplasm. They have a diameter of about 3–4 µm and contain no nucleus. Yet, platelets contain an endoplasmatic reticulum, a Golgi apparatus, mitochondria, a cytoskeletal structure of microtubules, the contractile proteins actin and myosin, intracellular granules, and membrane receptors like glycoprotein Ib (GPIb) and glycoprotein IIb/IIIa (GPIIb/IIIa), hence providing high functionality and response. Platelets have a half-life of around 8–10 days. The main functions of platelets are the formation of platelet plugs and catalysing coagulation reactions. [47]
Platelets are already sensitively responding to little stimulation. If activated, they become sticky and change their shape to non-uniform spheres. Platelets are able to

adhere to injured blood vessels. GPIb binds to collagen and other connective tissue elements, that are exposed at sites of injury. Von Willebrand factor (vWF), also binding to GPIb, is a crucial cofactor for this event. An internal contraction of the platelets leads to, inter alia, an extrusion of the intracellular granules' content into the extracellular environment (*degranulation*), including coagulation factors for fibrin fibre formation (e. g. thrombin, factor V and stabilising factor XIII), platelet activating factors (e. g. thromboxane $A_2$, thrombin and adenosine diphosphate (ADP)) and the rod-shaped blood plasma protein fibrinogen. Fibrinogen has two binding sites per molecule, which bind to GPIIb/IIIa on the platelets' external surface, thus each linking two platelets together. The presence of more platelet activating factors in combination with fibrinogen ultimately leads to a platelet plug formation by aggregation. [47] Although this platelet plug is important for hemostasis, it has been shown in recent experiments that platelets are not essential for crucial signalling to direct the regular repair process. [48]

Aggregated platelets express negatively charged membrane phospholipids. These combined with membrane associated proteins within tissue thromboplastin initiate the extrinsic pathway of the coagulation cascade. It is shown together with the intrinsic pathway in Figure 2.3. The intrinsic pathway is initiated by surface-mediated reactions, that happen for example by contact with diseased endothelial cell membranes or negatively charged surfaces of implants. The 13 plasma proteins' series of reactions (factor I–XIII) leading to blood clotting is covered in the following sections.

#### 2.2.1.1 Intrinsic Pathway

The intrinsic pathway is initiated by the adsorption of factor XII to a negatively charged surface, where it is activated to factor XIIa. This activated factor converts prekallikrein to kallikrein, which generates more factor XIIa and kallikrein reciprocally. Factor XI binds along with prekallikrein to high molecular weight kininogen (HMWK) and is therewith anchored to the charged surface. Factor XIIa is able to activate this factor XI to factor XIa. [47]

The next step is the activation of factor IX to factor IXa by factor XIa. This happens only in the presence of calcium ions. Previously released thrombin independently activates factor VIII to factor VIIIa. With factor VIIIa and calcium as cofactors, factor IXa finally activates factor X to factor Xa. This is catalysed on a phospholipid surface, e. g. surface of platelets, which makes the clotting more likely to happen on the surface phase. [47]

#### 2.2.1.2 Extrinsic Pathway

The extrinsic pathway is initiated by the activation of factor VII to factor VIIa. Necessary cofactor for this activation is the membrane protein tissue factor. Tissue factor is expressed by activated white blood cells and endothelial cells; It becomes accessible when underlying vascular structures are exposed to blood, which happens at the location of a vessel injury. Tissue factor is also a necessary cofactor for factor VIIa to finally activate factor X to factor Xa. [47]

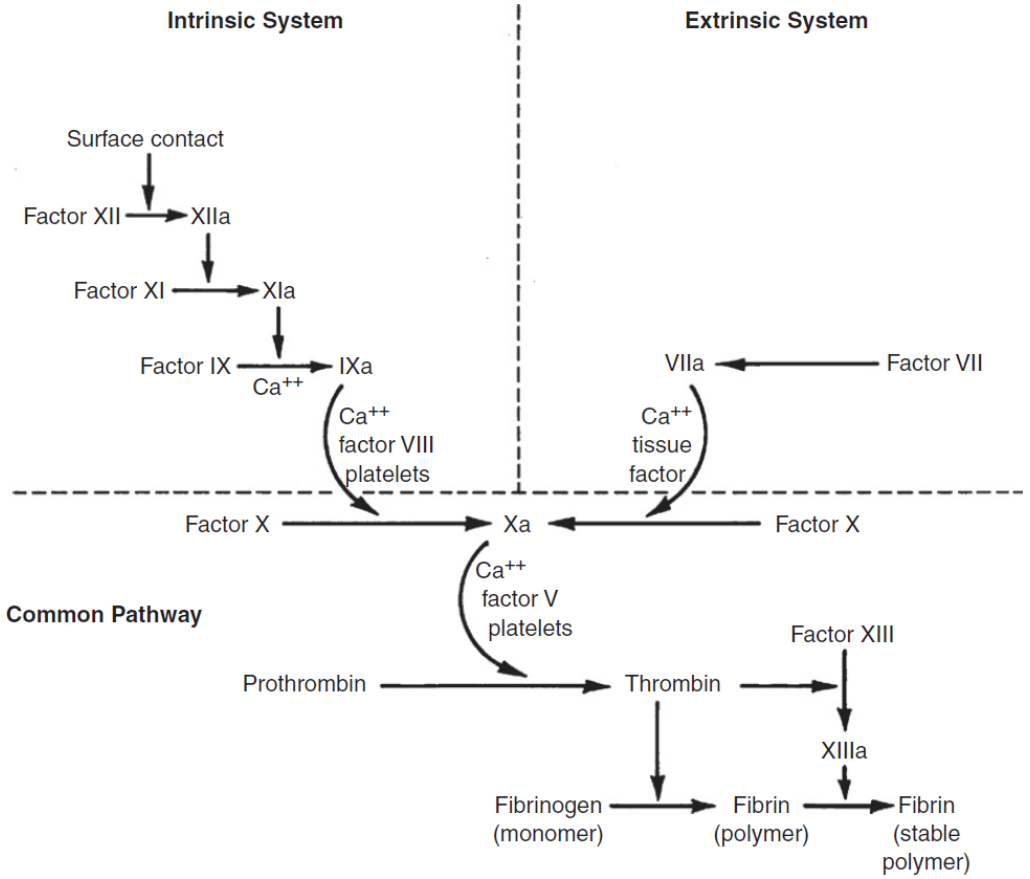


**Figure 2.3:** Mechanisms of clotting factor interactions. Shown are the extrinsic and the intrinsic pathway of the clotting factors (proenzymes). Both pathways converge upon a common path. These coagulation cascades lead to a production of the enzyme thrombin, which cleaves fibrinogen in order to form a stabilising fibrin polymer. (Hanson and Tucker, 2013 [47])

#### 2.2.1.3 Common Pathway

Both pathways finally lead to a common pathway by resulting in factor Xa. Factor V gets activated to factor Va independently by thrombin, just like factor VIII before in the intrinsic pathway (compare section 2.2.1.1). Factor Va and calcium as well as platelet phospholipids serve as cofactors for factor Xa to convert factor II (prothrombin) to thrombin. This conversion is like the activation of factor X before in the intrinsic pathway (compare section 2.2.1.1) through catalysis on phospholipid surfaces more likely to happen on the surface phase. Besides the already mentioned ability of thrombin to activate platelets (compare section 2.2.1), factor VIII (compare section 2.2.1.1) and factor V (see above), thrombin additionally acts on fibrinogen and activates previously by platelets released factor XIII (compare section 2.2.1) to factor XIIIa. It cleaves small peptides from fibrinogen and so forms fibrin monomers that polymerise to a gel. This gel transforms into a tough and insoluble fibrin polymer by interaction with factor XIIIa. [47]

#### 2.2.1.4 Control Mechanisms

There are a number of mechanisms, including, but not limited to, interactions between platelets, the coagulation system and endothelium. This grants hemostasis to occur only localised and not to initiate a generalised thrombosis. The most important ones will be covered in the following paragraphs.
Mentioned shall also be fibrinolysis, which naturally occurs after 24–48 hours after the coagulation. Fibrinolysis is the enzymatic breakdown of the blood clot. It happens mainly by the enzyme plasmin, which originally circulates inactively as plasminogen. Plasminogen is likely to adhere to fibrin and is so readily enclosed inside the blood clot already. Plasminogen activators activate the inactive plasminogen to plasmin; The main role for this occurrence is played by tissue plasminogen activator. Plasmin is able to digest fibrin and releases fibrin degradation products into the blood. [49]

**Blood Flow** The blood flow reduces the local concentration of factors and other precursors and dilutes these activated materials into a larger volume. Removal eventually happens by passing through the liver. Furthermore, blood flow confines thrombin layers to the walls. [49]

**Surface Catalysing** Several clotting reactions like the previously mentioned conversion of prothrombin to thrombin (compare section 2.2.1.3) and activation of factor X in the intrinsic pathway (compare section 2.2.1.1) are catalysed by phospholipid surfaces like surfaces of platelets. But also the factor Xa formation by factor VIIa (compare section 2.2.1.2) is catalysed at cell membranes. This confines blood clotting locally. [49]

**Endothelium and Glycocalyx** Endothelial surfaces are coated by the glycocalyx. This is a smooth mucopolysaccharide layer made of glycoproteins and proteoglycans that are integral membrane proteins. It consists of core proteins with covalently attached glycosaminoglycan (GAG) chains. GAGs are long, linear carbohydrate polymers and negatively charged due to e. g. sulphate groups. Examples are heparin and heparan sulfate. This coating makes the glycocalyx neither adhesive to platelets nor to protein adsorption. [49]
Moreover, the endothelium secretes soluble mediators like prostacyclin (prevention of platelet activation) and vWF (binds factor VIII besides platelet activation and aggregation, compare section 2.2.1). [49]

**Coagulation Inhibitors** Another control mechanism for blood clotting are natural inhibitors of thrombin and other coagulation enzymes. As discussed earlier (compare section 2.2.1.3), fibrin binds up free thrombin. One more occurring inhibitor is antithrombin III, which builds complexes with heparin. This complex is a potent inhibitor that binds and inhibits not only thrombin, but also inactivates many factors in the coagulation cascade. Tissue factor pathway inhibitor is a further example of an inhibiting protein. In association with factor Xa, it inhibits the tissue factor-factor VII complex. [49]

**Degradation of Cofactors** There are several enzymes generated during the coagulation process which are able to degrade cofactors. Plasmin, which has been mentioned before (compare section 2.2.1.4), degrades fibrinogen and fibrin. It furthermore inactivates factors V and VIII. Thrombomodulin is a protein found on surfaces of endothelial cells, which binds to thrombin. This complex activates a specific plasma protein, protein C, that is able to degrade activated factors Va and VIIIa. [49]

## 2.2.2 Inflammatory Phase

A phase that is considered to happen subsequently after blood coagulation is the inflammatory phase. Removal of cellular and tissue debris from the injury's site, destruction of foreign material such as microorganisms, and secretion of signalling molecules in order to attract cells to the wound for the production of new tissue are exemplary important events happening during this phase. [50] However, there are also critical views onto the importance of inflammatory cell types which question their significance for the actual wound repair process. [51]
An inflammation is well defined via the Latin expression "*Rubor et tumor cum calore et dolore*" which describes the cardinal signs of an inflammation: Redness, swelling, heat, and pain. Nowadays, loss of function is considered as an additional sign of inflammation, however, leaving the original definition unaffected. One main factor causing these typical inflammatory signs is local vasodilation and increased vascular permeability. During the previous coagulation phase, products such as bradykinin, serotonin, and prostaglandins are produced. These are strong vasodilators and increase vascular permeability, which directly links the inflammatory phase to the coagulation phase. [50]
Mainly two cell types are involved in the inflammation progress, those are neutrophils and monocytes. Both are myelocytic leukocytes (in the following referred to as *leukocytes*) and derive from myeloblasts (neutrophils) and monoblasts (monocytes). Myeloblasts and monoblasts originate like erythrocytes and megakaryocytes (compare section 2.2.1) and unlike lymphatic leukocytes from the myeloid stem cell line of differentiated pluripotent hematopoetic stem cells. Other important cells for the inflammatory response are basophils and eosinophils. Both differentiate like neutrophils from myeloblasts and are called granulocytes due to intracellular granules. [50, 52]
Leukocytes circulate in the bloodstream and migrate into tissues in order to kill, consume or destroy foreign objects such as bacteria, pieces of damaged tissue, and dead cells. The roles of the different cell types will be discussed later in section 2.2.2.3. Main mechanism for the attraction of leukocytes is via chemotaxis; Main mechanism for the removal of foreign objects is via phagocytosis. [50] Both are covered in the following.

### 2.2.2.1 Chemotaxis

Chemotaxis is the migration along a chemical gradient. Cells tend to move up a concentration gradient of a specific chemotactic factor (chemotractant) to the source of the molecule. Therewith, this mechanism helps leukocytes to migrate to

the injury's site. Chemotractants (also known as *chemokines*) occur during different events; They can originate from injured tissue (e. g. tissue plasminogen activator, compare section 2.2.1.4), from coagulation (e. g. kallikrein, compare section 2.2.1.1), from fibrinolysis (e. g. fibrin degradation products, compare section 2.2.1.4), and from the immune response (e. g. various cytokines). Since many of the leukocytes' chemotractants are produced during injury and coagulation, here, inflammation is considered to be the next stage of wound healing after the coagulation phase. [53]

#### 2.2.2.2 Phagocytosis

Phagocytosis is mainly executed by macrophages, but also by neutrophils (compare section 2.2.2.3 below). Microbes bind to specific antibodies and C3b (*opsonisation*). Thereby marked microbes can be recognised by phagocyte receptors in the cells' membrane, which triggers a zipping up of the cells membrane around the microbe and enclosing it within. This ingests the microbe into a phagosome. The phagosome fuses with one of the cells' lysosomes and activates the cell. An activation leads to a metabolic burst of reactive oxygen and nitrogen species, including superoxides, free radicals, hydrogen peroxide, and nitric oxide. Lysosomal enzymes and these chemicals are usually very effective ways to kill the phagocytosed microbe. [54]
Activated phagocytic cells also secrete many cytokines, growth factors, and proteases that attract more leukocytes, but also lymphocytes to the area, that begin the tissue repair process. [54]

#### 2.2.2.3 Inflammatory Cell Types

**Neutrophils** are dominant during the first days after injury occurrence and therefore involved in the initial, acute phase of the inflammatory response. These cells are with a disappearance after 24–48 hours rather short-lived, however, their emigration to the tissue is quick due to early in the inflammatory phase activated chemotractants for neutrophil migration. Aforementioned enables neutrophils to be one of the first cells to arrive at the injury's location. Neutrophils are capable of phagocytosis by ingestion and consumption of foreign objects. [52, 53] Despite being one of the first cell types on-site, surprisingly it has been shown by Dovi et al. in 2003 that the repair process of a wound occurred more rapidly by neutrophil knockdown. [55] Chemotractants secreted by neutrophils seem therefore to be compensated by other ways.
**Basophils** are responsible for a release of heparin into the blood. They are also involved in an allergic immune response via immunoglobulin E antibody binding to surface receptors. Basophils' granules contain, inter alia, histamine, bradykinin and serotonin. A release of these may cause systemic and local allergy symptoms and furthermore vasodilation of blood vessels. [52]
**Eosinophils** are able to attach to parasites and release lethal chemical compounds from their granules. Among those are enzymes, superoxides and proteoglycan 2, which is a cytotoxic major basic protein. Moreover, eosinophils are involved in the consumption of antibody-antigen complexes. [52]
**Monocytes** are immature cells during circulation in the blood stream. Chemotractants for monocytes are usually activated over a longer duration of days up to weeks.

By migration into tissues, adhesion and differentiation are triggered. They swell from a diameter of maximum 20 µm to 80–100 µm and become granular which finally turns them into macrophages; The whole process until full maturity takes about 8 hours. This matured cell type is rather long-lived with a lifespan of 2–4 months and secretes important cytokines for following wound healing mechanisms. [56] Macrophages are also referred to as phagocytes due to their greater involvement in phagocytosis compared to neutrophils. Macrophages are even able to keep indigestible particles in their cell until cell death. Eventually, a new macrophage will phagocytose the dead one, including the indigestible particles. A continuing of this process or if a too large particle shall be phagocytosed, macrophages are capable of fusing together with each other to form a large, multinucleated foreign body giant cell (FBGC). Little is known regarding biological responses that might trigger the FBGC development. [52,53] Although the importance of these phagocytosing cell type is obvious, there are studies that also indicate that the absence of macrophages during the wound healing process might enhance re-epithelialisation. [57, 58]

#### 2.2.2.4 Four Lines of Defence

Defence against infection and foreign objects during the inflammatory phase can be divided into four lines.
**Tissue macrophages** are the first line of defence. These cells are readily waiting in the tissue for foreign objects to come along and attack them immediately.
**Neutrophil diapedesis** is the second line of defence. Endothelial cells express more cell-cell adhesion receptors and retract from each other the closer to the site of injury. Neutrophils stick weakly to the endothelial cells; The strong adhesion near the injury's site finally squeezes them between endothelial cells. Neutrophils then migrate through the tissue to the site of injury and start phagocytosis.
**Monocytes and macrophages** are the third line of defence. New monocytes migrate out from the blood circulation to the tissues in order to replace dying tissue macrophages (first line of defence).
**Production of leukocytes** is the fourth line of defence. It is up-regulated in the bone marrow, however, this last line of defence takes 3–4 days for new granulocytes to enter the bloodstream.

#### 2.2.2.5 Pathophysiology of the Inflammatory Phase

An inflammation is called chronic if the inflammatory phase is lasting for months or even years. Several reasons are leading to such an outcome. Main factors are necrotic tissue sealing the wound, contamination with pathogens, and content of foreign material which cannot be phagocytosed or solubilised during the previous acute inflammation, which usually lasts for approximately two weeks. The previously described cardinal signs of an inflammation (compare section 2.2.2) may not be suitable for a chronic inflammation. A chronic wound is characterised by through lysis disappearing granulocytes and instead appearing lymphocytes as well as more monocytes and macrophages, respectively, whose extensive local proliferation represents the response of the host. The increased presence of macrophages, which also appears due to aforementioned FBGCs, attracts increased quantities of fibroblasts,

that produce higher amounts of collagen. Fibrous, encapsulated tissue is the result, known as *granuloma*. [59]

### 2.2.3 Later Phases

Several phases of the wound healing process occur after the initial coagulation phase and the following inflammatory phase (compare Table 2.1). A summarising of those is shown in the following sections.

#### 2.2.3.1 Proliferative Phase

In the proliferative phase, which usually starts after 48 hours after injury, proteases not only degrade provisional matrix and ECM, but also growth factors that are initially released by blood platelets. New cells which are attracted to the wound, including fibroblasts and endothelial cells, secrete new growth factors, including epidermal growth factor (EGF). These initiate mitosis of connective tissue cells at the wound margin. In this way, a prepopulation with new tissue cells develops at the wound site. [50]

#### 2.2.3.2 Repair Phase

The transition between the proliferative and repair phase is fluent. It is considered to happen up until 5–7 days after injury.
For **ECM production**, fibroblasts are able to synthesise necessary proteins (e. g. type III collagen, later type I collagen, compare section 2.2.3.3 below) and to deposit them from the wound margin inwards. Specific growth factors such as EGF and transforming growth factor beta (TGF-$\beta$) further promote the ECM synthesis. The growth factors accumulate in the ECM, which in turn controls the availability and bioactivity of them. [60]
The formation of new blood vessels, referred to as **angiogenesis**, requires an established ECM as well as stimulation of endothelial cells. Growth factors released by macrophages like vascular endothelial growth factor (VEGF) represent such stimulators. Endothelial cells proliferate, maturate and organise themselves into capillary tubes; The process stops when the wound is filled with new tissue. [61]
The newly vascularised tissue contains many small blood vessels, including capillaries. This gives the wound a *granular* appearance. Through further ECM production by fibroblasts' and myofibroblasts' activity, the connective tissue compacts to **granulation tissue**, which makes the wound contract. [62]

#### 2.2.3.3 Remodelling Phase

The remodelling phase takes weeks until up to months or even years. After collagen deposition at the wound site reaches a high enough concentration, apoptosis decreases the cell content. This results in acellular tissue. The collagen synthesis changes from type III to type I and therefore remodels the whole ECM, which includes the degradation of the granulation tissue. [63]

#### 2.2.3.4 Scar Formation

The formation of scars is one outcome of the remodelling phase. It is caused by changes in relative amounts, type, and structures of collagen, which might be arranged in dense parallel bundles. Scar tissue is usually not as strong as the original tissue and may not have the same functional characteristics as the original tissue. [50]

## 2.3 Bacteria

Nowadays, life is separated into three fundamental taxa, which are bacteria, archaebacteria, and eukaryotes. Bacteria, as well as archaebacteria, are unicellular microorganisms without a nucleus (*prokaryotes*). Their deoxyribonucleic acid (DNA) is floating in compressed form inside a nucleoid in the cytoplasm. Besides this bacterial chromosome, additional DNA fragments can be found in the cytoplasm, that are called plasmids. Those are multiplied independently of the bacterial chromosome and also passed on during reproduction, which happens via cell division. Moreover, plasmids can be transferred from one individual to another, which is crucial for the widely discussed development of resistance. Protein biosynthesis takes place at bacterial ribosomes, however, bacteria lack the complex compartmentalisation into cell organelles. [64]
Bacteria can be categorised in many different ways, examples are morphological properties (e. g. shape and flagellation) and aerobic consumption and tolerance, respectively (e. g. aerobic, anaerobic, aerotolerant and microaerotolerant). [64] A classification into only two categories is possible due to the bacteria's complex cell walls and was discovered by Hans Christian Gram in 1884. [65] This method uses a special stain consisting of hexamethyl pararosaniline chloride (crystal violet) and potassium triiodide (Lugol's iodine) and a counterstain consisting of rosaniline hydrochloride (fuchsine) and has been widely used in research. [66–68]
**Gram-positive bacteria** have a cell wall with high contents of peptidoglycan. These bacteria commonly have only a single cell membrane (*monoderm*) that is surrounded by a thick layer of peptidoglycan. After staining, peptidoglycan restrains crystal violet during washing with ethanol. These bacteria keep a blue stain. No counterstain is necessary. [65]
**Gram-negative bacteria** exhibit a low content of peptidoglycan in their cell wall. They generally possess two cell membranes (*diderm*) with an only thin intermediate layer of peptidoglycan. After staining, crystal violet can be washed out via ethanol. By counterstaining, these bacteria appear red. [65]
Bacteria are commonly considered as pathogenic, if they form a parasitic association with other organisms. Pathogenic bacteria may cause several infections, diseases and even death on human hosts. Pathogenicity is defined by characteristic spectra of interactions. [64] Species of *Staphylococcus* and *Streptococcus* may cause diseases ranging from skin infections and pneumonia up to a systemic inflammatory response and death. [69] *Chlamydia* contains types of bacterial parasites which are involved in pneumonia, urinary tract infections or even coronary heart diseases. [70] *Pseudomonas* include some organisms that are called opportunistic pathogens, which means diseases can only affect people bearing from e. g. immunosuppression or cys-

tic fibrosis. [71, 72]
Some types of bacteria are able to form a *biofilm*. A biofilm is derived from the bacteria themselves. The involved bacteria are irreversibly attached to either a substratum, an interface, or to each other. It is an embedding into an extracellular matrix that consists of polymeric substances produced by the bacteria. These include polysaccharides, extracellular DNA and supportive proteins. Host components like fibrin, blood platelets, or immunoglobulins can be integrated into the matrix as well. Biofilms may be unimicrobial or polymicrobial. Key characteristic for bacteria inside of a biofilm is the presentation of an altered phenotype concerning growth rate and gene transcription compared to planktonic bacteria. They can communicate via quorum sensing. Biofilm bacteria are protected from several of the host's defences and moreover significantly less susceptible to antimicrobial agents. [73–75]

### 2.3.1 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa*, in short *P. aeruginosa* (P. a.), is one type of the aerobic, Gram-negative genus *Pseudomonas*. It is rod-shaped and usually 2–4 µm long and 0.5–1 µm thick. Purulent infections result in the occurrence of blue-green pus, which is where *P. aeruginosa* got its name *aerugo* from (lat.: *cupric acetate*). [76]
*P. aeruginosa* prefers humid environment, but is able to survive in dry environments as well. It can be found in ventilation hoses, air humidifiers, incubators, dialysis machines, and pharmacy, which makes *P. aeruginosa* to be considered as a major hospital germ. [76]

#### 2.3.1.1 Interfered Wound Healing by *P. aeruginosa*

Like mentioned earlier, infections with *P. aeruginosa* often need to be favoured by a weakened immune system due to its opportunistic behaviour. However, a high risk also exists for patients with defects at skin or mucous membranes. Pathogenicity then affects the mechanisms of wound healing (compare section 2.2) and is caused by various virulence factors. [77]
*P. aeruginosa* possesses numbers of thin, diaphanous fimbria emanating from the organisms' cell wall, as well as a single, corkscrew-shaped flagellum; both visualised on the cover figure. Fimbria are thread-shaped cell attachments that are typical for Gram-negative bacteria and consist of adhesins. They allow *P. aeruginosa* to adhere to target cells. The flagellum provides for the bacteria's unipolar mode of motility. [78] In situ, *P. aeruginosa* does not only express enzymes (e. g. elastase) for breaking down tissue building blocks, but also a whole variety of toxins against cells. The two most important ones are haemolysin and exotoxin A. Haemolysin relieves haemoglobin and therefore actively haemolyses erythrocytes. Exotoxin A disables the cells' ability of protein synthesis by blocking elongation factor-2 with ADP-ribosylation. Other cytotoxins expressed by *P. aeruginosa* are the secreted enzymes exoenzyme S and exoenzyme U. [79]
*P. aeruginosa* owns antiphagocytic mechanisms. Main factor is the lipopolysaccharide layer around the bacteria, which makes their surface smooth and additionally protects against opsonisation. [80] *P. aeruginosa* is also able to build a biofilm. [81]

### 2.3.2 *Staphylococcus aureus*

*Staphylococcus aureus*, in short *S. aureus* (S. a.), is one type of the aerobic, Gram-positive genus *Staphylococcus*. *S. aureus* usually occurs in piled colonies; sometimes in tetrades and pairs. The size of one round-shaped *S. aureus* bacterium is around 1 µm in diameter. Breeding of *S. aureus* on blood agar results in characteristic yellowish-golden colonies, therefore the designation *aureus* (lat.: *golden*). The colour is clearly visible in Figure 2.4. [76]

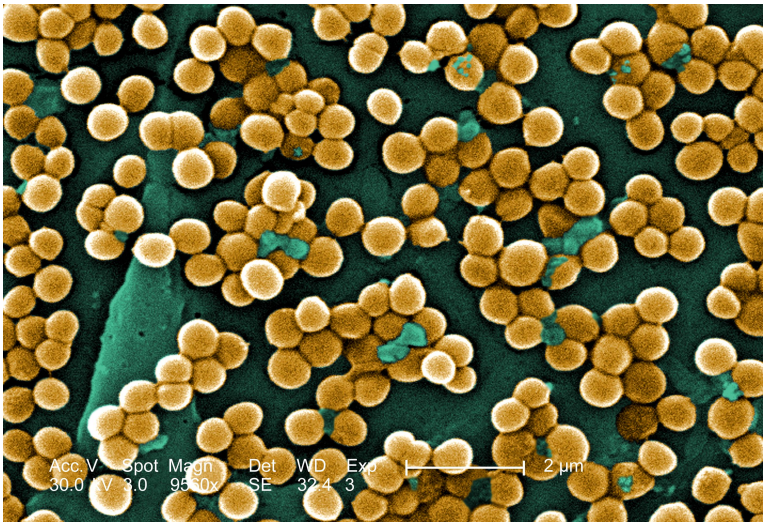


**Figure 2.4:** *Staphylococcus aureus* recorded with a scanning electron microscope. It involves the methicillin-resistant *Staphylococcus aureus* (MRSA). Clearly visible is the characteristic yellowish-golden colour. Magnification is 9,560x. MRSA occur increasingly since the 1960s and cannot be treated with common antibiotic therapy. (CDC, 2005 [82])

*S. aureus* occurs at up to 50% of the population, it can be found on the skin, but also in the nasal atrium, perineum, colon, and vagina. The main way of infection is by smear infection, crucial is dermal contact via the hands. [76]

#### 2.3.2.1 Interfered Wound Healing by *S. aureus*

Previously described mechanisms of wound healing (compare section 2.2) can be significantly affected by a pathogenic invasion of *S. aureus*, which holds several pathogenicity factors that may lead to a chronic wound. Main factor are integral parts of the *S. aureus*'s cell wall, including, but not limited to, peptidoglycan. These parts activate the complement system and trigger chemotaxis (compare section 2.2.2.1), which in turn attracts, inter alia, neutrophils (compare section 2.2.2.3). These cells start to phagocytose *S. aureus* and perish in doing so; Pus is formed. [83] Furthermore, *S. aureus* is able to adhere to tissue by the adhesin clumping factor A, which specifically binds to fibrinogen, and other collagen-binding proteins. Clumping factor A is furthermore enabling *S. aureus* to build a fibrin wall around the abscess they live in. This allows protected reproduction. [84] However, *S. aureus* also expresses the enzyme fibrinolysin for breaking down the fibrin wall in order to

spread. The additionally expressed enzymes collagenase, lipase, and hyaluronidase enable *S. aureus* to degrade building blocks of the tissue and to eventually invade it. [85]
*S. aureus* also holds, besides the building of a fibrin wall, several other antiphagocytic properties. So does it not only express clumping factor A, but also clumping factor B (Panton-Valentine leukocidin). [86] This kind of leukocidin is cytotoxic and destroys phagocytosis practising cells, which is why the above mentioned perishing of those occurs. Further mechanisms are the utilisation of protein A, that blocks the Fc portion of antibodies. [85] Therefore, opsonisation by docking at the bacteria's site is prevented and, in turn, also the accumulation of phagocytosing cells, which require docked antibodies for recognition. Another mechanism to prevent phagocytosis is the formation of a glycocalyx capsule that acts antiphagocyticly. This eventually enables *S. aureus* to build a biofilm. [87]

## 2.4 Antimicrobial Substances

For the treatment of bacterial infections or prevention of those, bacteriocidal substances are necessary in order to kill bacteria. However, also bacteriostatic substances might be considered. Commonly used are many different kinds of antibiotics that all attack a pathogen in a different way; One example is the specific assault of the bacterial ribosome. Since the human, eukaryotic ribosome is structurally different to the bacterial, prokaryotic one, it remains not affected. [88]
Biochemical as well as physiologic effects of antibiotics and other drugs are, among other things, dependent on *pharmacodynamics*. Pharmacodynamics highlight dose-response relationships, which covers the relationship between drug concentration and effect such as drug-receptor interactions. This includes the study of how a drug may affect an organism. Together with *pharmacokinetics*, which is the study of how an organism may affect a drug, it has an impact on dosing, benefit, as well as adverse effects. Parameters that are often discussed to be crucial in this field are the maximum (peak) serum concentration ($C_{max}$), the area under the concentration-time curve (AUC), and the time above the minimum inhibitory/bactericidal concentration (T>MIC/T>MBC) of a drug (in the following referred to as the *time above the minimum effective concentration T>MEC*), which are visualised in Figure 2.5. [89]
$C_{max}$ is the concentration's maximum of a drug in a test area after administration of it. It is related to the time at which $C_{max}$ is observed as a pharmacokinetic parameter. A therapeutic effect of a drug that is dependent on its $C_{max}$ value (*$C_{max}$-driven*) is not dependent on the duration of the drug's presence, meaning the higher the maximum concentration over a specific time, the more significant the therapeutic effect during this time. [91]
AUC is the definite integral of the plotted concentration of a drug in a test area against time after administration of it. AUC is interpreted as the total drug exposure over time and is proportional to the amount of drug absorbed by the test area. Furthermore, AUC tells the average concentration over a time interval. Drugs, whose therapeutic effect is dependent on AUC (*AUC-driven*), are concentration independent, but depend on the duration of the drug's presence. This means, the longer a drug is present in the test area, the higher the AUC will be, which in turn

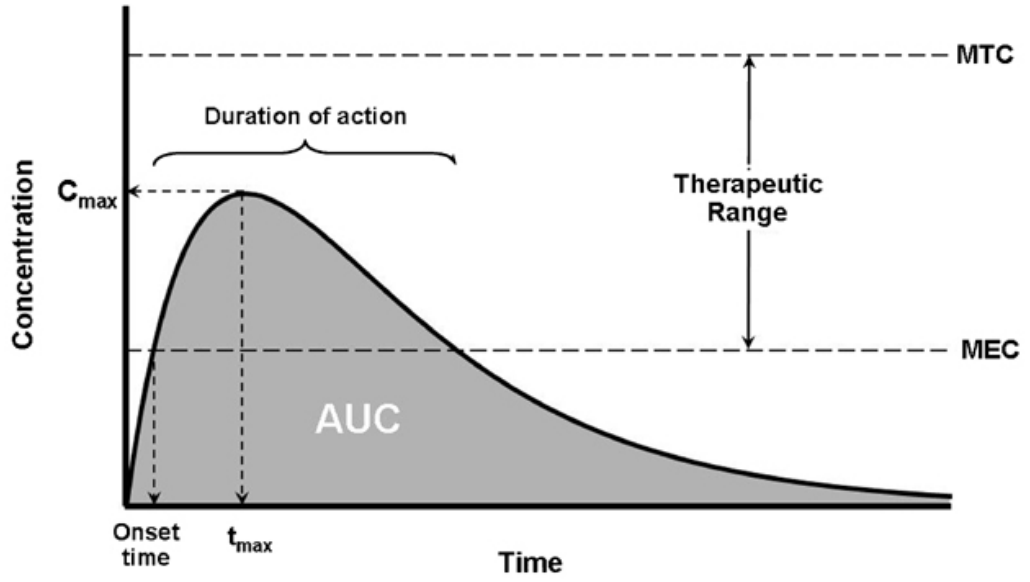


**Figure 2.5:** Pharmacokinetic/pharmacodynamic parameters that describe a concentration time profile, in this example after an oral administration. Visualised are the maximum concentration ($C_{max}$), the time at which $C_{max}$ is observed ($t_{max}$), the area under the concentration-time curve (AUC), the minimum effective concentration (MEC, equivalent to MIC or MBC, respectively) and the maximum tolerated concentration (MTC). The duration of action complies with the time above the minimum inhibitory/bactericidal concentration (T>MEC). (Mehrotra et al., 2007 [90])

increases the significance of the therapeutic effect. [91]
T>MEC dependence is a combination of concentration and time dependence. If a drug's therapeutic effect is dependent on T>MEC (*T>MEC-driven*), there is no significant therapeutic effect unless the MIC/MBC has been reached (concentration dependence). After reaching this value, the concentration plays a subordinate role only and rather the duration of how long the concentration can be held above the MIC/MBC is significant and determines the therapeutic effect. [92]

## 2.4.1 Conventional Treatment of Bacterial Infections

Most common treatment of bacterial infections is via antibiotics. In the following, examples against the aforementioned bacteria *P. aeruginosa* and *S. aureus* shall be given.

### 2.4.1.1 Treatment of Infections with *P. aeruginosa*

A completely different structure between Gram-positive and Gram-negative bacteria, especially in the cell membranes, which was described earlier (compare section 2.3), leads to necessary treatments with antibiotics using different types of mechanims of actions for infections with *P. aeruginosa*. Three main types are: *Piperacillin* in combination with *tazobactam*, *ceftazidime*, and *ciprofloxacin*.
Piperacillin is a $\beta$-lactam antibiotic. It does not penetrate the cell membrane but binds to a range of specific penicillin-binding proteins that are responsible for cell

wall synthesis. These proteins are located inside the bacterial cell wall. Piperacillin inhibits the third and last stage of bacterial cell wall synthesis. As a result, cell lysis is initiated by bacterial cell wall autolytic enzymes such as autolysins. [93] It is considered to be dependent on the AUC. [94]
Ceftazidime is another $\beta$-lactam antibiotic. Its pathway of the mechanism of action is the same as for piperacillin; It also inhibits bacterial cell wall synthesis. [95] Ceftazidime is dependent on both the AUC and the T>MEC. [96]
Ciprofloxacin is an antiinfective agent of the fluoroquinolone class. The mechanism of action of ciprofloxacin is different from that of other antimicrobial agents such as $\beta$-lactams. It penetrates the cell membrane and inhibits the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV. [97] These enzymes are essential for the bacterial DNA replication, transcription, repair, strand supercoiling repair, and recombination. Ciprofloxacin's dependence is believed to be based on the AUC. [98]

#### 2.4.1.2 Treatment of Infections with *S. aureus*

Three main types are usually the method of choice against *S. aureus*: *Vancomycin*, *tigecycline* and *daptomycin*.
Vancomycin is a glycopeptide and inhibits cell wall peptidoglycan synthesis. Similar to piperacillin, it interferes with the last stage in the bacteria's biosynthetic pathway of peptidoglycan at the outer wall site, since it is not able to penetrate the cytoplasmic membrane. Vancomycin binds to peptidoglycan components of the wall. Eventually, all the peptidoglycan binding sites will be saturated, which resins their function in ion-exchange and inhibits peptidoglycan synthesis and bacterial growth. [99] Nowadays, vancomycin is considered to be pharmacodynamically and -kinetically dependent on the AUC, whereas it was formerly believed to be dependent on the T>MEC. [100]
Tigecycline is a glycylcycline and a rather newer antibiotic compared to vancomycin. It has a characteristic four-ring carbocyclic skeleton and a specific alkyl-glycylamido group side chain and is able to penetrate the cytoplasmic membrane. Tigecycline acts by inhibiting protein translation. It binds to a specific ribosomal subunit and therefore blocks the entry of amino-acyl transfer RNA molecules into the ribosome. This prevents elongating peptide chains with amino acid residues. Tigecycline's critical therapeutic parameter is the AUC. [101]
Daptomycin is a lipopeptide. It disrupts the cell membrane's function by inserting itself into the membrane and aggregating there. This is dependent on phosphatidylglycerol and calcium. The aggregation alters the shape of the membrane and forms an ion channel which in turn causes depolarisation by ion efflux. Therefore, synthesis of proteins, DNA and RNA is inhibited and eventually causes cell death. [102] Daptomycin is considered to be dependent on the AUC. [103]
However, inter alia, also the usage of many antibiotics in intensive farming to support animal growth increases the development of antibiotic resistance against in particular antibiotics that have been used for a long time (e. g. vancomycin). [104] This represents a huge drawback of the usage of antibiotics and suggests alternative substances instead. Alternative drugs are continuously under development. One example is acyldepsipeptides, that instead of affecting reactions in the bacteria's cytoplasm attacks within the bacteria's metabolism leading to a dysfunction of an

important enzyme which is important for cell division. [105] Furthermore, an endolysin has been discovered that specifically lyses *S. aureus* only. [106] And in a very recent study from 2016, a drug called *lugdunin* was found in humans' noses. It is produced by a specific kind of bacterium (*Staphylococcus lugdunensis*) and probably attacks the synthesis of the cell membrane. [107] Possible side effects are to be investigated.

### 2.4.2 Definition of Resistance, Tolerance and Persistence

Although the development of resistance has been discussed often and widely, it is important to know about the differences of bacterial behaviour against pharmaceutical treatment. By distinguishing between the terms *resistance*, *tolerance* and *persistence*, it is possible to describe this behaviour more precisely. The definitions in the following are based on a recent opinion by Brauner et al. from 2016; A visualisation of them is shown in Figure 2.6. [108]

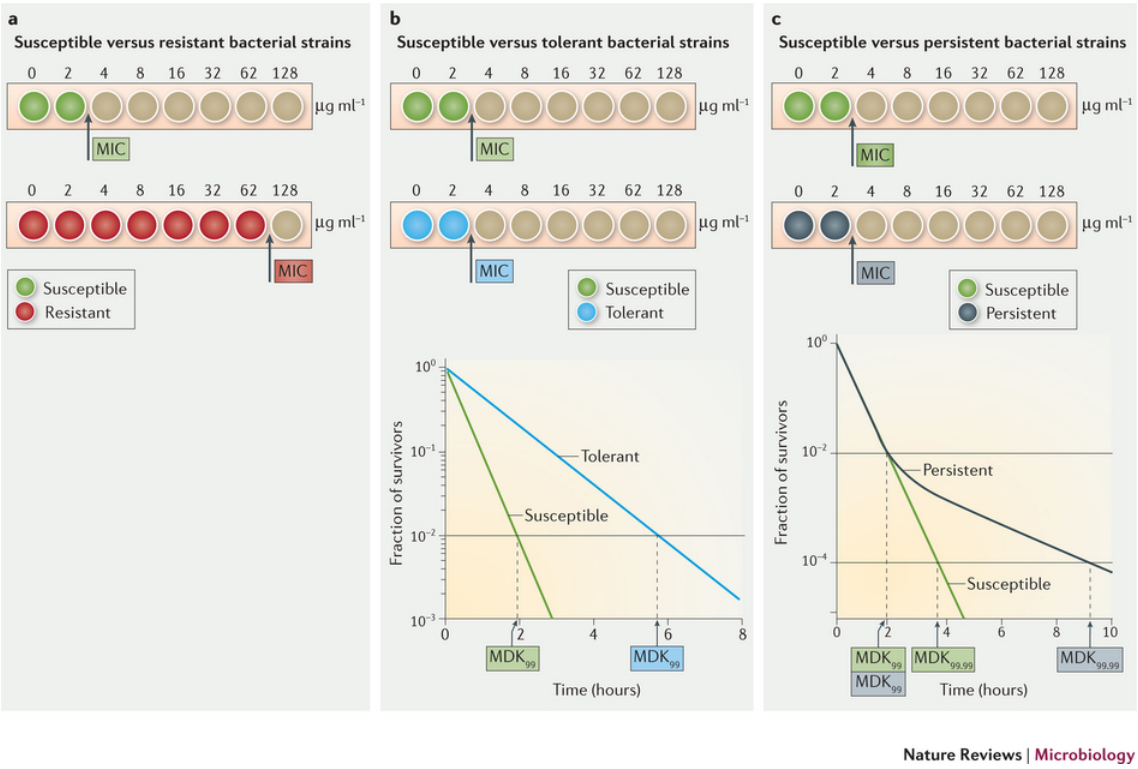


**Figure 2.6:** Exemplary drug responses of resistance, tolerance and persistence. Coloured wells symbolise bacterial growth. **a)** The MIC for a resistant strain is higher than the MIC for a susceptible one. **b)** The MIC for a tolerant strain is similar to the MIC for a susceptible one. However, the minimum duration for killing (referred to as *MDK* in the figure) is higher for a tolerant strain. **c)** The MIC for a persistent strain is similar to the MIC for a susceptible one. However, the MDK is higher for a persistent strain when looking at the time-kill curve for the last 1% of a bacterial population. (Brauner et al., 2016 [108])

#### 2.4.2.1 Resistance

Resistance describes the microorganisms' ability to grow despite of high drug concentrations and is caused by inheritable mutations. [109] Mechanisms of resistance on a molecular level include mutations in the drug target, in enzymes that inactivate the drug and activation of efflux pumps in order to pump out the drug. [109, 110] Genes that are involved in the changed phenotype responsible for these mechanisms are called *resistomes*. [111]
Resistance of bacteria results in a decreased effectiveness of the drug and therefore requires a higher concentration of it in order to produce the same effect compared to a susceptible strain. [112] Time-independence is an important characteristic when looking at the MIC, which is used to quantify resistance between a resistant and a susceptible strain. A higher MIC compared to another strain is regarded as resistant. [109]

#### 2.4.2.2 Tolerance

Tolerance describes the microorganisms' ability to survive transient high concentrations of a drug that would otherwise be lethal. [113, 114] Tolerance in bacteria can be a result of genetic mutation or environmental conditions, therefore it can be either inheritable or not. [115]
Tolerance is rather time-dependent than concentration-dependent. [116] This suggests a MIC not being meaningful to quantify tolerance since tolerant bacteria can have the same MIC as non-tolerant strains. [117,118] A quantification by comparing the minimum duration for killing values of two strains seems more reasonable.

#### 2.4.2.3 Persistence

Persistence describes a subpopulations' ability of a bacterial population to survive high concentrations of a drug that rapidly kills the majority of the bacterial population whereas the subpopulation persists much longer, which results in a biphasic time-kill curve. [119, 120]
Persistence occurs in a subpopulation only, which is a major difference to resistance and tolerance. [121] It is non-heritable, that could be repeatedly shown by growing surviving subpopulations and receiving the same time-kill curve as with the bacterial population before. [122, 123] Persistence means killing at a slower rate and is due to a switching between a susceptible and a persistent phenotype. The ratio of the persistent phenotype is typically less than 1%. [119] There is time-dependent persistence, meaning the subpopulation is tolerant, and there is dose-dependent persistence, meaning the subpopulation is resistant. [120, 124, 125]

### 2.4.3 Polyhexamethylene Biguanide

Polyhexamethylene biguanide, in short polyhexanide (PHMB), is an alkaline compound that is known for its broad-spectrum biocidal activity against bacteria, fungi, and parasites. [126] Therefore, it is widely utilised in clinics, homes, and industry. [127] More recently, it also finds applications in antimicrobial wound dressings. [35,36] An advantage of PHMB in this application is its inability to penetrate

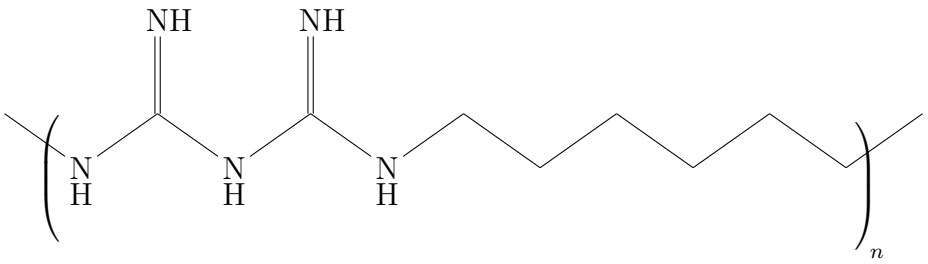


**Figure 2.7:** Structural chemical formula of polyhexamethylene biguanide. Cationic sites of this polymer are the upper nitrogen atoms. Counter-ions (usually originating from hydrochloric acid) are not shown.

tissue to deeper regions. [128] Its structure of biguanide units connected by hexamethylene hydrocarbon chains is visualised in Figure 2.7. A huge advantage of PHMB is that even though it has been used for several decades, no resistant behaviour of bacteria to PHMB has been reported yet. [129]
The investigation of PHMB in this project was chosen due to this promising properties along with its difference in its below described mechanism of action to most antibiotics.

#### 2.4.3.1 Mechanism of Action of Polyhexanide

The mechanism of action of PHMB has been previously described in a review of 2005. [130] It binds to the coat of both Gram-positive and Gram-negative bacteria and displaces stabilising calcium. [131] PHMB binds directly to the cytoplasmic membrane as well as to lipopolysaccharides and peptidoglycan components of the cell wall. Bridging of adjacent phospholipids occurs by the interaction of PHMB with the cell membrane. [132–134] The adsorption to the membrane leads to a sequestration of these phospholipids, which ultimately leads to a fragmentation into fluid and liquid crystalline regions. [131–135] A consequence is cellular leakage of intracellular ions and pool materials, eventually causing a total loss of the membrane's permeability barrier. [134, 136] It has been shown that this mechanism is a function of the polymer's chain length, since longer polymers are able to form larger phospholipid domains during the sequestration. [137]
A very recent study by Chindera et al. from 2016 rejects this widely accepted mechanism of action and could show much less membrane activity to Gram-negative bacteria than expected. [138] Instead, they could prove that PHMB not only enters the bacterial cells, but also condenses bacterial chromosomes, leading to cell division inhibition. This happens due to PHMB's ability to directly bind to DNA fragments. [139] Whereas the previously described mechanism of action of PHMB was believed to not attack mammalian cells because of a lack of interaction with the structural different mammalian cell membrane and because the cationic molecules are repelled by it, the harmlessness towards mammalian cells rather appeared to be due to an entrapping of PHMB within endosomes after entering mammalian cells in this recent study. This restricts entry into the cells' nuclei.

# 3
# Methods

MATERIALS such as antimicrobial substances, culture broth media, other substances, and bacterial strains that have been used shall be presented in this chapter. Furthermore, the preparation of materials that were not acquired externally is demonstrated, followed by an explanation of used standard methodology. Lastly, the developed experimental method for the simulation of release kinetics is described by a performance and experimental plan.

## 3.1 Materials

Isotonic saline solution containing either 0.1% peptone (in the following referred to as *peptone water*; Lot# 314, 570, 703 and 4498) or 0.5% peptone, 0.5% polysorbate 80 and 0.4% dextran sulphate (in the following referred to as *dextran sulphate*; Lot# 229), Tryptic soy broth (TSB; Lot# 3495 and 4211), and simulated wound fluid (SWF; Lot# 542, 637, 734, 788 and 829) were acquired from Sahlgrenska Universitetssjukhuset Bakteriologiska Laboratoriet (Gothenburg, Sweden).
Peptone is a mixture of peptides and amino acids and has very low molecular weights which prevents precipitation and coagulation by salts. Peptone water served as dilution medium. Dextran sulphate acted as neutralising solution for PHMB due to its ion exchanging properties. TSB consists of 17 g Tryptone, 3 g Phytone, 5 g sodium chloride, 2.5 g dipotassium phosphate and 2.5 g glucose per 1 l distilled water; SWF consists of 50% fetal bovine serum and 50% peptone water. Both TSB and SWF were used as culture medium.
Resazurin sodium salt (Lot# MKBF7963V) was purchased from Sigma-Aldrich LLC (St. Louis, US) at a level of purity of >99%. PHMB was bought as Cosmocil (20% solution; Lot# 14GR099214) from Lonza Group AG (Basel, Switzerland). A Petrifilm™ Plate Reader (including Petrifilm™ Plate Reader software, version 3.0.0) and Petrifilm™ Aerobic Count Plates (Lot# 2018-01 TJ, 2018-06 TN and 2018-07 TI) were obtained from 3M™ (Maplewood, US).
The 12 channel IPC-N tubing pump (ISM 937) and corresponding Tygon® pump tubing with 0.19 mm inner diameter and 40 cm length (SC0001; Lot# 14194789), Tygon® extension tubing with 0.19 mm inner diameter and 10 m length (SC0025; cut to pieces of 1 m (±5 cm); Lot# 1620760) and stainless steel tube connectors with 0.3 mm inner diameter (ISM 580; Lot# 47662) were procured from Cole-Parmer LLC (Vernon Hills, US).
Mechanical pipettes from Thermo Fisher Scientific Inc. (Waltham, US) and electrical pipettes from Sartorius AG (Göttingen, Germany) were worked with, standard

laboratory equipment like Falcon® tubes, Erlenmeyer flasks and 96 well microtiter plates were acquired from VWR International (Radnor, US).
Static humidified incubators from Termaks AS (Bergen, Norway) and a shaking non-humidified incubator from Infors AG (Bottmingen, Switzerland) were used. The operated centrifuge was a Sigma 3-18K from Sigma Laborzentrifugen GmbH (Osterode am Harz, Germany).

### 3.1.1 Preparation of Solutions and Dilutions

A stock resazurin suspension with a concentration of around 5 g/l was created by suspending 0.5 g of resazurin sodium salt in 100 ml of distilled water. Additionally, a stock PHMB solution with a concentration of 2% was generated by diluting 25 ml of Cosmocil with a dilution factor of 1/10 in 225 ml of peptone water.

### 3.1.2 Bacterial Strains

The two pathogenic bacteria *P. aeruginosa* and *S. aureus*, which are commonly found in wounds, were used in order to simulate a wound like environment. In particular, the bacterial strains *Pseudomonas aeruginosa* ATCC® 15442 (in the following, *P. aeruginosa* refers to this strain only) and *Staphylococcus aureus* ATCC® 6538 (in the following, *S. aureus* refers to this strain only) were worked with.

#### 3.1.2.1 Culturing of Bacteria

Bacteria colonies originated from at -70°C cryopreserved bacteria that have then been spread and growing on Tryptic soy agar plates. All experiments began with an inoculation of a Falcon tube containing 3 ml TSB with 2–3 colonies of *P. aeruginosa* or *S. aureus*, respectively, token from the Tryptic soy agar plates. The Falcon tubes were vortex mixed and then statically incubated at 35°C for 17 h (±1 h; in the following referred to as *overnight culture*).
The overnight cultures were expected to have an approximate colony-forming unit (CFU) concentration of around $3 \cdot 10^9$ CFU/ml for *P. aeruginosa* or $6 \cdot 10^8$ CFU/ml for *S. aureus*, respectively. Subsequently, firstly a tenfold dilution by transferring 0.5 ml into 4.5 ml peptone water was executed and secondly the diluted bacteria were further diluted into SWF in a manner to achieve the respective desired concentration for each experiment.

## 3.2 Viable Count of Colony-Forming Units

For determination of viable counts, the spread-plate method was used. 3M™ Petrifilm™ Aerobic Count Plates consist of a cover film and a bottom film. The cover film consists of adhesive, a cold water-soluble gelling agent, and the redox indicator triphenyl tetrazolium chloride (TTC). [140] Bacterial metabolism reduces TTC to produce a visible red dye. [141] The bottom film contains a cold water-soluble gelling agent and dried nutrients. [140]

The Petrifilm™ culture plate method was performed according to the manufacturer's instructions in order to assess the mesophilic aerobic bacteria. [142] In short, at first a tenfold dilution was created by transferring either 0.5 ml or 0.1 ml of the bacterial suspension into 4.5 ml or 0.9 ml of dilution medium, respectively. If the bacterial suspension contained PHMB, dextran sulphate served as dilution medium and the suspensions was given 10 min at room temperature for the neutralising broth to react. If the bacterial suspension contained no PHMB, peptone water served as dilution medium. Thereafter, they have been vortex mixed and serially tenfold diluted by transferring 0.5 ml into 4.5 ml of peptone water until the estimated concentration was lying at 10–50 CFU/ml. The transparent cover film of a Petrifilm™ was opened, after which 1 ml of the lowest decimal dilution was added to the bottom film of it and 1 ml of the second lowest decimal dilution was added to the bottom film of another Petrifilm™. After the bottom films were covered by the cover films, the medium was pressed with a plastic spreader in order to spread the sample circularly and uniformly. The Petrifilms™ were incubated at 35°C for 24 h for *S. aureus* and 48 h for *P. aeruginosa*, respectively. The appearance of red dots, which can be seen in Figure 3.1, was considered to be the mesophilic aerobic CFU of bacteria and either counted automatically with a Petrifilm™ Plate Reader for *S. aureus* or manually for *P. aeruginosa*, respectively.

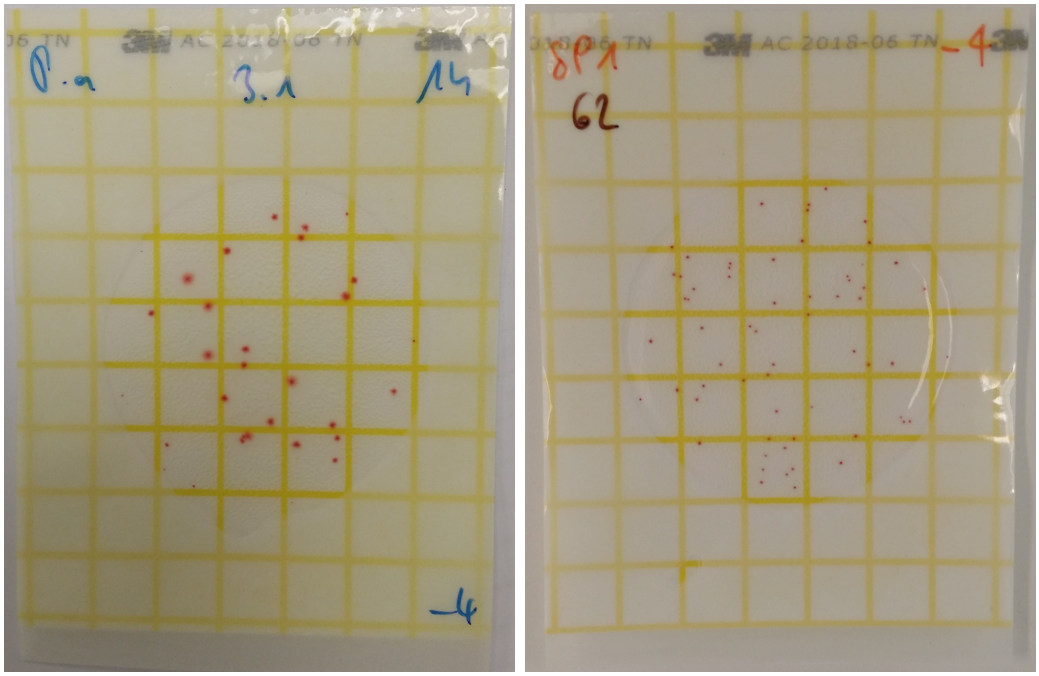

**Figure 3.1:** Exemplary representation of red dots on Petrifilms™ after application of inoculum and following incubation. Each dot is considered to be a colony-forming unit of respective bacteria. Left: Petrifilm™ inoculated with *P. aeruginosa* Right: Petrifilm™ inoculated with *S. aureus*

Due to the necessary first tenfold dilution step, the limit of detection for this viable count method is 10 CFU/ml and increases tenfold for each further dilution step.

## 3.3 Minimum Inhibitory Concentration

Testing the MIC for bacteria is based on the colour change of the non-toxic blue resazurin stain. By chemical reduction, resazurin turns into resorufin, which is of pink colour. [143] The reaction of resazurin to resorufin is irreversible and proportional to aerobic respiration. [144] Normally metabolising cells, including bacteria, as well as fungi, reduce resazurin and therefore induce a colour change of the medium as an indication of the microorganisms' metabolism and thereby viability. [145, 146]
A detailed protocol of the MIC testing can be contemplated in section A.1 of the Appendix. Tenfold dilution testing and ray design of the protocol was not necessary and not executed. In short, test media consisting of SWF (instead of Mueller-Hinton broth in the protocol) supplemented with stock resazurin suspension to a final concentration of 0.02 g/l was created. Stock PHMB solution was diluted in test media to a concentration of 0.2 mM by assuming a molecular weight of PHMB of around 3,000 g/mol. [128] 200 µl of the media containing PHMB was filled into the first column of a 96 well microtiter plate (well volume of 300 µl). Three replicates per bacterial strain were carried out, each replicate demanding one row. Twofold serial dilution was performed by moving 100 µl of the first column to 100 µl test media in the adjacent well etcetera until 12 wells have been diluted. Bacteria were diluted to a desired concentration of around $6 \cdot 10^5$ CFU/ml (instead of 1–3·$10^6$ CFU/ml in the protocol) like described earlier in section 3.1.2.1, but into test media instead of SWF. Next, 100 µl of the bacterial dilution were added to all wells, which halved the PHMB's as well as the bacterial concentration in each well to 100–0.048828125 µM PHMB and $3 \cdot 10^5$ CFU/ml, respectively. The well plate was then stirred with 500 rpm for 30 s and statically incubated at 35°C and 90% relative humidity for 24 h (±1 h). Viable counts of the inoculum were confirmed by duplicate Petrifilm™ plate counting like described earlier in section 3.2. The lowest concentration of PHMB needed to inhibit colour change from blue to pink was considered to be the MIC, if at least two of the replicates corresponded.
For testing of inactivation of PHMB, another MIC testing with the following further deviations was run in parallel to the just described testing. The medium containing PHMB was created one day in advance and stored protected against light at 4°C for 24 h. 1 h prior to the testing, it was stored unprotected against light at room temperature. The readily prepared well plate was then stored unprotected against light in a non-humidified shaking incubator at 35°C and 100 rpm.

## 3.4 Simulation of Release Kinetics and Time Dependence of the Minimum Inhibitory Concentration

For the simulation of release kinetics of an actual wound dressing that contains an antimicrobial substance, an initial concentration of 0 µM of antimicrobial substance was assumed. For reasons of easier feasibility, a linear increase of its concentration was striven for, i. e. the concentration gradient should be constant. The final con-

centration should be the respective MIC, regarding each combination of PHMB and a respective bacterial strain.

### 3.4.1 Minimum Inhibitory Concentration of Polyhexanide

As a first step, the MIC of PHMB against *P. aeruginosa* and *S. aureus* was determined, following the description in section 3.3. The MIC was estimated three times for each bacterial strain, meaning a result with $3 \times 3$ replicates per strain was obtained.

### 3.4.2 Increasing the Concentration of Polyhexanide Over Time

The previously in section 3.4.1 determined MIC of PHMB for *P. aeruginosa* was decided to shall be reached after 3, 6, 9 and 24 h. A fifth group should not receive any PHMB and serve as a control group. Each of the five groups consisted of three replicates that in turn consisted of 50 ml Erlenmeyer flasks and were inoculated with 10 ml of bacterial suspension with around $3 \cdot 10^5$ CFU/ml in SWF which was prepared like described earlier in section 3.1.2.1. All samples were then stored in a shaking incubator at 100 rpm and 35 °C.
The IPC-N tubing pump was set and calibrated to the lowest possible flow rate of 0.7 µl/min in order to keep the change in volume of the samples negligibly low. The outflow of the pump tubing was connected to extension tubing by the use of tube connectors; The outflow of the extension tubing was then in turn fixed inside of the Erlenmeyer flasks by closing them with an untight stainless steel lid. The inflow of the pump tubing was connected to four beakers, each containing around 40 ml SWF with a respective concentration of PHMB in order to reach the MIC after the desired time points for the four groups by simultaneously keeping the same flow rate for all groups. The desired concentrations were found by using the following equation 3.1.

$$c[\text{mM}] = \frac{(V_{\text{sample}} + Q \cdot t) \cdot MIC}{Q \cdot t} \tag{3.1}$$

with $V_{\text{sample}}$ = volume of one replicate [ml]
$Q$ = flow rate [ml/min]
$t$ = duration after which MIC shall be reached [min]
$MIC$ = minimum inhibitory concentration [mM]

With the in section 3.4.1 determined MIC, the four respective desired concentrations for the four beakers could be calculated. Stock PHMB solution was then diluted in 40 ml SWF per beaker to these desired concentrations by assuming a molecular weight of PHMB of around 3,000 g/mol. [128] Figure 3.2 displays the whole experimental setup.
Hourly samples of 100 µl were taken from each replicate, the first one at 0 h and the last one at 9 h. Two additional samples were taken after 24 and 48 h. This

loss in volume affected the respective actual concentrations of PHMB in each group to differ from the calculated one. However, the deviation was considered to be low enough to be neglected. The samples were first diluted in 900 µl of dextran sulphate, thenceforth diluted with a dilution factor that was estimated to match the bacterial concentration and plated by following the methodology described earlier in section 3.2.
Thereafter, this experiment was repeated with the exact same setup but by using *S. aureus* instead of *P. aeruginosa* as an inoculum.

## 3.5 Development of Resistance

For an investigation of possible development of bacterial resistance, tolerance or persistence, respectively, the two combinations between bacterial strains and PHMB (i. e. *P. aeruginosa* vs. PHMB and *S. aureus* vs. PHMB) should be incubated until bacterial growth recovered to the stationary phase.
Therefore, each combination group consisted of three replicates which in turn consisted of 50 ml Erlenmeyer flasks. Stock PHMB solution was diluted in 30 ml SWF to the twofold MIC that has been determined earlier in section 3.4.1 by assuming a molecular weight of PHMB of around 3,000 g/mol; [128] 5 ml of the antimicrobial solution has been filled into one Erlenmeyer flask of each respective replicate.
Both bacterial strains were diluted to a desired concentration of approximately $6 \cdot 10^5$ CFU/ml like described earlier in section 3.1.2.1. Next, 5 ml of each bacterial dilution were added to the respective Erlenmeyer flasks, which halved the PHMB's as well as the bacterial concentration in each flask to the respective MIC and to $3 \cdot 10^5$ CFU/ml, respectively. All Erlenmeyer flasks were then stored in a shaking incubator at 35°C and 100 rpm for 72 h (±1 h).
After incubation, one sample of 0.5 ml of each replicate was taken and diluted tenfold in a Falcon® tube that contained 4.5 ml of dextran sulphate. The dilutions were vortex mixed and thereafter centrifuged with 4,700 rpm for 10 min. The supernatant was withdrawn and 5 ml of peptone water were added to each sample. The dilutions were again vortex mixed and centrifuged with 4,700 rpm for 10 min. Anew, the supernatant was withdrawn, 5 ml of peptone water were added to each sample, and the samples were vortex mixed. Thenceforth, after these two washing steps, the bacterial dilutions were further diluted in SWF to an estimated concentration of around $6 \cdot 10^5$ CFU/ml. Finally, stock resazurin suspension was added to each sample to a concentration of 0.02 g/l. These samples were then used as bacterial dilutions in a following MIC testing, which has been described earlier in section 3.3. Testing of inactivation of PHMB was not carried out.

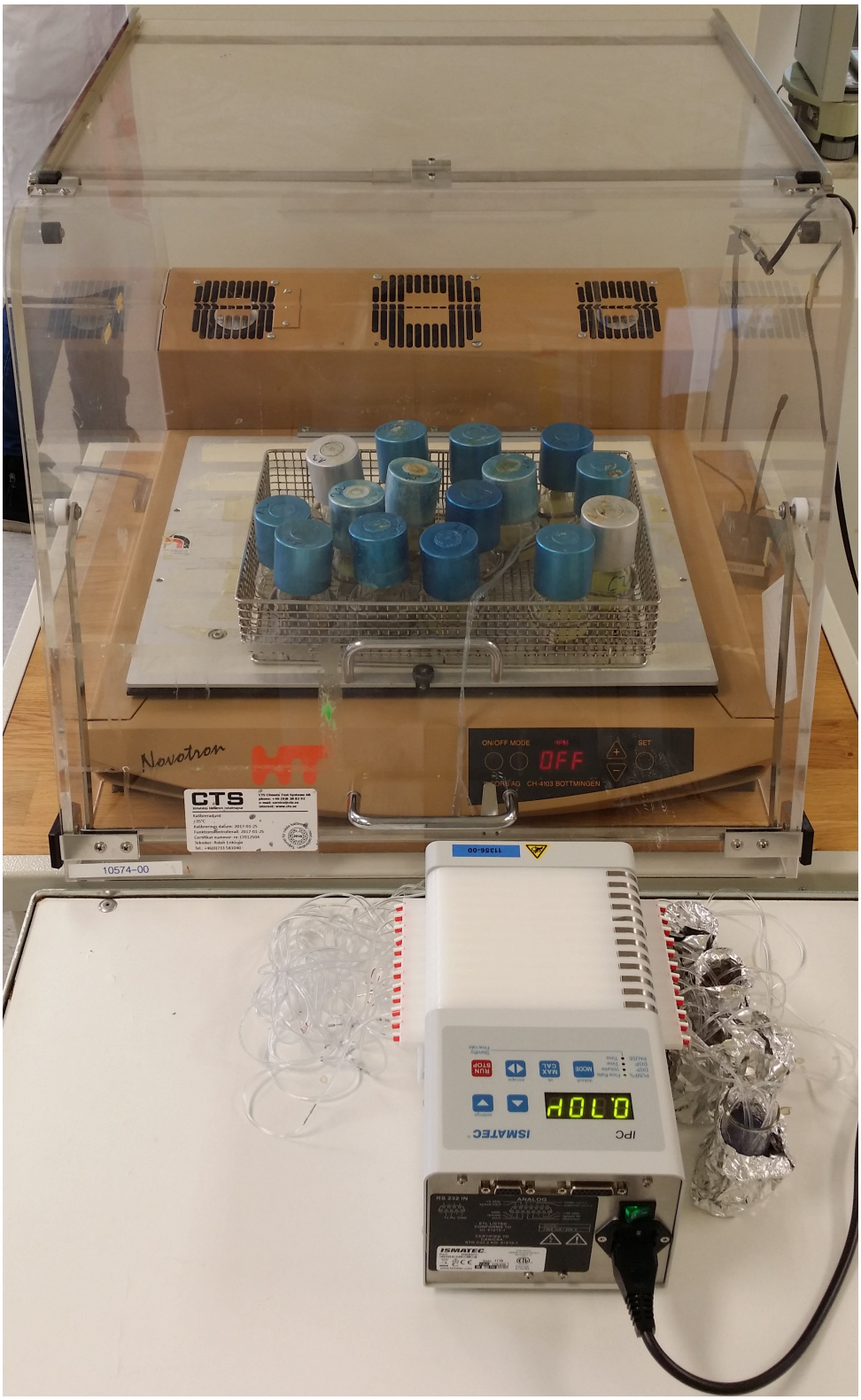


**Figure 3.2:** Experimental setup for dynamic dosage of antimicrobial compounds. Four beakers (foreground, right) which contained antimicrobial substances were connected to the IPC-N pump (foreground, centre). Pump tubing was connected to respective Erlenmeyer flasks, which represented the experimental groups and replicates. Erlenmeyer flasks were stored in a shaking incubator (background, centre).

# 4

# Results

Results of sections 3.4 and 3.5 are presented in this chapter. Thereby, the same chronological order of chapter 3 is maintained. Results are shown by figures and graphs, smaller data sets may be displayed as well. Larger raw data are not presented.

## 4.1 Minimum Inhibitory Concentration of Polyhexanide

The final concentration of the following dynamic dosage experiments was decided to be the respective MIC, whereof the results are presented in this section.
Concentrations of PHMB in the wells of the 96 well microtiter plates were starting at 100 µM in the first column and decreasing twofold until 0.048828125 µM in the last column. Bacterial start concentrations of *P. aeruginosa* and *S. aureus*, respectively, were almost identical in all replicates and approximately $3 \cdot 10^5$ CFU/ml.

### 4.1.1 Minimum Inhibitory Concentration against *P. aeruginosa*

A visual inspection of the wells with the lowest concentration of PHMB needed to inhibit colour change from blue to pink was executed in Figure 4.1 (left). A concentration was considered to be the MIC, if at least two replicates corresponded. Results were consistent throughout the three repeated lines and all replicates, no deviations were visible. The lowest concentration of PHMB needed to inhibit colour change from blue to pink was 0.78125 µM in each row. Values can be seen in Table 4.1.

**Table 4.1:** Individual MIC values of PHMB against *P. aeruginosa* in 96 well microtiter plates. The median was considered to be the MIC, if it occurred at least twice per repeated line.

| Line | Replicate 1 MIC [µM] | Replicate 2 MIC [µM] | Replicate 3 MIC [µM] | Median [µM] |
|---|---|---|---|---|
| I | 0.78125 | 0.78125 | 0.78125 | 0.78125 |
| II | 0.78125 | 0.78125 | 0.78125 | 0.78125 |
| III | 0.78125 | 0.78125 | 0.78125 | 0.78125 |

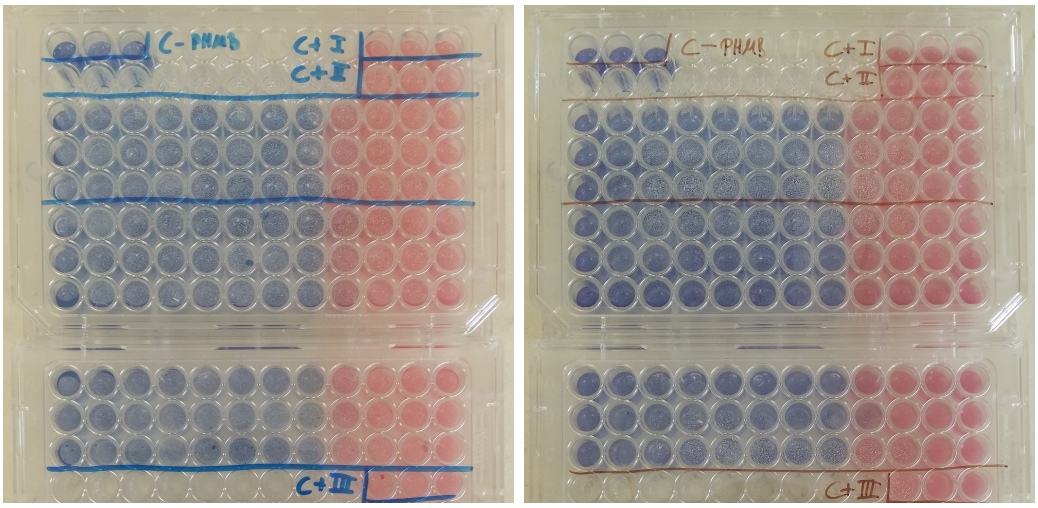


**Figure 4.1:** MIC testing of PHMB against *P. aeruginosa*; 96 well microtiter plates contained different concentrations of PHMB, starting at 100 µM in the first column and decreasing twofold until 0.048828125 µM. Wells that are appearing pink visualise metabolising bacteria. Left: MIC testing following the protocol. Right: MIC testing with changed conditions to test possible inactivation of PHMB against *P. aeruginosa*

A verification of viable counts of the starting inoculum is shown in Table 4.2.

**Table 4.2:** Individual CFU counts of *P. aeruginosa* in the starting inoculum for MIC testing of PHMB. Resulting concentrations need to be multiplied with 0.5, which was the dilution factor of the individual wells of the 96 well microtiter plates.

| Line | Dilution factor | Count 1 [CFU/ml] | Count 2 [CFU/ml] | Mean [CFU/ml] | STDEV [CFU/ml] |
|---|---|---|---|---|---|
| I | $\frac{1}{1{,}000}$ | 556 | - | $4.85 \cdot 10^5$ | $\pm 5.56 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 42 | 48 | | |
| II | $\frac{1}{1{,}000}$ | 543 | - | $5.48 \cdot 10^5$ | $\pm 8.17 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 65 | 45 | | |
| III | $\frac{1}{1{,}000}$ | 535 | - | $5.42 \cdot 10^5$ | $\pm 5.33 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 48 | 61 | | |
| Average | | | | $5.25 \cdot 10^5$ | $\pm 7.07 \cdot 10^4$ |

Since the MIC of PHMB was the same for all replicates of the three tested cell lines and therefore also for all three mean concentrations of *P. aeruginosa*, a MIC of 0.78125 µM can be considered as reliable for the average mean concentration of *P. aeruginosa*, which was $5.25 \cdot 10^5$ CFU/ml in the starting inoculum. Because of the dilution factor of 0.5 in the wells, this concentration has to be multiplied with it. This results in a concentration of $2.62 \cdot 10^5$ CFU/ml, for which the MIC of 0.78125 µM is valid.

$$MIC_{\text{PHMB vs P.a.}} = 0.78125\,\mu\text{M}$$

#### 4.1.1.1 Inactivation of Polyhexanide against *P. aeruginosa*

The results of the MIC testing with possible inactivation of PHMB against *P. aeruginosa* due to changed conditions, which was run in parallel, showed no changes of the MIC. Figure 4.1 (right) displays the microtiter plate and possible colour changes from blue to pink. Anew, a concentration was considered to be the MIC, if at least two replicates corresponded.
Results were another time consistent throughout the three repeated lines and all replicates, without any deviations and the lowest concentration of PHMB needed to inhibit colour change from blue to pink was 0.78125 µM in each row. Values can be seen in Table 4.3.

**Table 4.3:** Individual MIC values of PHMB against *P. aeruginosa* in 96 well microtiter plates after testing of possible inactivation of PHMB. The median was considered to be the MIC, if it occurred at least twice per line.

| Line | Replicate 1 MIC [µM] | Replicate 2 MIC [µM] | Replicate 3 MIC [µM] | Median [µM] |
|---|---|---|---|---|
| I | 0.78125 | 0.78125 | 0.78125 | 0.78125 |
| II | 0.78125 | 0.78125 | 0.78125 | 0.78125 |
| III | 0.78125 | 0.78125 | 0.78125 | 0.78125 |

The used starting incoulum was the same as for the in parallel run MIC testing above, which was following the protocol. Therefore, the verification and determination of viable counts in Table 4.2 is also valid here.
Since the MIC of PHMB was the same for all replicates of the three tested lines and coincided with the previously determined MIC of PHMB of 0.78125 µM against *P. aeruginosa*, it was considered as stable for the tested conditions.

### 4.1.2 Minimum Inhibitory Concentration against *S. aureus*

A visual inspection of the wells with the lowest concentration of PHMB needed to inhibit colour change from blue to pink was executed in Figure 4.2 (left). A concentration was considered to be the MIC, if at least two replicates corresponded. Results were consistent throughout the three repeated lines and all replicates, no deviations were visible. The lowest concentration of PHMB needed to inhibit colour change from blue to pink was 0.1953125 µM in each row. Values can be seen in Table 4.4.
A verification of viable counts of the starting inoculum is shown in Table 4.5.
Since the MIC of PHMB was the same for all replicates of the three tested cell lines and thus also for all three mean concentrations of *S. aureus*, a MIC of 0.1953125 µM can be considered as reliable for the average mean concentration of *S. aureus*, which was $6.82 \cdot 10^5$ CFU/ml in the starting inoculum. Because of the dilution factor of 0.5 in the wells, this concentration has to be multiplied with it. This results in a concentration of $3.41 \cdot 10^5$ CFU/ml, for which the MIC of 0.1953125 µM is valid.

$$MIC_{\text{PHMB vs S.a.}} = 0.1953125\,\mu\text{M}$$

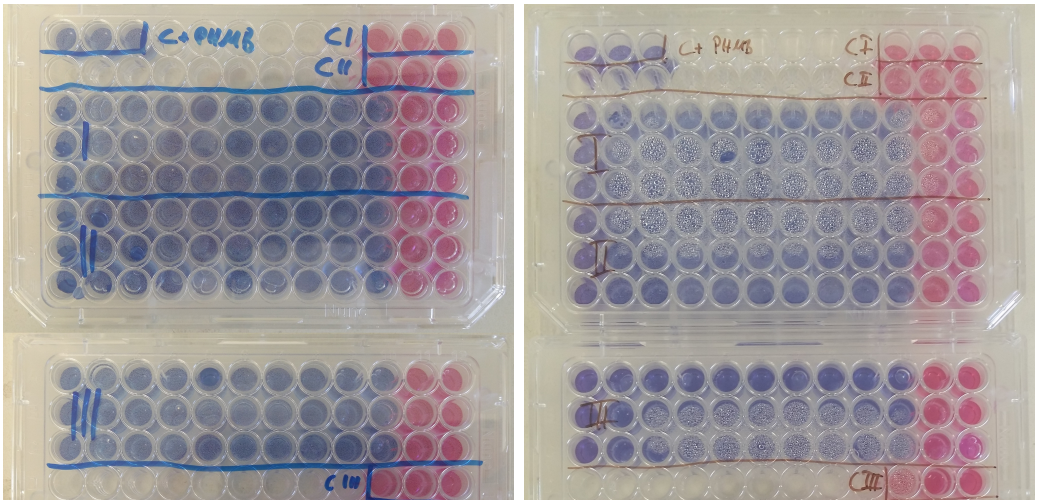


**Figure 4.2:** MIC testing of PHMB against *S. aureus*; 96 well microtiter plates contained different concentrations of PHMB, starting at 100 µM in the first column and decreasing twofold until 0.048828125 µM. Wells that are appearing pink visualise metabolising bacteria. Left: MIC testing following the protocol. Right: MIC testing with changed conditions to test possible inactivation of PHMB against *S. aureus*

**Table 4.4:** Individual MIC values of PHMB against *S. aureus* in 96 well microtiter plates. The median was considered to be the MIC, if it occurred at least twice per repeated line.

| Line | Replicate 1 MIC [µM] | Replicate 2 MIC [µM] | Replicate 3 MIC [µM] | Median [µM] |
|---|---|---|---|---|
| I | 0.1953125 | 0.1953125 | 0.1953125 | 0.1953125 |
| II | 0.1953125 | 0.1953125 | 0.1953125 | 0.1953125 |
| III | 0.1953125 | 0.1953125 | 0.1953125 | 0.1953125 |

**Table 4.5:** Individual CFU counts of *S. aureus* in the starting inoculum for MIC testing of PHMB. Resulting concentrations need to be multiplied with 0.5, which was the dilution factor of the individual wells of the 96 well microtiter plates.

| Line | Dilution factor | Count 1 [CFU/ml] | Count 2 [CFU/ml] | Mean [CFU/ml] | STDEV [CFU/ml] |
|---|---|---|---|---|---|
| I | $\frac{1}{1{,}000}$ | 609 | - | $6.13 \cdot 10^5$ | $\pm 2.87 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 65 | 58 | | |
| II | $\frac{1}{1{,}000}$ | 659 | - | $6.90 \cdot 10^5$ | $\pm 7.27 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 79 | 62 | | |
| III | $\frac{1}{1{,}000}$ | 638 | - | $7.43 \cdot 10^5$ | $\pm 7.50 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 81 | 78 | | |
| Average | | | | $6.82 \cdot 10^5$ | $\pm 8.21 \cdot 10^4$ |

#### 4.1.2.1 Inactivation of Polyhexanide against *S. aureus*

The results of the MIC testing with possible inactivation of PHMB against *S. aureus* due to changed conditions, which was run in parallel, showed no changes of the MIC. Figure 4.2 (right) displays the microtiter plate and possible colour changes from blue to pink. Anew, a concentration was considered to be the MIC, if at least two replicates corresponded.
Results were another time consistent throughout the three repeated lines and all replicates, without any deviations and the lowest concentration of PHMB needed to inhibit colour change from blue to pink was 0.1953125 µM in each row. Values can be seen in Table 4.6.

**Table 4.6:** Individual MIC values of PHMB against *S. aureus* in 96 well microtiter plates after testing of possible inactivation of PHMB. The median was considered to be the MIC, if it occurred at least twice per line.

| Line | Replicate 1 MIC [µM] | Replicate 2 MIC [µM] | Replicate 3 MIC [µM] | Median [µM] |
|---|---|---|---|---|
| I | 0.1953125 | 0.1953125 | 0.1953125 | 0.1953125 |
| II | 0.1953125 | 0.1953125 | 0.1953125 | 0.1953125 |
| III | 0.1953125 | 0.1953125 | 0.1953125 | 0.1953125 |

The used starting incoulum was the same as for the in parallel run MIC testing above, which was following the protocol. Therefore, the verification and determination of viable counts in Table 4.5 is also valid here.
Since the MIC of PHMB was the same for all replicates of the three tested lines and coincided with the previously determined MIC of PHMB of 0.1953125 µM against *S. aureus*, it was considered as stable for the tested conditions.

## 4.2 Increasing the Concentration of Polyhexanide Over Time

The results of time-kill studies during the dynamic, linear increase of the concentration of PHMB are presented in this section, by covering first *P. aeruginosa* and *S. aureus* second. Results are presented without replicates that were affected by leaking tubing and without outliers (in the following referred to as *cleaned*).

### 4.2.1 Time-Kill Behaviour of *P. aeruginosa*

The calculated concentration-time curves that represent the dosage of PHMB against *P. aeruginosa* can be seen in Figure 4.3. Concentration changes due to volume reduction (i. e. taking samples) and volume increase (i. e. adding diluted PHMB) were taken into account. The previously in section 4.1.1 determined 0.78125 µM of PHMB as MIC against *P. aeruginosa* was roughly reached after 3, 6, 9 and 24 h by

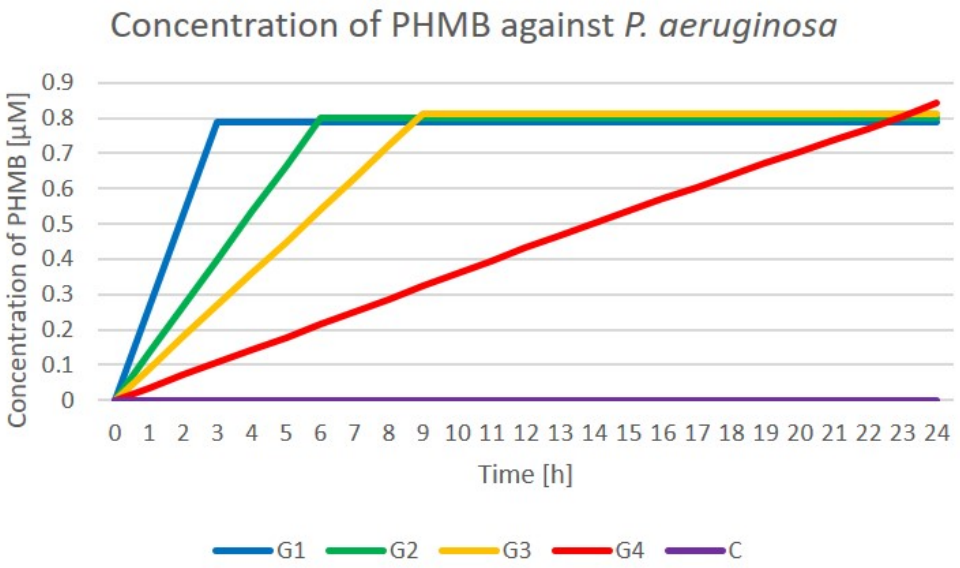


**Figure 4.3:** Calculated concentration-time curve of PHMB in SWF against *P. aeruginosa* for different test groups. G1 shall reach the MIC after 3 h, G2 after 6 h, G3 after 9 h and G4 after 24 h. C is the control group with no PHMB added at any time. The concentrations of each respective group were kept at their current value after 24 h.

group 1 (G1), group 2 (G2), group 3 (G3) and group 4 (G4), respectively. After 24 h, no further concentration changes in any group were performed.
Results of growth behaviour of *P. aeruginosa* after dynamic, linear increase of the concentration of PHMB can be seen in Figure 4.4. Group 1 started decreasing in its concentration of *P. aeruginosa* after 2 h. It reached its minimum at 6 h with a reduction of 3 $\log_{10}$-units and thereafter started to increase again. At 24 h, the bacterial concentration was almost precisely equal to the initial bacterial concentration of the inoculum and after 48 h, the bacteria could recover to roughly the same bacterial concentration as the control group. Group 2 showed an almost constant bacterial concentration until 9 h and increased afterwards. At 24 h, it was increased by 3 $\log_{10}$-units and after 48 h, it had fully recovered to the area of the control group. Group 3 showed a slightly inhibited growth behaviour until 9 h, although the bacterial concentration gradient could never be pushed down to 0 or even into negative. After 24 h, group 3 showed no difference from the control group anymore. Group 4 and the control group showed typical bacterial growth during the whole period, i. e. lag phase (0–1 h), exponential phase (1–24 h) and stationary phase (24–48 h).

### 4.2.2 Time-Kill Behaviour of *S. aureus*

The calculated concentration-time curves that represent the dosage of PHMB against *S. aureus* can be seen in Figure 4.5. Concentration changes due to volume reduction (i. e. taking samples) and volume increase (i. e. adding diluted PHMB) were taken into account. The previously in section 4.1.2 determined 0.1953125 µM of PHMB

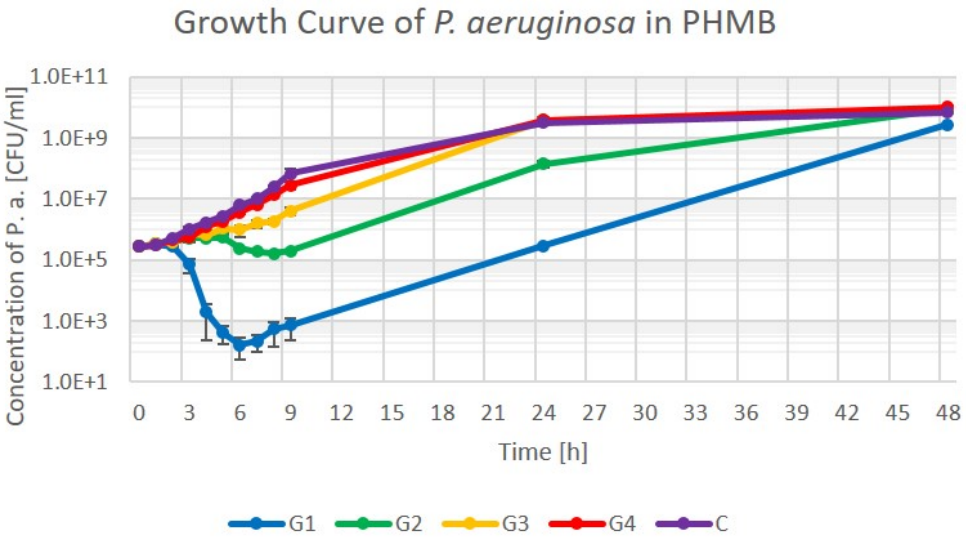


**Figure 4.4:** Growth behaviour of *P. aeruginosa* in an increasing concentration of PHMB. Concentrations of PHMB were increased with different gradients, according to Figure 4.3; Colours are matching. Error bars represent standard deviation. Missing error bars are due to cleaned results or wrongly estimated dilution factors during viable counting.

as MIC against *S. aureus* was roughly reached after 3, 6, 9 and 24 h by group 1 (G1), group 2 (G2), group 3 (G3) and group 4 (G4), respectively, and no further concentration changes in any group were performed after 24 h.
Results of growth behaviour of *S. aureus* after dynamic, linear increase of the concentration of PHMB can be seen in Figure 4.6. The concentration of *S. aureus* in the groups 1 and 2 subsequently started decreasing after 2 and 4 h, respectively. Group 1 reached its minimum at 7 h with a reduction of 2.5 $\log_{10}$-units, group 2 reached its minimum at 9 h with a reduction of 1 $\log_{10}$-unit. Afterwards, bacterial concentrations started increasing again and were 0.5 $\log_{10}$-units below (G1) and above (G2), respectively, the initial inoculum's bacterial concentration at 24 h. Both groups had fully recovered to the area of the control group after 48 h. Group 3 showed an almost constant bacterial concentration until 9 h and increased afterwards. After 24 h, group 3 had fully recovered to the area of the control group. Group 4 showed no significant difference from the control group, both groups showed the typical bacterial growth phases, including lag phase (0–2 h), exponential phase (2–9 h) and stationary phase (9–48 h).

### 4.2.3 Comparison between *P. aeruginosa* and *S. aureus* against Polyhexanide

A comparison between the respective groups 1 of *P. aeruginosa* and *S. aureus* against PHMB can be seen in Figure 4.7. The bacterial population of *P. aeruginosa* could be decreased faster and also close to 1 $\log_{10}$-unit lower compared to *S. aureus*, so was the minimum reached already after 6 h (7 h for *S. aureus*). However, *P. aeruginosa*

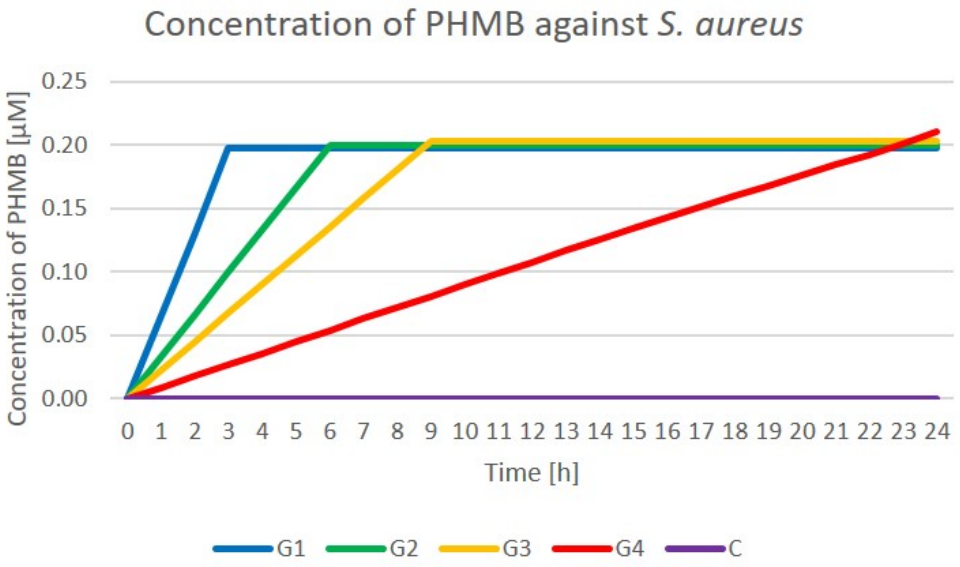


**Figure 4.5:** Calculated concentration-time curve of PHMB in SWF against *S. aureus* for different test groups. G1 shall reach the MIC after 3 h, G2 after 6 h, G3 after 9 h and G4 after 24 h. C is the control group with no PHMB added at any time. The concentrations of each respective group were kept at their current value after 24 h.

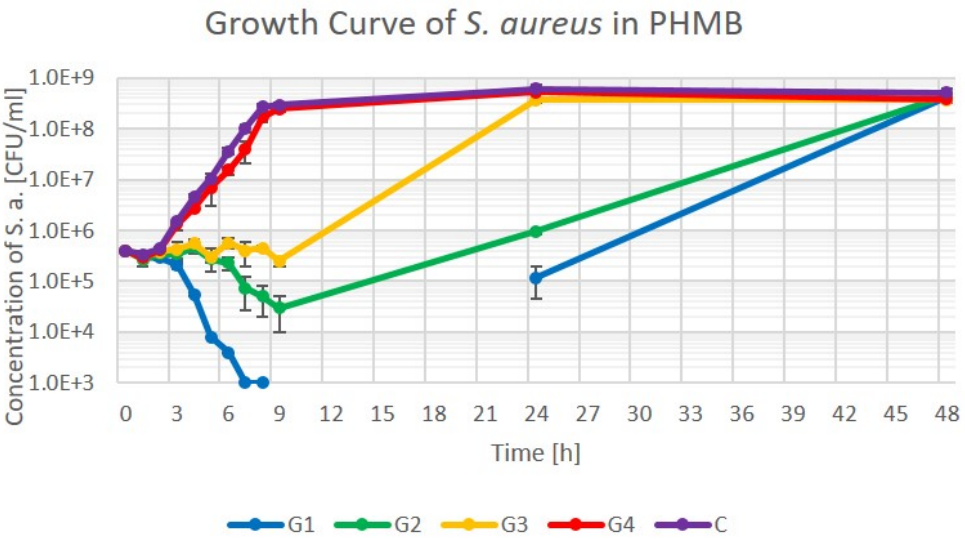


**Figure 4.6:** Growth behaviour of *S. aureus* in an increasing concentration of PHMB. Concentrations of PHMB were increased with different gradients, according to Figure 4.5; Colours are matching. Error bars represent standard deviation. Missing data points or error bars are due to cleaned results or wrongly estimated dilution factors during viable counting.

started to grow again already after these 6 h, whereas *S. aureus* started growing only after 8 h. Both bacterial strains reached roughly the initial bacterial concentration after 24 h and both fully recovered to their respective control group's value after 48 h.

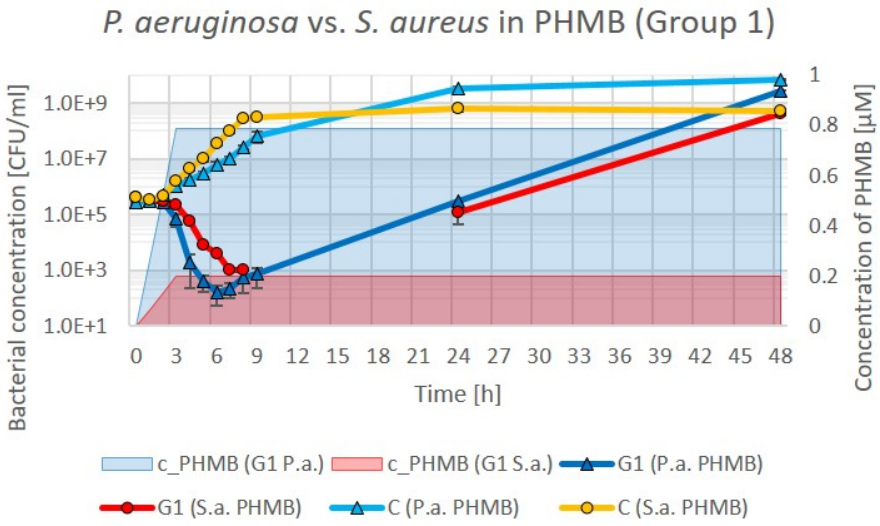


**Figure 4.7:** Comparison between the respective groups 1 of *P. aeruginosa* and *S. aureus* against PHMB. The respective concentration-time curves of PHMB are drawn in the background and match the colour of the corresponding bacterial concentration-time curve. Error bars represent standard deviation. Missing data points or error bars are due to cleaned results or wrongly estimated dilution factors during viable counting.

The respective groups 2 of *P. aeruginosa* and *S. aureus* against PHMB are compared in Figure 4.8. *P. aeruginosa* could be held roughly constant in their bacterial concentration, whereas the one of *S. aureus* decreased around 1 $\log_{10}$-unit. Moreover, after 24 h, the bacterial concentration of *S. aureus* was only 0.5 $\log_{10}$-units above the initial bacterial concentration, but the population of *P. aeruginosa* almost recovered to less than 1 $\log_{10}$-unit below its control's bacterial concentration. Both groups recovered fully to their respective control group's values after 48 h.

Figure 4.9 shows a comparison between the respective groups 3 of *P. aeruginosa* and *S. aureus* against PHMB. The growth in concentration of *P. aeruginosa* could only be slightly inhibited until 9 h, but never be stopped, whereas the concentration of *S. aureus* could be held almost constant during the first nine hours. However, both groups showed no significant difference from the control group anymore as of 24 h.

A comparison between the respective groups 4 of *P. aeruginosa* and *S. aureus* against PHMB revealed no difference from their corresponding control group during the whole period; Typical bacterial growth phases, including lag phase, exponential phase and stationary phase could be observed.

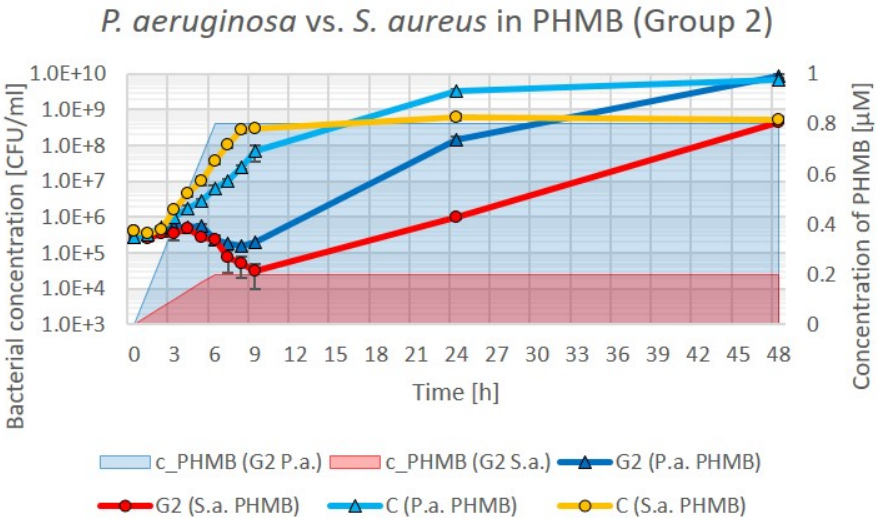


**Figure 4.8:** Comparison between the respective groups 2 of *P. aeruginosa* and *S. aureus* against PHMB. The respective concentration-time curves of PHMB are drawn in the background and match the colour of the corresponding bacterial concentration-time curve. Error bars represent standard deviation. Missing error bars are due to cleaned results or wrongly estimated dilution factors during viable counting.

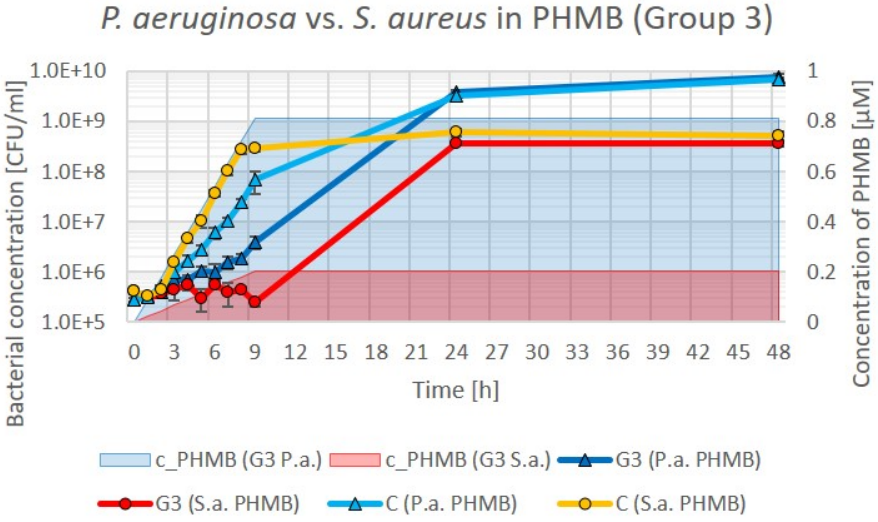


**Figure 4.9:** Comparison between the respective groups 3 of *P. aeruginosa* and *S. aureus* against PHMB. The respective concentration-time curves of PHMB are drawn in the background and match the colour of the corresponding bacterial concentration-time curve. Error bars represent standard deviation. Missing error bars are due to cleaned results or wrongly estimated dilution factors during viable counting.

## 4.3 Development of Resistance against Polyhexanide

Bacteria were grown for 72 h in their in section 4.1 determined respective MIC. After dilution of bacteria to the estimated concentration that was used in section 4.1 as well, a MIC testing was repeated by following the description in section 3.3, without investigating a possible inactivation of PHMB. Anew, concentrations of PHMB in the wells of the 96 well microtiter plates were starting at 100 µM in the first column and decreasing twofold until 0.048828125 µM in the last column.

### 4.3.1 Resistance of *P. aeruginosa*

A visual inspection of the wells with the lowest concentration of PHMB needed to inhibit colour change from blue to pink was executed in Figure 4.10. A concentration was considered to be the MIC, if at least two replicates corresponded.

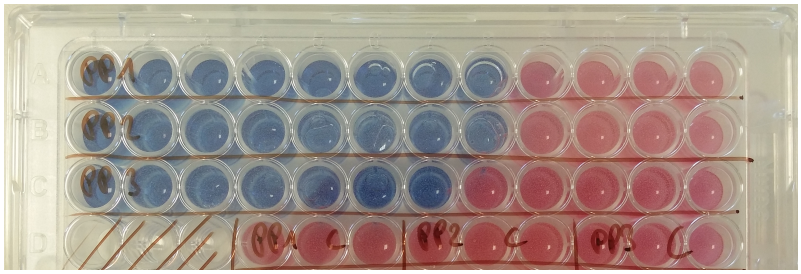

**Figure 4.10:** Retesting of the MIC of PHMB against *P. aeruginosa* after culturing for 72 h in the MIC of PHMB; 96 well microtiter plates contained different concentrations of PHMB, starting at 100 µM in the first column and decreasing twofold until 0.048828125 µM. Wells that are appearing pink visualise metabolising bacteria.

Results were consistent throughout the first two replicates, the third replicate differed twofold. The lowest concentration of PHMB needed to inhibit colour change from blue to pink was 0.78125 µM in the first two replicates. Values can be seen in Table 4.7.

**Table 4.7:** Individual MIC values of PHMB against *P. aeruginosa* in 96 well microtiter plates after culturing for 72 h in the MIC of PHMB. The median was considered to be the MIC, if it occurred at least twice.

| Replicate 1 MIC [µM] | Replicate 2 MIC [µM] | Replicate 3 MIC [µM] | Median [µM] |
|---|---|---|---|
| 0.78125 | 0.78125 | 1.5625 | 0.78125 |

The MIC resulted in 0.78125 µM of PHMB against *P. aeruginosa* and coincides with the initial MIC testing in section 4.1.1. A verification of viable counts of the starting inoculum is shown in Table 4.8.

**Table 4.8:** Individual CFU counts of *P. aeruginosa* in the starting inoculum for MIC testing of PHMB after culturing for 72 h in the MIC of PHMB. Resulting concentrations need to be multiplied with 0.5, which was the dilution factor of the individual wells of the 96 well microtiter plates.

| Replicate | Dilution factor | Count 1 [CFU/ml] | Count 2 [CFU/ml] | Mean [CFU/ml] | STDEV [CFU/ml] |
|---|---|---|---|---|---|
| I | $\frac{1}{1{,}000}$ | 708 | - | $6.56 \cdot 10^5$ | $\pm 6.13 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 57 | 69 | | |
| II | $\frac{1}{1{,}000}$ | 876 | - | $6.79 \cdot 10^5$ | $\pm 1.40 \cdot 10^5$ |
| | $\frac{1}{10{,}000}$ | 58 | 58 | | |
| III | $\frac{1}{1{,}000}$ | 456 | - | $5.45 \cdot 10^5$ | $\pm 6.52 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 61 | 57 | | |
| $\text{Average}_{\text{I \& II}}$ | | | | $6.67 \cdot 10^5$ | $\pm 1.08 \cdot 10^5$ |

Since the MIC of PHMB was determined with the first two replicates, it can be considered as reliable for the average mean concentration of *P. aeruginosa* between those two replicates, which was $6.67 \cdot 10^5$ CFU/ml in the starting inoculum. Because of the dilution factor of 0.5 in the wells, this results in a concentration of $3.34 \cdot 10^5$ CFU/ml. This is around 127% of the concentration used in the initial MIC testing (compare section 4.1.1).

$$MIC_{\text{PHMB vs P.a.; 72h}} = 0.78125\,\mu\text{M}$$

### 4.3.2 Resistance of *S. aureus*

A visual inspection of the wells with the lowest concentration of PHMB needed to inhibit colour change from blue to pink was executed in Figure 4.11. A concentration was considered to be the MIC, if at least two replicates corresponded. The second replicate was not available, since its bacterial population died during culturing. The lowest concentration of PHMB needed to inhibit colour change from blue to pink was 0.1953125 µM. Values can be seen in Table 4.9.

**Table 4.9:** Individual MIC values of PHMB against *S. aureus* in 96 well microtiter plates after culturing for 72 h in the MIC of PHMB. The median was considered to be the MIC, if it occurred at least twice.

| Replicate 1 MIC [µM] | Replicate 2 MIC [µM] | Replicate 3 MIC [µM] | Median [µM] |
|---|---|---|---|
| 0.1953125 | - | 0.1953125 | 0.1953125 |

The MIC resulted in 0.1953125 µM of PHMB against *S. aureus* and coincides with the initial MIC testing in section 4.1.2. A verification of viable counts of the starting inoculum is shown in Table 4.10.

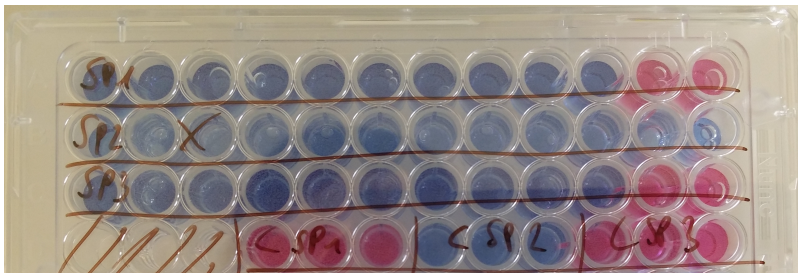

**Figure 4.11:** Retesting of the MIC of PHMB against *S. aureus* after culturing for 72 h in the MIC of PHMB; 96 well microtiter plates contained different concentrations of PHMB, starting at 100 µM in the first column and decreasing twofold until 0.048828125 µM. The second replicate was not available, since the bacterial culture died during culturing. Wells that are appearing pink visualise metabolising bacteria.

**Table 4.10:** Individual CFU counts of *S. aureus* in the starting inoculum for MIC testing of PHMB after culturing for 72 h in the MIC of PHMB. Resulting concentrations need to be multiplied with 0.5, which was the dilution factor of the individual wells of the 96 well microtiter plates.

| Replicate | Dilution factor | Count 1 [CFU/ml] | Count 2 [CFU/ml] | Mean [CFU/ml] | STDEV [CFU/ml] |
|---|---|---|---|---|---|
| I | $\frac{1}{1{,}000}$ | 652 | - | $6.31 \cdot 10^5$ | $\pm 1.51 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 62 | 62 | | |
| II | $\frac{1}{1{,}000}$ | - | - | - | - |
| | $\frac{1}{10{,}000}$ | - | - | | |
| III | $\frac{1}{1{,}000}$ | 612 | - | $5.17 \cdot 10^5$ | $\pm 7.13 \cdot 10^4$ |
| | $\frac{1}{10{,}000}$ | 44 | 50 | | |
| Average | | | | $5.74 \cdot 10^5$ | $\pm 7.66 \cdot 10^4$ |

The MIC of PHMB can be considered as reliable for the average mean concentration of *S. aureus*, which was $5.74 \cdot 10^5$ CFU/ml in the starting inoculum. Because of the dilution factor of 0.5 in the wells, this results in a concentration of $2.87 \cdot 10^5$ CFU/ml. This is around 84% of the concentration used in the initial MIC testing (compare section 4.1.2).

$$MIC_{\text{PHMB vs S.a.; 72h}} = 0.1953125\,\mu\text{M}$$

# 5
# Discussion

Analysing and comparing results with expectations and literature is the goal of this chapter, as well as outlining decisions to possible respective further or superseding investigations. Finally, potential limitations will be examined.
Already the determined MIC of PHMB revealed a potential discussion. PHMB (compare section 2.4.3.1) seems to work more potently for *S. aureus* as compared to *P. aeruginosa* since the needed concentration of PHMB to inhibit growth of *S. aureus* resulted in a four times lower concentration as for *P. aeruginosa* in section 4.1. As mentioned, recently it has been claimed a different mechanism of action of PHMB, at least against Gram-negative bacteria, which states binding to DNA fragments instead. [138] A penetration of the cell wall is necessary for doing so. However, PHMB is a molecule with polar sites, which makes it being repelled from the bacteria's membrane. Therefore, passive permeation can considered to be very unlikely. Endocytosis as a mechanism of uptake is likelier and could be more difficult for Gram-negative bacteria due to their diderm membrane, which might slow down penetration as compared to monoderm Gram-positive bacteria. This would explain the necessary higher concentration against Gram-negative *P. aeruginosa* for this.
Assuming the formerly believed mechanism of action by sequestration of the bacteria's cell wall needs further discussion. Since PHMB would bind, inter alia, directly to a bacterium's cell membrane and fragment it, the double membrane of Gram-negative bacteria could protect them by simply having a second shield against the compound. PHMB might need to destroy the bacteria's membrane twice in order to lead to bactericidal results. Nevertheless, as a matter of the age difference of around 30 years between the literature for this mechanism compared to the topicality of the literature of the new mechanism, the latter one is believed to be more reliable. [131,138] Conceivable would also be an actual effect of both mechanisms of action.
A measurement of the MIC is a widely used method to gather information about the antimicrobial effect, however, it has been found that a MIC can vary in vitro according to the experimental conditions such as the used inoculum, growth medium and time. [147] Although it has been found consistency within the results of the MIC testing during this whole project, this limits the MIC testing as a metric and makes it a rather relative one.
The time-kill curve of increasing concentration of PHMB against *P. aeruginosa* in section 4.2.1 mainly shows three noteworthy findings. First, reaching the same bacterial concentration after 24 h as in the initial inoculum includes a decreasing bacterial population in between, i. e. the killing rate is higher than the growth rate. This showed group 1, which drastically reduced its bacterial concentration before reaching

the initial one after 24 h. Second, holding a bacterial population at a constant level seems to require a constantly increasing concentration of PHMB. This was shown by group 2, where during the first 6 h of increasing concentration of PHMB and another 3 following hours it resulted in a constant bacterial concentration, however, after continuing to hold the concentration of PHMB at a constant level thereafter, bacterial recovery seemed initiated. Third, reaching the MIC of PHMB after 9 h or later seems to have no remarkable impact on bacterial growth of *P. aeruginosa* as compared to the control group. This behaviour was shown by group 3 and 4.

Higher vulnerability of Gram-positive bacteria against PHMB could be proven again in sections 4.2.2 and 4.2.3. Group 3 of *S. aureus* showed to be held constant in its bacterial concentration as long as the concentration of PHMB was rising, instead of showing close to no difference of group 3 of *P. aeruginosa*. This is rather comparable to group 2 of *P. aeruginosa*, however, here a higher concentration gradient was used. The same gradient causes in *S. aureus* the effect of ending up at the initial bacterial concentration, which was only reachable with an even higher gradient in *P. aeruginosa*. Since this higher gradient in group 1 of *S. aureus* led to a steeper fall in bacterial concentration but only do a difference of 1 $\log_{10}$-unit after 24 h as compared to group 2, this might be an indication that bacterial recovery is initiated earlier in a population that has been reduced faster.

As an applicable antiseptic in wound care, this suggests the use of PHMB, especially if the known bacterial infection is Gram-positive. For preventative measures, a combination with an antiseptic of higher potency against Gram-negative bacteria might be conceivable.

All results suggest a strong time-dependence of the MIC. The same final concentration led to completely different growth behaviours. Nevertheless, there are publications that contain investigations of growth behaviour that indicate similar growth curves than the obtained ones, however, antimicrobial substances have never been supplied in a dynamic manner, but full dosage in the beginning. [148,149] Moreover, the decided times of investigation vary a lot and are often rather short-term, meaning 6 h and less. Possible recovery of the respective bacterial populations might not be covered by this. In both ways, the here presented results are unique.

The ability of all replicates to at least show growth behaviour or even having recovered already as of 48 h together with the fact that a MIC testing over 24 h required initial bacterial killing was therefore surprising and finally led to further hypotheses, which pushed the initial one slightly into the background: Is PHMB inactivated by certain experimental conditions and therefore not available anymore after a specific time, and if not, are bacteria able to develop resistances against it? Instead of developing the used method about dynamic dosage further, it was decided to investigate into those directions instead.

PHMB has been used in conditions that simulated the used experimental conditions of the time-kill investigations during the respective MIC test. As it has been shown in sections 4.1.1.1 and 4.1.2.1, there was no inactivation affecting the results after this simulation and therefore can be excluded.

As discussed in section 2.4.2, the method for testing resistance is a retesting of the MIC. Withal, it should be noted that a MIC test only allows conclusions about resistance, but not about tolerance or persistence, as mentioned earlier. [108]

The difficulty of the MIC tests in section 4.3 after 72 h of incubation is the bacteria being in their death phase already. The concentration of a culture must be estimated as precisely as possible in order to dilute the bacterial concentration to the same as in the initial MIC testing. Only this grants reasonable comparisons. A decreasing population drastically increases the difficulty of a correct estimation of bacterial concentration. Furthermore, the washing steps, including vortex mixing, diluting in neutralising media, and centrifugation, affected the actual received bacterial concentration. Nevertheless, 127% of the concentration of *P. aeruginosa* used initially for the MIC testing are considered as equal enough to compare. Since the MIC was identical, no signs of developed resistances of *P. aeruginosa* against PHMB can be seen. The same observation goes for *S. aureus*, where 84% of the initially used bacterial concentration were tested and resulted in an equal MIC of PHMB. Uncertainties about the MIC test metric that have been discussed above relativise such a statement to some extent.

An explanation for bacteria to recover can then only be tolerance or persistence. As described earlier in section 2.4.2, tolerance is defined by surviving transient higher concentrations. Since this was not the case, bacterial persistence appears to be the explanation to the ability to recover. A small subpopulation that is able to withstand the respective antimicrobial substance's mechanism of action seems to be responsible for creating a new stationary phase population. Recovery due to a deactivation of antimicrobial substances can be excluded since it has not shown any effect on the MIC testing.

Statements about pharmacodynamic and pharmacokinetic parameters are difficult due to a used concentration profile that is only increasing but not decreasing in concentration. Since the actual concentration has never been above the MIC, a dependence on T>MIC can be excluded. The AUC differed among the groups, however, the difference over 48 h was very little. The longer the experiment would run, the smaller the difference in the respective AUC would differ and could not explain the different growth curves anymore. $C_{max}$ was the same in each group, but one has to consider that a $C_{max}$ value is just as a MIC value dependent on the used bacterial concentrations. As soon as bacteria start growing, a necessary $C_{max}$ value to inhibit growth would increase as well, which would set up different necessary $C_{max}$ values for the respective groups. A dependence on $C_{max}$ seems conceivable with this background, because the earlier the MIC was reached, the higher was the reached effect. Furthermore, a simple dependence on the time until the MIC is reached could also be reasonable. Such a dependence has to be seen together with the fact, that every group started with a low bacterial concentration that naturally needs to go into exponential phase first in order to finally reach stationary phase. A dependence on the time until the MIC is reached for cultures in stationary phase is less conceivable, if a bacterial strain is not able to develop resistances against an antimicrobial substance. In that case, time is important to prevent this development. To make a specific statement here, investigations into more complex concentration profiles are necessary. A general lack of knowledge about such concentration profiles in wounds, including PHMB, further impedes the possibility of investigating concentration profiles.

A translation to release kinetics of wound dressings would suggest a high initial boost

of PHMB to a determined bactericidal concentration. Especially in order to prevent bacterial infections, such profiles seem to have the highest effect. In particular by considering the apparent ability of bacteria to recover despite the presence of PHMB, it is very important to be as effective as possible with an early killing of bacteria, before bacteria are able to recover.
Moreover and nevertheless, the findings in this project might be useful for other applications. In fact, the effectiveness of any application that covers the prevention of bacterial growth depends on pharmacodynamic and pharmacokinetic knowledge of the used respective antimicrobial compound against a specific bacterial strain. In particular, it could concern medical devices such as catheters and implants to prevent bacterial infections, medication to treat such infections, but also cosmetics that promise to act antimicrobially or even for the preservation of foodstuffs.
Miniaturisation of experimental setups often goes with advantages like great space savings and easy transport. For this reason, it is conceivable to miniaturise the used experimental setup by the use of microfluidic systems such as a lab-on-a-chip device. This would allow to accommodate the used macroscopic methodology on a credit card-sized plastic substrate. Small flow rates are implemented via capillary forces. Biological, chemical, and physical processes take place in various reaction and analysis chambers. Good control over concentrations and gradients is a further advantage of microfluidic systems that makes them suitable to the used methodology. The technology has already been used in order to culture or detect bacteria and even allowed to study single cells. [150, 151] If bacteria would behave different in smallest space regarding growth and expressed phenotype compared to the macroscopic methodology remains to be investigated.

## 5.1 Limitations

Apart from the already mentioned issues about the MIC testing, the biggest limitation during the project was leaking tubing during the experimental phase. Since only triplicates were used, a failing replicate already meant a loss in statistical significance. Leaking tubing occurred during all runs and therefore, statistical calculations have not been carried out. Research about the cause of leaking tubing resulted in the autoclave sterilisation step. In order to keep the bacteria not affected by impurities of the environment, the whole system needed to be kept sterile, which was realised by autoclaving all parts, including the tubing. Tubing was sealing tight with the respective connectors before, however, in the autoclaving step, a temperature of 121°C was reached, which is below the melting temperature of Tygon® tubing. Still, testing revealed that all tightness was lost after autoclaving, which suggested the temperature being above the glass transition temperature, even though below the melting temperature. This would explain a relaxation of the material, which so adjusts to the connectors' shape and cannot grant a tight sealing any longer. A solution to this limitation could be fulfilled in two ways. First, the material could be exchanged with another one, that allows higher temperatures without affecting the material's properties. A possible tubing material could be the thermoplastic elastomer PharMed®. Second, the sterilisation technique could be changed. An appropriate alternative could be ethylene oxide sterilisation, but conceivable is also

just a simple flushing with an ethanol-propanol-mixture. Radiation sterilisation is not recommended, since it might change the materials properties by cross-linking the polymer.
A further limitation was the used flow rate of 0.7 µl/min. It is challenging to calibrate a small flow like this, since already small measuring inaccuracies might have a major influence onto the actual flow. A solution for this could be the use of a higher flow rate, which would also mean a higher increase in volume of the samples. To counteract this, the initial sample volume could be increased as well, which would keep the higher increase in volume negligible.

# 6
# Conclusion

Bringing all findings together to a final statement by covering the initial hypothesis leads to a likely pharmacodynamic and pharmacokinetic dependence on $C_{max}$ of PHMB regarding the time-kill behaviour of both used bacterial strains. Furthermore, dependence on the time until an effective concentration is reached, i. e. MIC or MBC, cannot be excluded and is conceivable.
Translating these findings to a suggestion for release kinetics of wound dressings urge to release the used antimicrobial compounds in a determined bactericidal dosage as fast as possible in order to reach highest efficacy. The shown higher potency of PHMB against Gram-positive bacteria suggests a combination with an antiseptic of higher potency against Gram-negative bacteria.
The additional investigation into the development of resistance proved that neither *P. aeruginosa* nor *S. aureus* are able to develop resistances against PHMB.
Therefore, the ability of bacteria to recover is a result of either persistence or tolerance, whereof persistence showed to be more probable. In fact, one can conclude that a MIC testing is only possible due to bacteria's persistence, since it appeared to be virtually impossible to keep a bacterial population constant over 24 h. It rather needs to be decreased in a first step and then recover to the initial concentration in a second step.
Farther-reaching conclusions can be drawn about the MIC. A MIC appeared to be a function of time and therefore is highly variable due to a high dependence on time but also on experimental conditions. These attributes make the MIC to a suboptimal basis of the experiment and one might even conclude that there is no such thing as a MIC.

## 6.1 Future Research

Further investigations need to be done into bacteria's ability to persist, tolerate or even resist concentrations of antimicrobial compounds. The important differences have been described before in section 2.4.2, the suggestions by Brauner et al. from 2016 could serve as guidance. [108]
Such bacterial behaviour would most likely be accompanied by a changed phenotype. This makes it reasonable to investigate into that as well, meaning a comparison of bacterial phenotype before and after the cultivation in an antimicrobial compound. A changed phenotype is also a characteristic of bacteria living in a biofilm, which can be the result of a long-term stable population in stationary growth phase. Bacterial biofilm formation has been moving into the limelight of science recently. This

experiment could deliver useful results for such treatments by using it on bacterial populations in stationary phase or even in biofilms.
However, the eventual next step would be the enhancement of the model in order to not only increase the concentration of an antimicrobial compound, but also to decrease it. Ultimately, a free adjustment of parameters is desirable so that any thinkable concentration profile can be implemented. For the application of this, the use of a Franz diffusion cell system can be considered. This system is made of two primary components, an upper donor chamber and a lower receptor chamber. The receptor chamber could contain a bacterial culture, whereas the donor chamber could introduce changing concentrations of antimicrobial substances. The two chambers are separated by a semi-permeable membrane, whose area exposed to the chambers (*orifice*) is defining the Franz diffusion cell size. Another possibility would be a hollow fibre bioreactor. Such bioreactors consist of multiple artificial tube membranes with a semi-permeable barrier that are packed into cartridges and can be used for liquid and gaseous separations. A cartridge could contain a bacterial population, whereas the fibres themselves carry an antimicrobial substance and could introduce concentration changes of this substance into the cartridge. Both methods allow a concentration change based on diffusion through a membrane.

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# A

# Appendix

## A.1 Protocol of Ray Design and MIC Testing

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## Purpose

Evaluate additive or synergistic effects of combinations of substances on antimicrobial effect

## Definitions

| | |
|---|---|
| SWF - | 50% fetal bovine serum in (MRD) |
| 96-well plate - | Sterile 96-well plate with well volume of at least 300 µL, 8 rows (A-H) and 12 columns (1-12) |
| Deep 96-well plate - | Sterile 96-well plate with well volume of at least 2000 µL, 8 rows (A-H) and 12 columns (1-12) |
| MIC - | Minimal inhibitory concentration; the lowest concentration of substance needed resulting in no growth, assessed by resazurin |
| Test media | cation adjusted MH broth supplemented with 10% FCS and 0.02 mg/ml resazurin |

## Materials and chemicals

Microorganisms:

*Pseudomonas aeruginosa* ATCC 15442

*Staphylococcus aureus* ATCC 6538

*Candida albicans* ATCC 10231

Sterile non-treated 96 well plates with well volumes of at least 300 µL and 2 ml

Sterile tips

Tryptic soy broth

Trypticase soy agar plates

Sabouraud agar plates

Maximum recovery diluent (MRD, 8.5 g NaCl/L and peptic digest of animal tissue, 1.0 g/L)

Fetal bovine serum

Resazurin sodium salt

Muller hinton broth, cation adjusted

## Test procedure

MIC of single substances is determined before evaluation of antimicrobial effect using combinations of substances.

### MIC of single substances

The MIC of each substance to be tested needs to be determined prior combining substances. This is done in two steps, first by ten-fold serial dilution of substances in MH-broth supplemented with 10%

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FCS and 0.02 mg/ml resazurin (Test media) using 96-well plates, and then narrowing the MIC concentration range by two-fold dilutions.

The highest final substance concentration in the system should be 100 mM, using test media as diluent. If a particular substance is not soluble in test media, DMSO could be used to make a high concentrated stock solution of the substance prior dilution in test media. The maximum amount of DMSO in the final system should not exceed 5% (w/w).

Perform 10-fold serial dilution by moving 10 µL substance dissolved in test media to 90 µL test media (3 replicates, i.e. 3 rows per substance) (figure 1). Next, add 90 µL microorganisms suspended in test media to all wells in a concentration so that the final amount of bacteria is 1-3 x $10^6$ CFU/ml and 1-3x$10^5$ CFU/ml for Candida. Gently stir the plate before transferring the plate to an incubator at 35 °C and 90% RH. Include a control with only test media and substance (highest concentration tested), this is to make sure that the substance alone does not generate a colour change of test media.

Confirm the viable counts of the inoculum by serial dilution of the inoculum in suitable diluent and plating on suitable substrate in duplicates followed by incubation in 35 °C to visualize colonies.

After 24 ±1 h of incubation of the 96-well plate, note the colour in the wells as blue (1) or purple/pink (2).

Repeat the test using two-fold dilutions (100 + 100 µL),3 replicates, the highest concentration of substance corresponds to the MIC value determined in the previous test based on 10-fold dilutions (figure 1).

Report the lowest concentration of substance needed to inhibit colour change from blue to purple/pink. At least two of the replicates need to correspond for the results to be valid.

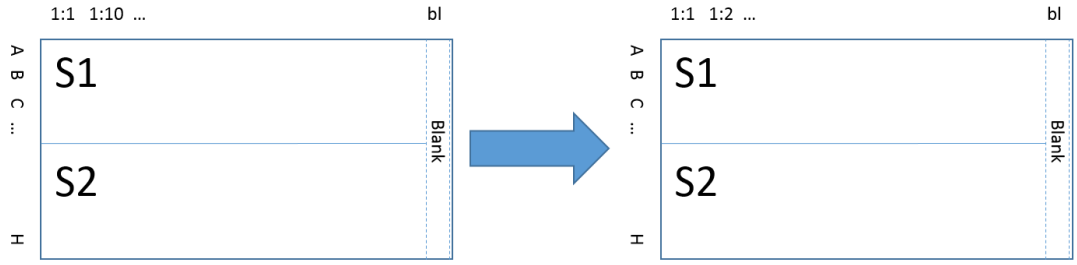


*Figure 1.* Ten-fold (left) and two-fold dilution series (right)

## Ray design using two substances

Prepare six stock solutions of substance 1 and 2 in test media according to excel sheet (“ray design calculation two substances”). Prepare two-fold dilutions series of each stock solution in a 96 well plate and dilute each stock solution 10 times, resulting in 11 different concentrations of each ray. Prepare 3 blank wells (A12, B12, C12) containing only test media and 3 positive control wells (E12, F12, G12) containing gentamicin in a suitable concentration. Mix 100 µL of these prepared solutions (rays and controls) with 100 µL microorganisms, using a concentration of cells so that the final

amount of bacteria is 1-3 x $10^6$ CFU/ml and 1-3x$10^5$ CFU/ml for Candida and the final well volume is 200 µL.

Gently stir the plate before transferring the plate to an incubator at 35 °C and 90% RH.

Confirm the viable counts of the inoculum by serial dilution of the inoculum in suitable diluent and plating on suitable substrate in duplicates followed by incubation in 35 °C to visualize colonies.

After 24 ±1 h of incubation of the 96-well plate, note the colour in the wells as blue (1) or purple/pink (2).

Note that the maximum final concentration of any substance should not exceed 100 mM, even though MIC or 2 x MIC might be above that limit.

### Ray design using three substances

For each microorganism to be tested, prepare four identical plates combining substance 1 and 2, according to the description above ("Ray design using two substances"). Prepare the dilutions of the six rays in deep well plates and aliquot 66 µL into four 96-well plates. Next add 66 µL substance 3 to the six rays in each plate, using fixed concentrations of substance 3 according to excel sheet ("ray design calculation three substances"). Finally, add 66 µL microorganisms using a concentration of cells so that the final amount of bacteria is 1-3 x $10^6$ CFU/ml and 1-3x$10^5$ CFU/ml for Candida and the final well volume is 200 µL. Incubate and analyse according to previous description.

**Note:**

If multiple species are tested on the same test occasion, dilution and combining substances can be done in deep 96-well plates and then aliquoted into 96-well plates to create identical setups to which different microorganisms are added.

**Reporting**

Raw data, based on the scoring system described previously, including information about:

- Type of microorganism
- Concentration of tested substances
- Amount of DMSO in system
- Appearance of substance suspensions (clear, i.e. dissolved, or precipitated)

Reporting will be done using excel template supplied by Mölnlycke Health Care and by photographing agar plates.