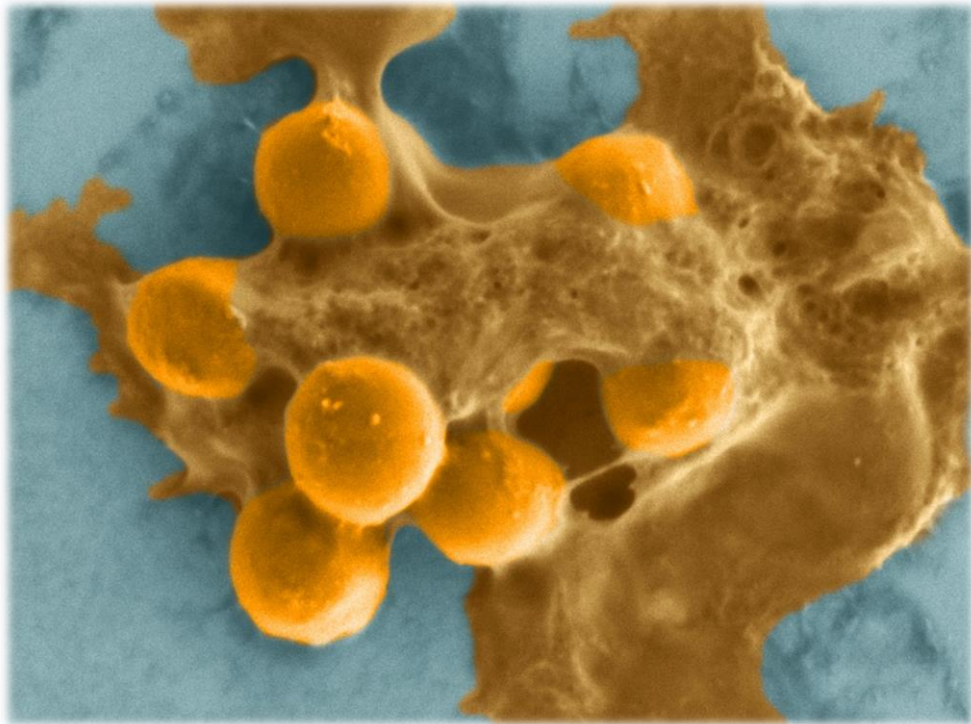# Towards Novel Antibiofilm Strategies

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A thesis submitted for the degree of

Doctor of Philosophy

at the University of Adelaide, Adelaide, Australia

Faculty of Health and Medical Sciences,

School of Medicine, Discipline of Surgery

April 2017

THE UNIVERSITY
of ADELAIDE

To my parents- who opened the world to me.

And to Nicky- who made my world complete.

Table of contents

I. Abstract

The rise of multidrug resistant bacteria has global implications posing a threat to human health. Bacteria naturally reside in biofilms as complex communities of cells encased in a self-assembled matrix. The biofilm state renders bacteria up to 1000-fold less susceptible to antimicrobial treatments, while unarming the body's immune response and promoting antibiotic resistance. Biofilms are recognised as the origin of devastating, antibiotic-refractory diseases and are associated with 80% of infections in the body, including chronic rhinosinusitis. The capability of bacteria in biofilms to resist current antibiotic therapies emphasises the need for novel therapeutic strategies.

Whilst oral drug delivery is frequently ineffective to treat biofilm-related infections, topical treatments have the potential to deliver higher drug concentrations to the infection-site while reducing systemic side-effects. In this thesis, the development of two innovative topical strategies against antibiotic resistant bacteria and bacterial biofilms were explored, specifically: (i) colloidal silver nanoparticles (AgNPs) and (ii) a treatment combining the iron chelator deferiprone (Def) and the haem analogue gallium-protoporphyrin (GaPP).

(i) Whilst the antimicrobial activity of spherical AgNPs is well described in planktonic bacteria, little is known about their antibiofilm effects and the influence of particle shape. AgNP spheres, cubes and stars were synthesised and their cytotoxicity towards human macrophages and human bronchial epithelial cells, as well as their activities against *S. aureus*, MRSA and *P. aeruginosa* biofilms were evaluated. While non-desirable toxicity and stability limited the utilisation of AgNP cubes and stars, AgNP spheres showed significant antibiofilm activity against clinically relevant biofilms *in vitro* and in an *in vivo* infection model in *C. elegans*. Moreover, AgNP spheres were physically stable in suspension for over 6 months with no observed loss of antibiofilm activity. This research has led to a phase I human clinical trial that commenced in October 2016 at The Queen Elizabeth Hospital, Woodville, SA, Australia.

(ii) The antibiofilm activity of a novel treatment combining Def and GaPP was investigated. These compounds interfere with bacterial iron metabolism, which presents a unique alternative target vital for all human pathogens. Def-GaPP demonstrated synergistic antibiofilm effects against a series of bacteria, including reference strains and multidrug resistant clinical isolates of *S. aureus*, *S. aureus* small colony variants, MRSA, *S. epidermidis*, *P. aeruginosa* and *A. johnsonii*. Furthermore, Def-GaPP potentiated the activity of antibiotics. *In vitro* cell culture studies confirmed no toxicity of Def-GaPP in murine fibroblasts and human bronchial epithelial cells. Moreover, a clinically used chitosan-dextran hydrogel for wound healing was used as a delivery vehicle for Def-GaPP, thereby complementing wound healing effects with strong antibacterial properties. The Def-GaPP gel showed significant antibiofilm activity in an *in vitro* wound model and in an *in vivo* infection model in *C. elegans*. This work resulted in a patent approval.

Two innovative strategies (i.e. colloidal AgNPs and Def-GaPP gel) have arisen from this thesis that hold significant promise as topical antibiofilm treatments. Both strategies have potential as alternatives to antibiotics or as adjuvants for the treatment of multidrug resistant bacteria and biofilm-associated infections and are advancing for clinical use.

II. Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

III. Acknowledgements

My PhD has been 3 years of excitement, frustration, happiness and chaos- the full rollercoaster of life. It has been quite a journey.

I met incredible people and inspiring characters, established networks all over the world and communicated with people of various backgrounds.

"It is the lives we encounter that make life worth living." -Guy de Maupassant-

My PhD has been an interdisciplinary project with a translational focus and is the result of successful teamwork and fruitful collaborations.

I am grateful for the supervision and guidance of Sarah Vreugde (ENT Surgery, Basil Hetzel Institute for Translational Health Research, University of Adelaide), Peter-John Wormald (ENT Surgery, The Queen Elizabeth Hospital, University of Adelaide) and Clive Prestidge (School of Pharmacy, University of South Australia).

Sarah's support as my principal supervisor was invaluable. Her guidance and encouragement have been an integral element of my PhD and I am grateful for all she has done. The on-going support and availability almost round the clock cannot be valued highly enough. I am happy to call you my "doctor mother".

I thank my co-supervisor PJ for exceptional opportunities throughout my PhD. PJ enabled the successful translation of my work to animal studies and pilot studies in humans, thereby making my work impact- and meaningful. This is an outstanding and unique outcome of a lab-based project and I am grateful for his support. PJ inspires through his professional and private achievements and his dedication to improve patient care.

My deepest thanks go to my family in Germany, in particular my beloved parents Marlene and Gunther Richter. They have provided me with a huge amount of love and encouragement over the years, they sparked my curiosity and stimulated me to reach for the stars. Who would have thought that my way lead me to seek the light of learning under the Southern Cross. My parent's exceptional support throughout my life has been unparalleled and proven regardless of kilometres apart.

Seek light

Sub cruce lumen

IV. Presentations arising from this thesis

13 oral presentations at national and international scientific conferences, including

- *'Silver nanoparticles as a topical treatment for biofilm-related infections'*
  2017 Annual Meeting of the Australian Society for Microbiology, Hobart, TAS, Australia
- '*Bug Wars- Battlefront Biofilms*'
  Invited speaker at the 2017 Nurses and Midwives Research Symposium, Adelaide, SA, Australia
- '*A surgical hydrogel to improve wound healing and fight bacterial biofilms*'
  2017 Annual Meeting of the Australian Society for Otolaryngology Head and Neck Surgery, Adelaide, SA, Australia
- '*Silver nanoparticles as a topical chronic rhinosinusitis treatment*'
  2017 Annual Meeting of the Australian Society for Otolaryngology Head and Neck Surgery, Adelaide, SA, Australia
- *'A topical antibiotic-free treatment to fight bacterial biofilms'*
  2017 Antimicrobials and StaphPath Symposium, Adelaide, SA, Australia
- *'Nanoparticles to tackle clinically relevant biofilms'*
  2017 Australian Colloid and Interface Symposium, Coffs Harbour, NSW, Australia
- *'Silver nanoparticles to tackle clinically relevant biofilms'*
  2016 Annual The Queen Elizabeth Hospital Research Day, Woodville, SA, Australia
- *'A topical treatment not based on antibiotics to fight bacterial biofilms'*
  2016 Antimicrobial Resistance in Microbial Biofilms and Options for Treatment Conference, Gent, Belgium
- *'A surgical hydrogel to combat MSSA and MRSA biofilms'*
  2016 Annual Meeting of the American Rhinologic Society, San Diego, CAL, USA
- *'Bug Wars- Battlefront Biofilms'*
  2016 Annual Meeting of the Australian Society for Microbiology, Perth, WA, Australia
- *'To sneeze or not to sneeze- a novel approach to combat sinonasal biofilms'*
  2015 Annual The Queen Elizabeth Hospital Research Day, Woodville, SA, Australia
- *'Mind "De GaPP": in vitro efficacy of deferiprone and gallium-protoporphyrin against Staphylococcus aureus biofilms'*
  2015 Annual Meeting of American Rhinologic Society, Dallas, TX, USA
- *'A Novel strategy to fight Staphylococcus aureus biofilms'*
  2014 Annual The Queen Elizabeth Hospital Research Day, Woodville, SA, Australia

6 poster presentations at national and international scientific conferences, including

- *'Silver nanoparticles as topical antibiofilm approach'*
  5th European Congress on Microbial Biofilms (EUROBIOFILMS 2017), Amsterdam, The Netherlands
- *'A non-antibiotic approach to combat S. aureus biofilms using deferiprone and gallium-protoporphyrin'*
  2016 Biofilms7 conference, Porto, Portugal
- *'A non-antibiotic strategy to combat S. aureus biofilms by targeting iron metabolism'*
  2016 Annual Meeting of the Australian Society for Microbiology, Perth, WA, Australia
- *'A non-antibiotic approach to combat S. aureus biofilms'*
  2016 Annual Meeting of the Australian Society for Medical Research, Adelaide, SA, Australia
- *'It takes 2 to tango- in vitro efficacy of deferiprone and gallium-protoporphyrin against S. aureus biofilms'*
  2015 7th American Society for Microbiology Conference on Biofilms, Chicago, IL, USA
- *'A Novel treatment combination to combat Staphylococcus aureus biofilms'*
  2014 Annual Florey International Postgraduate Research Conference, Adelaide, SA, Australia

V. Awards and prizes arising from this thesis

2017

- Channel 9 **Young Achiever of the Year Award, Finalist** in "Science & Technology"
- **Conference Attendance Grant**, European Society of Clinical Microbiology and Infectious Diseases
- **Research Travel Award**, School of Medicine, University of Adelaide

2016

- **People's Choice Winner of the 3 Minute Thesis Competition** and University Finalist, University of Adelaide (youtube video: https://www.youtube.com/watch?v=ZE2q0L2fl8g)
- **Health Award**, Northern Communities Health Foundation
- **Trevor Prescott Memorial Scholarship**, Freemasons Foundation
- Channel 9 **Young Achiever of the Year Award, Finalist** in "Science & Technology"
- **Winner Best 3 Minute Thesis Presentation**, Australian Society for Microbiology
- **International Travel Award**, School of Medicine, University of Adelaide
- **Conference Attendance Grant**, European Society of Clinical Microbiology and Infectious Diseases
- **Student Award**, Australian Society for Microbiology, SA/NT Branch
- **Research Abroad Scholarship**, University of Adelaide

2015

- **D R Stranks Travel Fellowship**, University of Adelaide
- **Winner Best Lay Description**, The Queen Elizabeth Hospital Research Day
- **Bertha Sudholz Research Scholarship** for Excellence in ENT Research, Florey Medical Research Foundation
- **International Travel Award**, The Hospital Research Foundation
- **3 Minute Thesis Competition Faculty Finalist**, University of Adelaide

2014

- **Winner Best Oral Presentation**, The Queen Elizabeth Hospital Research Day

VI. List of tables

VII. List of figures

VIII. Abbreviations

AgNPs	Silver nanoparticles
AJ	*Acinetobacter johnsonii*
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BK	Biofilm killing
CF	Cystic fibrosis
CFU	Colony forming units
CRS	Chronic rhinosinusitis
Def	Deferiprone
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
FDA	Food and Drug Administration (USA)
GaPP	Gallium-protoporphyrin
LDH	lactate dehydrogenase
MDR	Multidrug resistant
MIC	Minimal inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MQ	Milly-Q (ultrapure) water
OD	Optical density
PA	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline

QS	Quorum sensing
QSI	Quorum sensing inhibitor
ROS	Reactive oxygen species
SA	*Staphylococcus aureus*
SCV	Small colony variant
SD	Standard deviation
SE	*Staphylococcus epidermidis*
SEM	Standard error of the mean
TEM	Transmission electron microscopy
UV-Vis	Ultraviolet-visible

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Chapter 1.

Introduction

1.1   Biofilms- a historic view

Bacterial biofilms have been known since 1684 when the Dutch scientist Antony van Leeuwenhoek

(Figure 1a) was the first to describe "aggregated bacteria on dental tartar" which was later defined

as plaque. With handcrafted microscopes (Figure 1b) he was the first who studied and described

microorganisms referring to them as "animalcules" (derived from Latin "tiny animal"). Antony van

Leeuwenhoek's pioneering work that included identifying structures of protozoa and bacteria,

examining spermatozoa and muscle fibres, observing cell division and the blood flow in capillaries

later resulted in his nickname "father of microbiology". However, as he never published a

manuscript or book, his legacy only survived due to the correspondence with the Royal Society of

London for Improving Natural Knowledge who printed around 190 of his letters about his
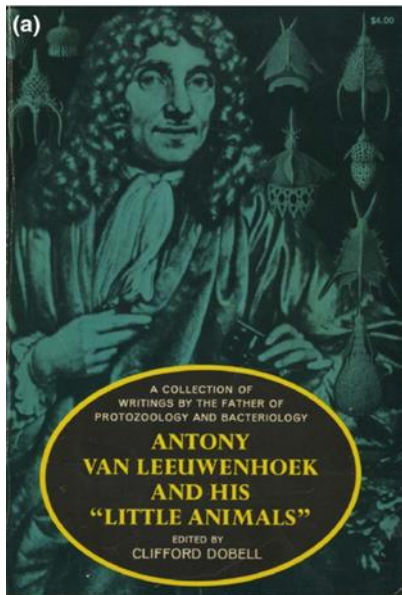
observations in a variety of fields, including microscopic findings of bacterial consortia[2].

Figure 1. (a) Antony van Leeuwenhoek (1632–1723) who was the first to observe and describe microbial biofilms in his own mouth. (b) A replica of his "microscope". Reprinted with permission[3].

Many centuries would pass until scientists investigate the role of bacterial biofilms on the aetiology

of infections. Only since the 1970s biofilm research started to become more and more important

after a paradigm shift in microbiology: based on the pioneering work of William Costerton and Niels

Høiby bacteria were not only seen as single, free-floating (planktonic) cells, but also as sessile cells

of single and multiple microbial species[3-6]. Biofilm cells are phenotypically different from planktonic

bacteria due to altered growth rates and gene transcription[7]. In 1978 the term biofilm was

introduced[8] and is now defined as "Aggregates of microorganisms in which cells that are frequently

embedded within a self-produced matrix of extracellular polymeric substances adhere to each other and/or to a surface" (definition by the International Union of Pure and Applied Chemistry, IUPAC[9]).

Biofilms can form on and coat various surfaces and materials, including metals, plastics, wood, rocks, medical devices, human and animal tissue, skin and bones- wherever the conditions allow microbial growth[10]. Biofilms occur in the natural environment and industrial settings, e.g. as slippery slime on rocks in rivers, as biofouling debris causing degradation on ship hulls or as "gunk" clogging household drains and water pipelines. Biofilms (Figure 2) furthermore have large implications in the medical field- it is now recognised that biofilms are associated with approximately 80% of microbial infections in the human body[11], including dental plaque, lung infections in cystic fibrosis, implant infections, chronic wound infections and chronic rhinosinusitis. However, exploiting the implications of biofilms on human health, particularly in infectious and chronic diseases, only commenced 40 years ago. Since then this clinically important field attracts increasing interest in the medical and scientific community[3].
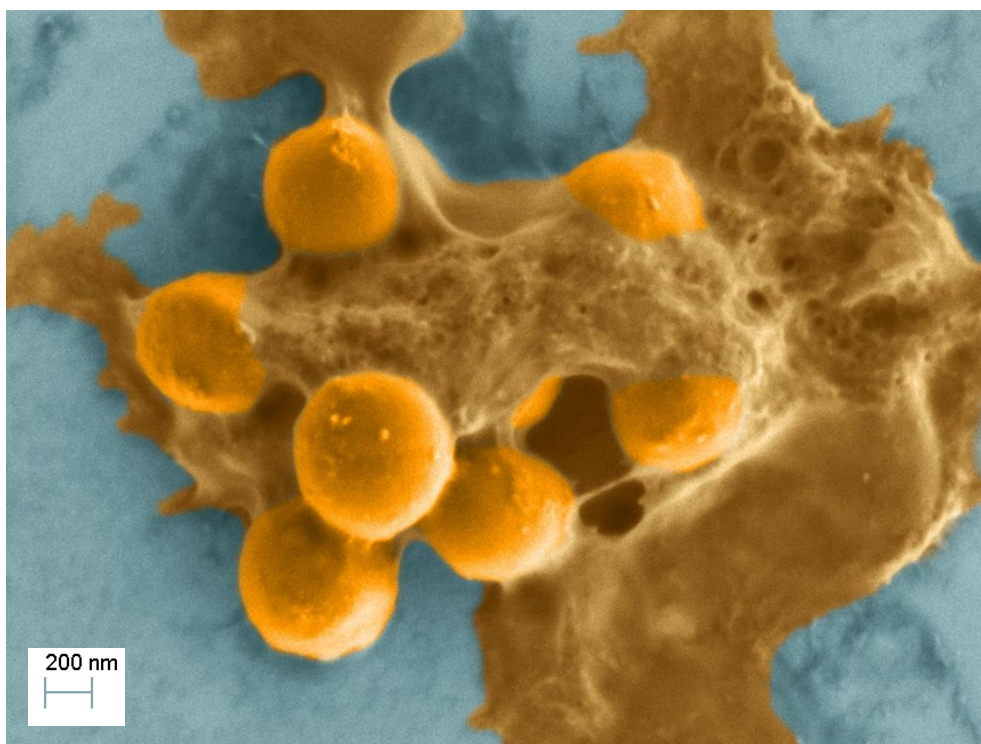
Figure 2. Scanning electron micrograph of Staphylococcus aureus biofilm.

1.2 Biofilm characteristics

The biofilm state is considered as the evolutionary default mode of bacteria[12,13] and it is known that 99% of bacteria can form and live in biofilms[14]. This lifestyle represents a survival advantage (Figure 3), enabling bacteria to adapt to diverse environments, survive harsh conditions and external stress, communicate within the community and streamline processes, such as nutrient acquisition and defence mechanisms[12]. Within the biofilm matrix bacteria are protected against hostile conditions, such as UV intensity, changes in water, oxygen, salt, nutrients and pH levels, or toxicity of metals and biocides[13,15]. The biofilm state furthermore facilitates withstanding the innate and adaptive immune response of the host and survival of antimicrobial therapies[16]. It is known that bacteria in biofilms are up to 1000-fold less susceptible to antimicrobial treatments than their planktonic counterparts[17].
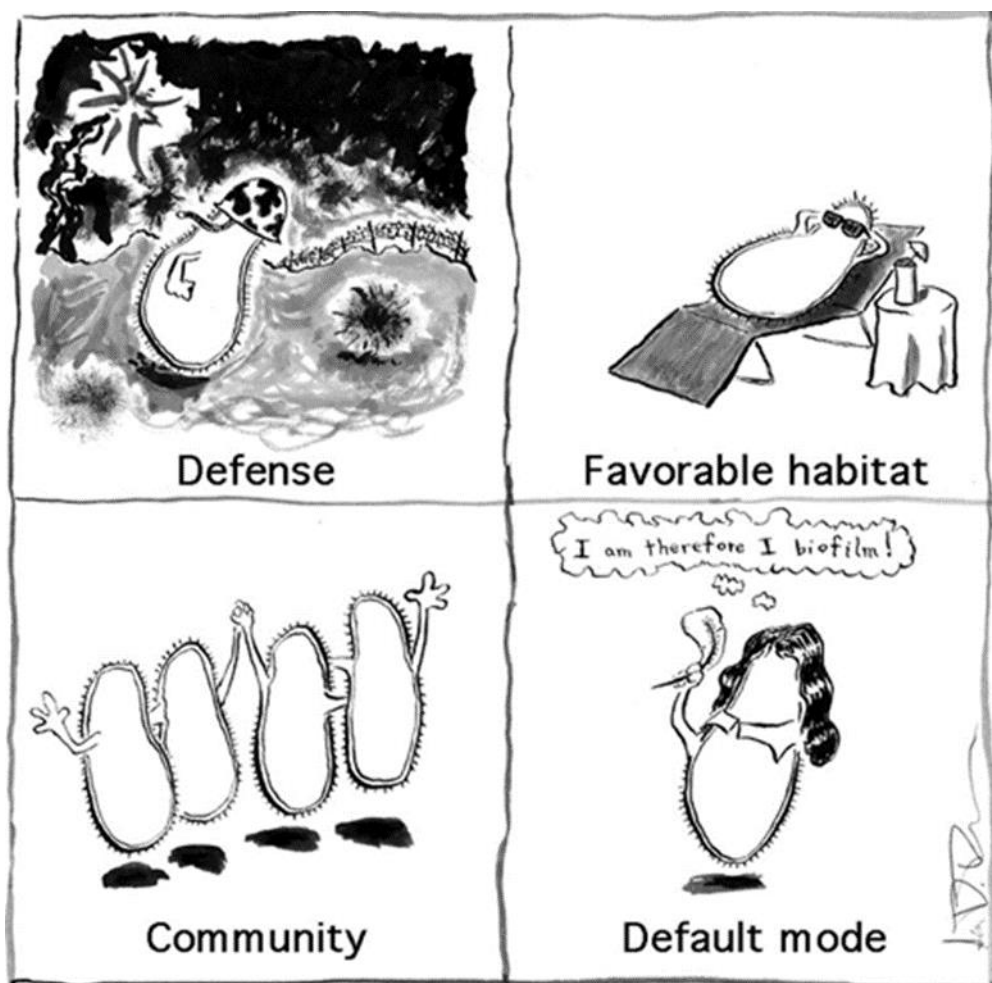
Figure 3. Dr. Sean D. Taverna's artistic interpretation of the four driving forces behind bacterial biofilm formation. Reprinted with permission[12].

The biofilm matrix mainly consists of polysaccharides, proteins, lipids and extracellular DNA and forms a 3-dimensional complex with dense areas, pores and water channels[18]. The latter provide nutrient and oxygen distribution within the biofilm and allow exchange and removal of metabolites[19,20]. Bacteria in biofilms adjust their gene expression according to alterations in the environment, nutrient supply and the presence of antimicrobials[13,21,22]. Bacterial strategies to adapt and resist to various conditions include:

- The biofilm matrix acts as a diffusion hindrance, lowering the amount of drug reaching the inner biofilm[18,23]

- Genotypic (horizontal gene transfer)[24,25] and phenotypic (physiological changes to address nutrient, oxygen or stress levels)[26-29] alterations of species within the biofilm

- Evolution to different growth states, i.e. fast growing/metabolic active states and slow growing/metabolic inactive states[11]

- Adaptive mutations[30]

- Quorum sensing (communication between bacterial cells)[31,32]

- Production of antibiotic degrading enzymes[33]

- Efflux pumps to remove antimicrobials from the biofilm[34]

- Multidrug tolerant persister cells that stay in niches not to be reached by antibiotics[17,35]

- Intracellular perseverance through the formation of small colony variants[36,37]

Despite increasing research interest to date, biofilms are still incompletely understood as they present dynamic consortia with complex social structures and are ever changing and evolving.

1.3 Biofilm life cycle

Biofilm formation (Figure 4) is a complex developmental process and adhesion, proliferation and detachment are the three major steps in biofilm formation[38]. The process is dynamic and starts with planktonic bacteria that attach reversibly to a surface and/or to each other. Pili, flagella, receptors

or other adhesive surface appendages make contact with biotic or abiotic surfaces. Adhesion is followed by the secretion of extracellular polymeric substances resulting in an irreversible attachment. Subsequently, cells proliferate resulting in the formation of microcolonies. The transcription of specific genes is activated and quorum sensing, or cell-to-cell signalling occurs, which plays an important role in the development of the biofilm[31,32]. The biofilm grows and cells differentiate according to nutrient supply and environmental conditions, resulting in a mature biofilm with multi-layered cell clusters. Within the biofilm an extreme genetic and phenotypic diversity is established. This heterogeneity can be based on a variety of different species, such as bacteria, fungi, algae, yeasts, protozoa, and other microorganisms, as well as multiple strains of the same species. Finally, cells disperse from the biofilm (actively due to space and nutrient limitations or passively due to fluid shear and starvation) and can colonise new areas commencing a new biofilm life cycle[16,18,39-41].
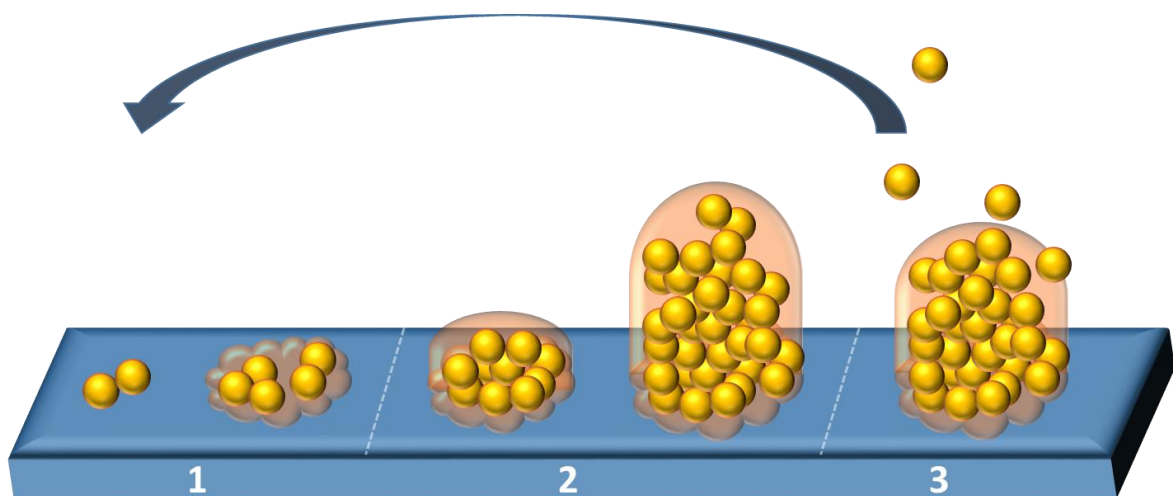
Figure 4. The biofilm life cycle exemplified by Staphylococcus aureus. 1: Adhesion. 2: Growth. 3: Detachment.

1.3.1   Adhesion mechanisms exemplified by *S. aureus*

Adhesion mechanisms vary in different species; they are specialised to activate species-specific virulence factors and influence host cell signalling, ultimately fostering bacterial growth and survival[42]. Herein, the specific attachment strategies are exemplified by *Staphylococcus aureus*, a

Gram-positive bacterium that plays a major role in the pathogenesis of both superficial and invasive infections, including chronic rhinosinusitis (CRS), sepsis, osteomyelitis and endocarditis[43]. Bacteria are able to attach to both biotic and abiotic surfaces where they can form biofilms[16]. In the case of CRS, *S. aureus* infects the sinuses and anchors itself on the nasal mucosa. For the irreversible attachment to surfaces *S. aureus* is equipped with a broad range of virulence factors, including surface proteins that are covalently attached to peptidoglycan in the cell wall[43]. Up to 24 cell wall-anchored (CWA) proteins are known for *S. aureus*, moreover, their repertoire on the surface varies among strains[44]. The expression of CWA proteins depend on growth conditions and nutrient supply, and is altered e.g. when iron is depleted. Metals like iron are essential for the viability and the pathogenesis of *S. aureus*[45], thus, *S. aureus* established pathways for iron acquisition from host haem/haemoglobin to survive when iron is restricted (this will be further discussed in the section "iron metabolism")[46,47]. As *S. aureus* is equipped with a limited number of proteins on the cell surface, the bacterium developed strategies to maximise the protein utilisation. Single proteins can be responsible for multiple functions which is subject to selective pressure, and several proteins can carry out the same functions (known as functional redundancy)[43]. Functions of CWA proteins include the attachment to surfaces, host extracellular matrix and cells, invasion, inflammation and immune evasion, as well as iron acquisition and biofilm formation.

It is known that CWA proteins are able to bind to various ligands like fibrinogen, fibronectin or collagen[48-51]. Furthermore, two distinctive attachment mechanisms of CWA proteins have been discovered[52,53]. However, the knowledge about attachment mechanisms of CWA proteins is far from complete and some ligands still remain unknown.

1.4   Biofilm matrix

Sessile bacteria reside in a self-excreted, hydrated matrix comprising of extracellular polymeric substances (EPS). The biofilm matrix (Figure 5) is a dynamic, multi-component extracellular compartment that provides architectural structure, cohesion and mechanical stability for the

embedded cells[54]. The gel-like matrix facilitates the adhesion to surfaces, allows for synergistic intra- and inter-species interactions in a three-dimensional microenvironment that transiently immobilises sessile bacteria[18]. It furthermore provides an external digestive system due to a variety of enzymes that metabolise biopolymers in close proximity to bacteria[55]. Moreover, the matrix protects bacteria from external stress, such as desiccation, UV radiation and toxic radicals. By working as a diffusion hindrance, the activity of immune cells as well as biocides is limited, which can ultimately lead to bacterial persistence, increased tolerance and resistance to antimicrobials[56]. The matrix evolves according to intrinsic and extrinsic fluctuations, including nutrient and gaseous level changes, and fluid shear. The high biodiversity within the biofilm greatly influences the matrix composition, EPS secretion and consequently the physico-chemical properties[55].
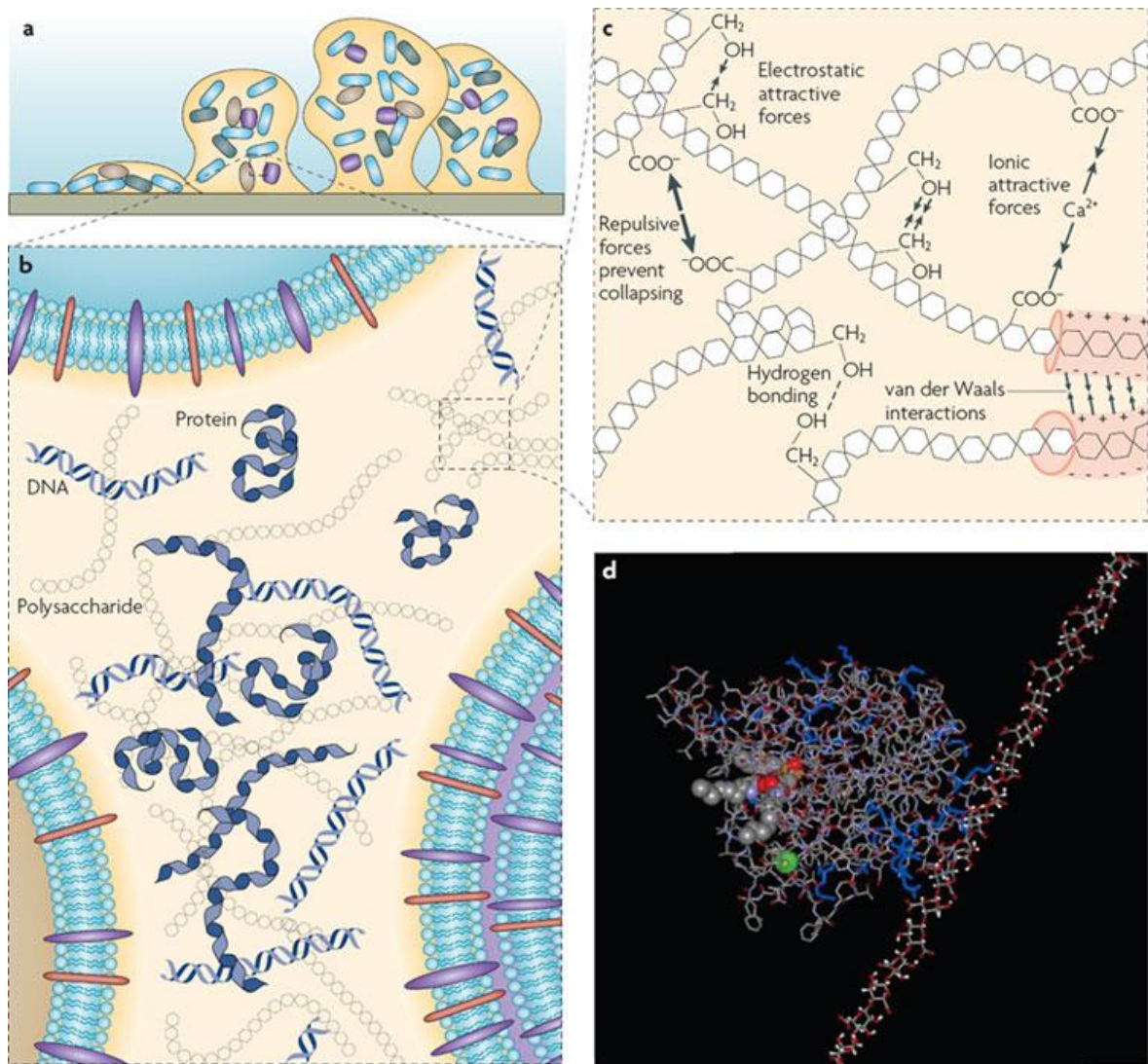
Figure 5. The extracellular polymeric substances matrix at different dimensions. (a) A model of a bacterial biofilm attached to a solid surface. (b) The major matrix components — polysaccharides, proteins and DNA — are distributed between the cells in a non-homogeneous pattern, setting up differences between regions of the matrix. (c) The classes of weak physico-chemical interactions and the entanglement of biopolymers that dominate the stability of the EPS matrix[57]. (d) A molecular modelling simulation of the interaction between the exopolysaccharide alginate (right) and the extracellular enzyme lipase (left) of Pseudomonas aeruginosa in aqueous solution. The coloured spheres represent 1,2-dioctylcarbamoyl-glycero-3-O-octylphosphonate in the lipase active site, except for the green sphere, which represents a Ca^{2+} ion. The aggregate is stabilised by the interaction of the positively charged amino acids arginine and histidine (indicated in blue) with the polyanionic alginate. Image courtesy of H. Kuhn, CAM-D Technologies, Essen, Germany. Reprinted with permission[18].

Microbial cells (from single and multi-species) constitute less than 10% of biofilms, while the matrix comprises over 90%[18]. Water presents the largest amount of the biofilm matrix accounting for up to 97%[56]. Water acts as solvent to facilitate mobility within the biofilm and enables diffusion, thereby influencing the biofilm ultrastructure[18]. Other matrix components are exopolysaccharides (1-2%), proteins and glycoproteins including secreted enzymes and signalling molecules (1-2%), extracellular DNA from lysed cells (1–2%), lipids and phospholipids, as well as various ions taken up

from the environment[54,58]. However, these numbers are only estimates as the specific ultrastructure and composition of a biofilm varies according to prevalent species and their physiology, fluid-flow dynamics, as well as physical and environmental conditions.

The biofilm matrix as arrangement of different microorganisms provides exchange of information between species, including quorum sensing and horizontal gene transfer. Seen as a functional, synergistic microconsortium bacteria can reside in biofilms according to nutrient, gaseous, pH and oxygen levels. Due to the flexible structure and pores in the matrix species can enter, move around and leave the matrix facilitating genetic exchange[18].

Voids and water channels are present in the biofilm matrix facilitating the flow of nutrients, oxygen, metabolites, signalling molecules, enzymes and waste products, resulting in localised gradients[56]. According to these gradients bacteria either proliferate leading to biofilm growth or remain in stationary phase surviving on internal resources such as iron storage proteins[55].

Physical forces play an important role for the mechanical stability of the biofilm matrix[18]. The EPS are entangled based on weak physico-chemical interactions such as hydrogen bonds, van der Waals interactions, electrostatic attractive forces, ionic attractive forces and repulsive forces (Figure 5c)[18]. These forces provide both strength and elasticity facilitating a coherent biofilm structure and simultaneously allowing dynamic adaptations to environmental changes[55].

1.4.1   Matrix components

The main components of EPS include (i) polysaccharides, (ii) proteins, (iii) nucleic acids and (iv) lipids- all of them display various functions in biofilms (Table 1).

(i) Exopolysaccharides play an important role for the cell attachment to colonise surfaces and for three-dimensional arrangements of different species forming the biofilm community[55]. The framework of the biofilm structure is built by exopolysaccharides responsible for the insertion of microbial cells and their bioactive products. Exopolysaccharides are found either capsular, i.e.

directly associated on the bacterial cell surface, or as loosely associated slime of the biofilm matrix[54].

(ii) Many proteins and enzymes are secreted by sessile bacteria. Structural, cell surface anchored proteins such as lectins can bind exopolysaccharides to bacteria, thereby stabilising the matrix. Some species including *S. aureus* produce biofilm-associated surface proteins (Bap) that play a role in biofilm formation and the infection process[59]. Moreover, proteinaceous bacterial appendages like flagella, pili and fimbriae contribute to the biofilm stability by cross-linking molecules of the EPS matrix[60].

Enzymatic and regulatory activities take place in the matrix creating a heterogeneous, functional microenvironment. The metabolic and physiologic capabilities diverts the biofilm bacteria from planktonic cells[61]. The composition and physical matrix properties are influenced by enzymatic and biopolymeric secretion (such as extracellular proteases, peptidases, hydrolases, glycosidases, esterases, lipases and other enzymes), shedding of cell surface material, cell lysis and interactions with macromolecules from the environment[18,54]. The enzymatic activity facilitates cell release from the biofilm complex by depolymerisation of structural matrix polymers to dispatch bacteria for colonisation of new sites. Moreover, secreted enzymes provide a digestive system supplying break-down products as carbon and energy sources for immobilised biofilm bacteria, but they can also act as virulence factors[18].

Proteins with diguanylate cyclase activity can synthesise cyclic di-guanosinemonophosphate (c-di-GMP), a secondary messenger ubiquitous in the bacterial world that controls the motile and sessile state of bacteria[62]. The release and degradation of c-di-GMP is influenced by environmental conditions, regulating c-di-GMP levels in cells. The c-di-GMP functions include the inhibition of bacterial motility, as well as the stimulation of adhesin production and other matrix components. Therefore, high levels of c-di-GMP are associated with EPS secretion and biofilm formation, while low levels cause biofilm dispersal and a subsequent release of motile, planktonic cells[55].

(iii) Extracellular DNA (eDNA) is a residue from lysed cells within the matrix that influences biofilm formation and visco-elastic properties, enhances the mechanical stability, increases adhesion to surfaces and contributes to the protection against aminoglycosides[63-65]. The effects of eDNA on the structure and function of biofilms depend on the eDNA origin[55].

(iv) Lipids are furthermore part of the biofilm matrix and exhibit diverse functions. Lipopolysaccharides can contribute to the attachment to lipophilic surfaces, while extracellular lipids can have surface-active properties. Exemplified by surfactin, viscosan and emulsan, surface-active lipids contribute to the dispersal of hydrophobic components resulting in bioavailability of the breakdown products[18].

Table 1. Functions of extracellular polymeric substances in bacterial biofilms. Adapted from[18].

Function	Relevance for biofilm organism	EPS components involved
Adhesion	Surface colonisation and long-term attachment	Polysaccharides, proteins, DNA, amphiphilic molecules
Aggregation of cells	Transient cell immobilisation, development of high cell densities, cell-cell recognition	Polysaccharides, proteins, DNA
Cohesion	Structural elements for matrix, mechanical stability, determination of EPS structure and biofilm architecture, matrix generation	Polysaccharides, proteins, DNA
Water retention	Hydrated microenvironment around organisms, desiccation tolerance	Polysaccharides, proteins
Protective barrier	Resistance and tolerance to host defences and biocides, protection from hostile conditions	Polysaccharides, proteins
Sorption of particles	Resource capture, accumulation of metal ions (detoxification), ion exchange	Polysaccharides, proteins
Enzymatic activity	External digestive system for nutrient supply, partial degradation of EPS for cell dispersal	Proteins
Nutrient source	Source of carbon, nitrogen and phosphorous compounds as biofilm food	All EPS components
Genetic information exchange	Horizontal gene transfer between cells	DNA
Intercellular information	Regulation of biofilm dynamics and responses, regulating c-di-GMP concentrations	Polysaccharides
Electron donor or acceptor	Redox activity and electron transport in matrix	Proteins
Export of cell components	Release of cellular material resulting from metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipids
Binding of enzymes	Enzyme accumulation, retention and stabilisation by polysaccharides	Polysaccharides, enzymes

1.5 Quorum sensing

Quorum sensing (QS), or cell-to-cell signalling, is the bacterial communication by which bacteria produce and detect signal molecules in a cell density dependent way, leading to a synchronised bacterial response. Based on small hormone-like signal molecules, termed autoinducers (Figure 6), bacteria streamline their behaviour in the entire population, thereby act as concerted multicellular organisms[66]. QS presents a survival advantage for bacteria and has been optimised in different species with variations in signal types, receptors, signal transduction pathways and signal target outputs. Three species-specific QS systems can be distinguished and include the acylhomoserine lactone (AHL) QS system in Gram-negative bacteria, the autoinducing peptide (AIP) QS system in Gram-positive bacteria and the autoinducer-2 (AI-2) QS system in both Gram-negative and Gram-positive bacteria[67].

(a) Acylhomoserin lactones
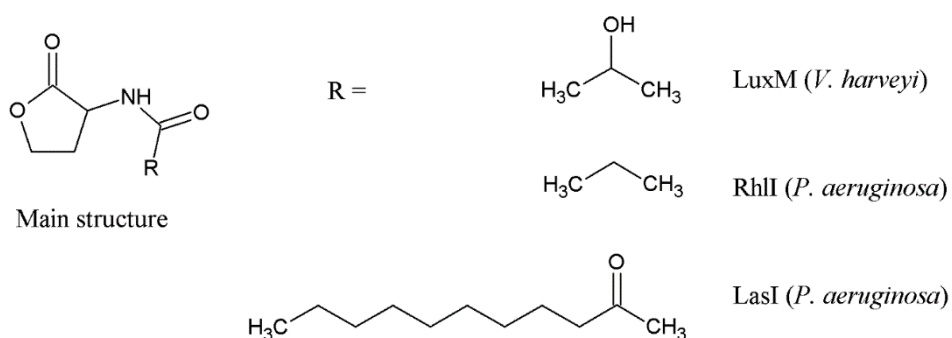
(b) Autoinducing peptides
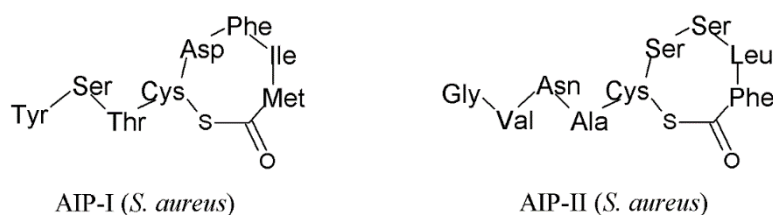
(c) Autoinducer-2 molecules
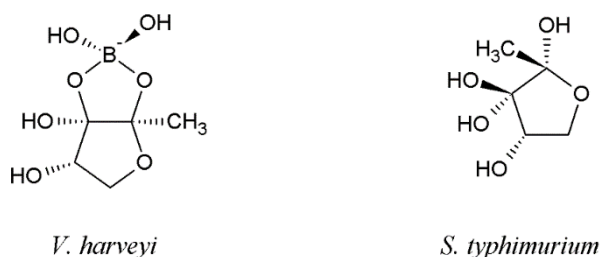
Figure 6. Examples of quorum sensing molecules of the (a) acylhomoserine lactone system, (b) autoinducing peptide system and (c) autoinducer-2 system.

QS plays an important role in biofilm formation, gene expression, enzyme excretion, the production of virulence factors and resistance[66]. Therefore, QS systems present a suitable target for novel antimicrobial strategies. Literature described QS inhibitors (QSIs) as promising antibiofilm agents, either alone or as potentiators of conventional antibiotics[67-69]. QSIs can interfere with the QS cascade on various levels, thereby impeding a coordinated bacterial response that makes bacteria vulnerable. Furthermore, QSIs can constrain the synthesis of signal molecules, initiate their degradation, prevent the signal's binding to specific receptors and hamper signal transduction. Examples of QSIs will be further discussed in the chapter "Innovative antibiofilm strategies".

1.6   Resistance, tolerance and persistence

It is generally accepted that sessile microorganisms show decreased susceptibility towards antimicrobial agents. This is due to decreased penetration of antibiotics, decreased growth rate of the biofilm cells and/or decreased metabolism of bacterial cells in biofilms. In addition, the presence of highly specialised survivor cells (so-called persister cells) and the expression of specific resistance genes (including efflux pumps) contribute to this tolerance and resistance[70-73].

Although tolerance and resistance to antimicrobials are of distinctively different mechanisms, both are commonly present in planktonic bacteria and biofilms. Tolerance and resistance are mostly intertwined processes operating together to increase bacterial survival[74].

Resistance is an inherited trait based on a genotypic alteration enabling bacteria to grow at high antibiotic concentrations, regardless of the treatment exposure length, and is reflected by the minimum inhibition concentration (MIC)[75]. Resistance relies on one of three origins, i.e. natural resistance, spontaneous mutation and acquired resistance. The high population density in biofilms facilitates an elevated production of resistance genes and an increased frequency of mutations induced by selective pressure from external stress[74]. The close proximity of cells allows for transfer of antibiotic resistance determinants and exchange of resistance genes by horizontal gene transfer between various strains and species, thereby enhancing resistance of the entire biofilm

15

population[72]. Once resistance has been established this survival advantage is passed on to next generations increasing population fitness. Resistance is observed as increased MICs of antimicrobials in planktonic cells and recalcitrance of biofilms[74]. Sub-MIC concentrations of antibiotics further promote biofilm growth, mutagenesis and virulence factor expression, thereby increasing resistance[76].

Bacteria in biofilms can establish various resistance mechanisms (Figure 7), including the production of antibiotic degrading enzymes such as beta lactamases. These enzymes can be released into the biofilm matrix to inactivate beta lactam antibiotics by hydrolysis of the beta lactam ring before bacteria are reached[77]. Bacteria can furthermore change specific target sites to prevent antibiotic attachment, reduce cell permeability, down regulate receptors to decrease antibiotic uptake and increase the expression of drug efflux pumps to expel antibiotics from bacteria (Figure 7)[73,78]. Further resistance strategies are bypassing pathways that are inhibited by antibiotics and the overproduction of antibiotic targets (Figure 7). In addition, the biofilm matrix contributes to resistance as it contains proteins and eDNA, which are able to induce the expression of operons to increase resistance against specific antibiotics. Negatively charged eDNA can immobilise positively charged antibiotics and host defence peptides by means of physico-chemical interactions[79]. The biofilm matrix as a diffusion barrier furthermore mediates the expression of resistance genes based on slow antibiotic penetration[80]. Moreover, hypoxic conditions in the biofilm interior are associated with increased resistance due to altered gene expression and increased drug efflux[81].
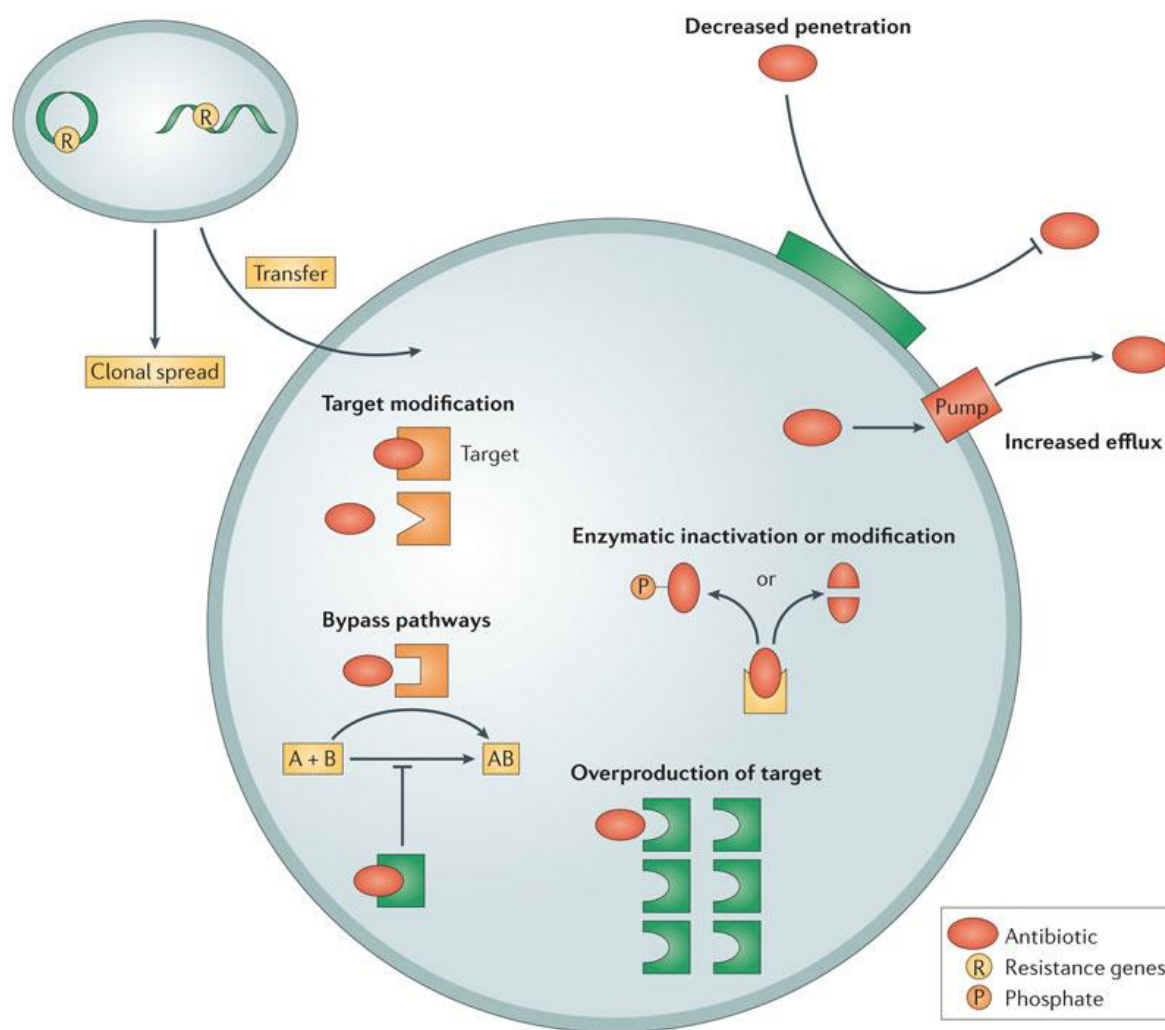
Nature Reviews | Drug Discovery

Figure 7. Major types of clinically relevant resistance mechanisms: target modification (of enzymes, ribosomes or cell-wall precursors, for example, mutation in the 30S ribosomal protein RpsL confers resistance to streptomycin), inactivation or modification of the antibiotic (for example, by beta lactamases), restricted penetration and/or increased efflux of the drug (for example, efflux of linezolid by the AcrAB–TolC multidrug pump), bypass of pathways inhibited by antibiotics and overproduction of targets[82,83]. Reprinted with permission[77,84].

In contrast to genotypic derived resistance, tolerance and persistence are transient features that facilitate bacterial survival based on reversible phenotypic change[78,85]. Although tolerant bacteria show similar MICs to susceptible strains, a longer treatment exposure is required to kill them (Figure 8). Similarly, persistent bacteria exhibit comparable MICs to susceptible strains, as well as an equivalent duration to kill 99% of bacteria. However, substantially prolonged treatment exposure is required to kill 99.99% of persistent cells (Figure 8). While resistance and tolerance are features of an entire biofilm community, persistence is only attributed to a subpopulation of clonal

bacteria[86]. Consequently, a biphasic time-kill curve results from populations where persistent and non-persistent bacteria are present[87] (Figure 8).

As resistant, tolerant and persistent bacteria share the trait of survival following antibiotic exposure, these terms frequently appear interchangeably in the literature without further classification.
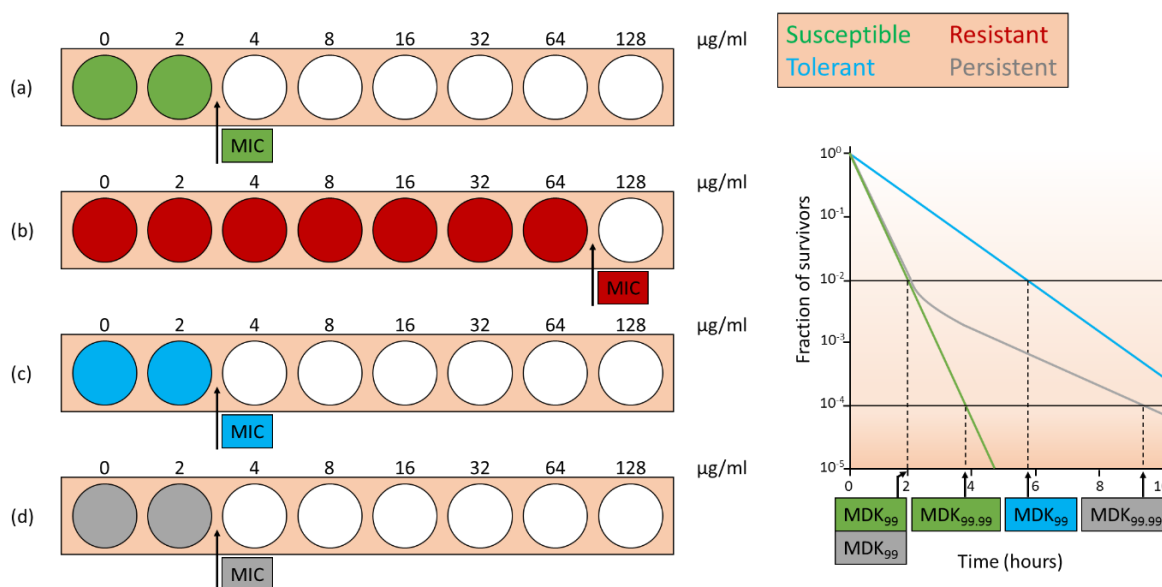
Figure 8. Left: Characteristic minimum inhibition concentration (MIC) for a (a) drug susceptible, (b) resistant, (c) tolerant and (d) persistent bacterial strain. Coloured wells indicate bacterial growth, white wells indicate a drug concentration dependent growth inhibition. Right: Characteristic minimum duration for killing of 99% (MDK$_{99}$) and 99.99% (MDK$_{99.99}$) of bacteria in the population for a susceptible (green), tolerant (blue) and persistent (grey) strain. Concentrations and timescales were chosen for demonstration purposes only. Adapted from[75].

The survival of both tolerant and persistent bacteria following transient exposure to high drug concentrations is accomplished by decelerating cellular bacterial processes. In biofilms tolerance and persistence can be observed as slow- or non-growing (dormant) bacteria, which can present as persister cells, small colony variants (both to be discussed in the following sections) or viable-but-non-culturable microorganisms[88]. The latter are dormant organisms that cannot be cultured on media that generally facilitates their growth, while cues of viability of cells, such as respiratory activity, can be measured[18].

A commonly shared feature of these slow- or non-growing cells is their low metabolic activity while membrane integrity is preserved[89]. Tolerance mechanisms furthermore include the failure of antimicrobial penetration through the biofilm matrix as it acts as a diffusion barrier. Negatively charged EPS components can interact with positively charged antimicrobials, such as aminoglycosides and polypeptides, thereby delaying drug penetration[63]. The heterogeneity in biofilms with a variety of phenotypes and genotypes and the associated localised gradients furthermore contribute to bacterial tolerance[15]. Aerobic, nutrient-rich conditions that account for high metabolic activity are prevalent on the biofilm surface, while anaerobic, nutrient-deprived and acidic conditions dominate in the inner biofilm, which can lead to low metabolic active cells and stationary phase like, dormant bacteria[11]. In biofilms at least 1% of bacteria in stationary phase establish tolerance to antibiotics[90]. This percentage increases over time, which subsequently leads to a considerably reduced killing efficiency of antimicrobial agents (such as silver nanoparticles[91]) and some antibiotics (e.g. vancomycin[92]) in mature biofilms. Many antibiotics target processes in metabolic active bacteria, such as the cell wall synthesis, DNA replication, transcription or translation and often require an active uptake[29]. Therefore, only a proportion of the biofilm is affected, while low metabolic active cells are only prevented from multiplying but are not actively killed. Hence, tolerant and persistent bacteria survive and can re-establish the colony with bacteria of elevated tolerance[93].

1.6.1   Persister cells

Persister cells are a small subpopulation of bacteria formed stochastically in the biofilm population[77]. Due to their non-growing or starving state and low metabolic activity, persister cells are considerably less susceptible to antibiotic mediated killing[94,95]. Persisters were first described in 1944 and are bacteria that survive stresses of compounds that eliminate metabolic active cells[96]. Outlasting the course of antibiotic treatment, hibernating persister cells "wake up" and re-establish the population, thereby are linked to persistent infections and recurrence of disease[94]. Persister

cells show a reduced energy production, down-regulated biosynthetic functions and can be equipped with a variety of toxin-antitoxin systems[73,97]. The latter are genes that encode both a toxin to inhibit essential cellular functions and an antitoxin to capture and antagonise the toxin[98,99]. During stress proteolytic degradation reduces the antitoxin concentrations, thereby free toxins can express their inhibitory functions, such as blocking protein synthesis, decreasing ATP levels and impeding translation, leading to a dormant state[100]. Several toxin-antitoxin modules have been linked to tolerance of persister cells, however, this tolerance is limited to specific antibiotics and toxin-antitoxins[74,101].

Persister cells, in particular their reversion to growing cells and pathways to efficiently destroy them are still not well understood. Only recently it was shown that a new antibiotic, ADEP4, is able to kill persisters by disrupting proteolysis[102]. This mode of action operates irrespective of ATP levels, hence, even persister cells with low ATP levels can be tackled. Conlon *et al.* showed significant activity of ADEP4 against *S. aureus* persisters and the treatment combined with rifampicin resulted in eradication of *S. aureus* persisters and biofilms[102,103]. This new class of antibiotics with a mechanism of action independent from bacterial energy production may lead to further innovations in antimicrobial therapies.

1.6.2 Small colony variants

Small colony variants (SCVs) are naturally occurring bacteria characterised by a 10-fold reduced colony size compared to the parent strain (Figure 9). Being part of the bacterial life cycle their formation is often induced by harsh conditions, such as antibiotics, starvation or cationic host defence peptides[104].
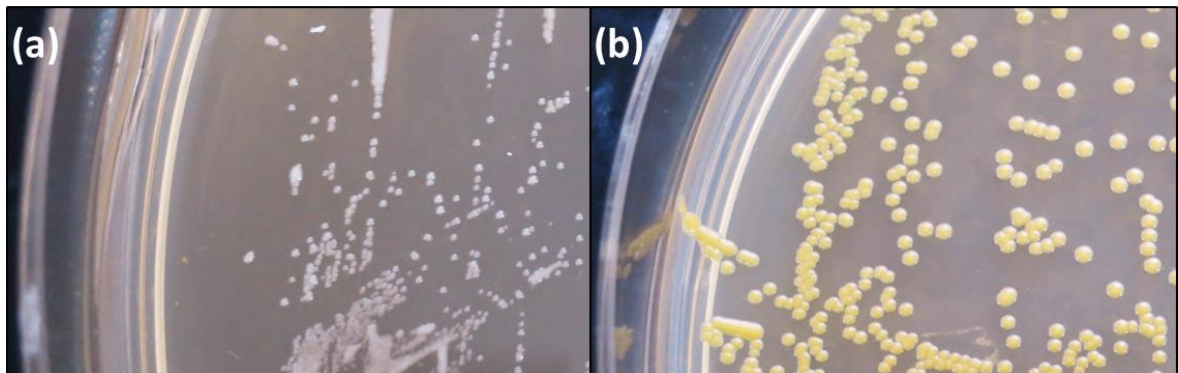
Figure 9. S. aureus small colony variants (a) and parent strain (b).

SCVs were first described in 1910 for *Salmonella typhi*[105] and since then have been found in other species, including *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *Burkholderia cenocepacia*, *Vibrio cholerae*, *Escherichia coli*, *Neisseria gonorrhoeae, Listeria monocytogenes* amongst others[106-111]. Associated with a plethora of diseases SCVs have been recovered from various infective sites, such as the lungs and sinuses, soft tissues, bones and joints[37,106]. However, due to their atypical morphological and physiological features that require tailored culture and identification procedures, SCVs frequently go undetected in routine clinical investigations and their prevalence is likely underestimated[36,112-114]. SCVs are characterised by distinctive phenotypic and pathogenic traits, such as a slow growth rate, low metabolic activity, decreased respiration, decreased ATP production, a frequent lack of pigmentation and haemolysis, decreased coagulase activity and decreased production of most virulence factors (except proteases and adhesins)[37,115]. These features can be inheritable or transient, thereby SCVs are linked to increased antibiotic tolerance and resistance[116].

SCVs can be described according to their auxotrophy- the inability of an organism to synthesise a particular organic compound required for its growth (definition by IUPAC[117]). There are two major forms, i.e. auxotrophy for the external growth factors menadione and/or haemin (and/or thiamine, required for the menadione synthesis), which is linked to defects in the bacterial electron transport, and auxotrophy for thymidine, an attribute of bacteria that rely on exogenous thymidine to survive as they lack its biosynthesis.

Menadione/haemin auxotrophs hold genetic mutations that cause the loss of menaquinone and the haem prosthetic group in cytochromes, which are essential for electron transport[37]. This type of SCVs are not able to activate the Krebs cycle, instead energy production relies on glycolytic and fermentation pathways[118]. The decreased ATP production leads to a slow growth, which makes SCVs less susceptible to antibiotics that require active bacterial metabolism, such as beta lactam antibiotics. A reduced membrane potential of menadione/haemin auxotrophs furthermore contributes to a decreased uptake of antibiotics[37].

Thymidine auxotrophs acquire thymidine by utilising DNase to digest external DNA of lysed cells[37]. Thereby, SCVs do not rely on the tetrahydrofolic acid pathway to produce thymidine for DNA synthesis and can circumvent biocidal effects of antibiotics targeting the bacterial folic acid synthesis (such as trimethoprim/sulfamethoxazole)[119]. Moreover, thymidine auxotrophs show a reduced Krebs cycle activity, similar to menadione/haemin auxotrophs, and the associated reduced ATP production results in a slow growth and diminished susceptibility to antibiotics[120,121].

Mediated by fibronectin bacteria are generally able to invade human cells, thereby escaping the host immune response and avoiding exposure to most antibiotics[116,122]. SCV phenotypes show an elevated rate of internalisation compared with the parent strain as SCVs express a higher amount of adhesins, such as fibronectin-binding proteins[123,124]. This facilitates an increased uptake into eukaryotic cells by attachment to integrins (transmembrane receptors) present on the cell surface[124-127]. Furthermore, a prolonged intracellular persistence of SCVs is mediated by an altered production of virulence factors. As an example, *S. aureus* SCVs were shown to express less α-toxin

(a pore-forming toxin) than the normal phenotype, hence, cell membrane damage and subsequent haemolysis were diminished, increasing the SCV survival in the host[123,128]. Furthermore, *S. aureus* SCVs can impede host cells to excrete hypoxia inducible factor, thereby prevent signalling to notify the host of intracellular pathogens[129].

The small colony morphology and the intracellular lifestyle are fundamental parts of the infection process, however, the switch between SCV and wild type is dynamic and reversible[130]. When SCVs leave their intracellular residence and in the presence of respective supplements (i.e. menadione, haemin or thymidine) SCVs can revert back to the normal, fully virulent wild-type form infecting other cells whilst causing disease relapse[37,131]. Even after aggressive antimicrobial therapies and surgery, infections recur after weeks, months and years due to intracellular SCVs[114,132]. Moreover, it is known that antibiotics like gentamicin and trimethoprim/sulfamethoxazole, or disinfectants like triclosan induce SCV formation, contributing to the emergence of multidrug tolerance and resistance[119,133,134]. Sub-therapeutic antibiotic exposure can trigger biofilm formation of SCVs and their parent strains, further complicating treatment[19,135,136]. Consequently, SCVs are associated with antibiotic-refractory and recalcitrant infections, such as chronic rhinosinusitis, respiratory tract infections in cystic fibrosis, chronic wound infections and prosthetic device infections[106,112,113]. As most antibiotics are not able to enter human cells, SCVs contribute to therapeutic failure and are a frequently unnoticed, persistent threat to human health.[112]

It is interesting to note that case reports and small clinical studies are the only studies to date reporting on SCV therapies and outcomes[112]. Despite the global significance of SCVs in the medical environment, there is a lack of efficient treatments and clinical guidelines. Further investigations and research towards optimised medical therapies remain to be conducted.

1.7 Biofilm infections

The pioneering work of William Costerton and Niels Høiby, the increasing biofilm research and the improvements in biomedical technology over the last decades have advanced our understanding

of the role biofilms play in health and disease[137]. Today we know that biofilms are not only responsible for "animalcules" living on our teeth, but they are also linked to various devastating, antibiotic-refractory diseases[39]. The majority of bacteria in the environment adapt to the biofilm state which presents a survival advantage. It is estimated that bacterial biofilms cause 65% of infections treated in the developed world and 80% of microbial infections in the human body[11]. In particular, biofilms are considered to be responsible for severity and recalcitrance of a plethora of infectious diseases, such as cystic fibrosis, osteomyelitis, endocarditis, chronic wound infections, urinary tract infections, otitis media and chronic rhinosinusitis (Figure 10). Moreover, biofilms form on implants, orthopaedic prostheses and other medical devices, causing infections that frequently require revision surgery and removal of the medical device[15,39,138-141].

DEVICE-RELATED INFECTIONS

- Ventricular derivations
- Contact lenses
- Endotracheal tubes
- Central vascular catheters
- Prosthetic cardiac valves, pacemakers and vascular grafts
- Tissue fillers, breast implants
- Peripheral vascular catheters
- Urinary catheters
- Orthopedic implants and prosthetic joints

TISSUE-RELATED INFECTIONS

- Chronic otitis media, chronic sinusitis
- Chronic tonsillitis, dental plaque, chronic laryngitis
- Endocarditis
- Lung infection in cystic fibrosis
- Kidney stones
- Biliary tract infections
- Urinary tract infections
- Osteomyelitis
- Chronic wounds

Figure 10. Examples of biofilm associated infections. Reprinted with permission[141].

The World Health Organization (WHO) recently announced a global priority pathogens list of 12 bacteria (Table 2) that pose greatest risk to human health[142]. All of them are associated with biofilm

formation, high antibiotic resistance, high mortality rates and high prevalence in communities, placing a tremendous burden on the health care system. The top 5 bacteria on the list are the so called ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species, which are recognised as the leading cause of nosocomial infections[143].

In this thesis, the focus is mainly on *S. aureus* in the context of chronic rhinosinusitis.

Table 2. The World Health Organization's global priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics[142].

Priority: critical	Priority: high	Priority: medium
Acinetobacter baumannii, carbapenem-resistant	*Enterococcus faecium*, vancomycin-resistant	*Streptococcus pneumoniae*, penicillin-non-susceptible
Pseudomonas aeruginosa, carbapenem-resistant	*Staphylococcus aureus*, methicillin-resistant, vancomycin intermediate and resistant	*Haemophilus influenzae*, ampicillin-resistant
*Enterobacteriaceae**, carbapenem-resistant, 3rd generation cephalosporin-resistant	*Helicobacter pylori*, clarithromycin-resistant	*Shigella* spp., fluoroquinolone-resistant
	Campylobacter, fluoroquinolone-resistant	
	Salmonella spp., fluoroquinolone-resistant	
	Neisseria gonorrhoeae, 3rd generation cephalosporin-resistant, fluoroquinolone-resistant	

* *Enterobacteriaceae* include: *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp. and *Providencia* spp., *Morganella* spp.

1.7.1 Chronic rhinosinusitis, a biofilm-associated condition

The importance of biofilms and SCVs has been widely recognised in the context of antibiotic-refractory diseases including chronic rhinosinusitis (CRS). CRS is a debilitating condition characterised by a persistent inflammation of the nasal cavity and paranasal sinuses that lasts for over 12 consecutive weeks[144]. It is grouped in two phenotypes: CRS with polyps and CRS without polyps. According to the Australian Institute of Health and Welfare CRS is highly prevalent in the community and one of the most frequently reported health conditions, comparable to asthma and diabetes[145]. Approximately 9.2 % Australians suffer from CRS (i.e. 1 in 6 people)[145] and the associated symptoms, including persistent nasal obstruction and blockage, facial pressure and pain, abnormal drainage, difficulties in breathing and a reduced sense of smell and taste. CRS affects people irrespective of age, gender and nationality and it is a worldwide common disease, however often underestimated. CRS patients have a low quality of life score and frequently require high-cost medical care, surgery and follow-up treatments with antibiotics and corticosteroids[146]. Long-term therapies with antibiotics contribute to the emergence of multidrug tolerance and resistance. The aetio-pathogenesis of CRS is multifactorial and includes systemic host factors, local host factors and environmental factors (Figure 11)[147].
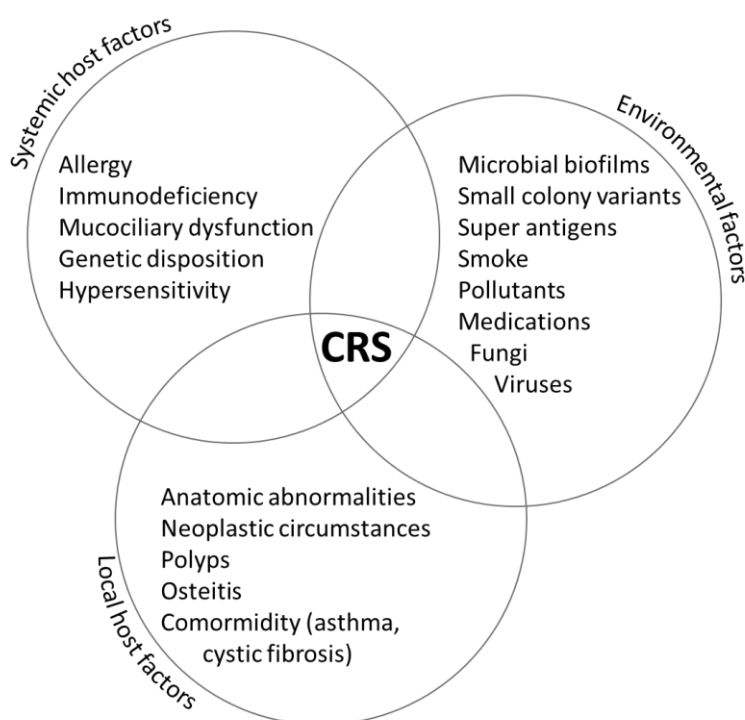
Figure 11. Contributing factors to chronic rhinosinusitis (CRS).

26

While bacterial biofilms commonly play a pivotal role in the pathogenesis and persistence of CRS, the exact role of sinonasal bacterial species is unclear. Microbiome analyses from various institutes around the world reported different absolute numbers of species prevalent in CRS. Nevertheless, most studies identified *Staphylococcus*, *Propionibacterium*, *Corynebacterium*, *Streptococcus* and *Actinobacteria* as the most abundant genera, whilst *Pseudomonas* and *Haemophilus* species were detected as well[148-151]. Interestingly, negative clinical outcomes were frequently associated with the presence of *P. aeruginosa* (Figure 12) in North America and the United Kingdom, while the relative abundance of *S. aureus* was linked to negative clinical outcomes in Australia and New Zealand[148,152-154].
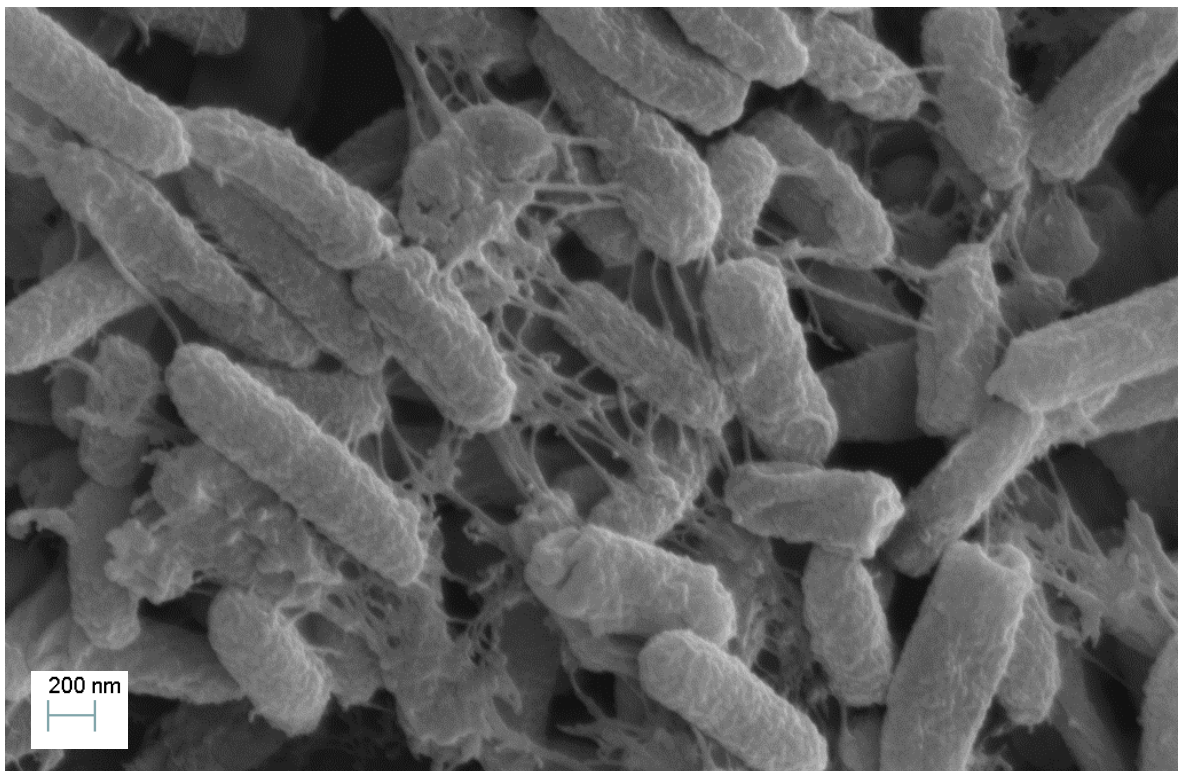
Figure 12. Scanning electron micrograph of Pseudomonas aeruginosa biofilm.

Whilst technological advances increased our knowledge of the bacterial heterogeneity in CRS, life of sessile bacteria in the polymicrobial neighbourhood is poorly understood. Much needs to be learned where and how different species anchor themselves in the sinonasal region to establish a sessile community, how they interact with each other and the host. Moreover, the role of different

species in CRS and their benefit to sinonasal health requires further elucidation. The presence of *Propionibacterium acnes* and *Acinetobacter johnsonii* has been associated with improved clinical outcomes following endoscopic sinus surgery[149,155]. Also, it has been suggested that *Burkholderia* and *Propionibacterium* species hold an important role as gatekeepers to maintain a balanced sinonasal bacterial community, however, further scientific evidence is required[148].

It has been observed that CRS is associated with a microbial imbalance, where certain species take over the bacterial community and disrupt the healthy ecological network[156]. This fosters a reduced bacterial diversity and elevated growth of certain pathogens such as *S. aureus*[148]. Indeed, microbiome studies of our group revealed that *S. aureus* is the most abundant species next to *Staphylococcus epidermidis*, *Propionibacterium acnes*, *Corynebacterium* species and *Acinetobacter* species[149] and that CRS is associated with *S. aureus* biofilms and SCVs[114,140,152].

1.8   Current antimicrobial strategies

Biofilms can be considered as a fortress that protects bacteria from attacks of antimicrobial compounds, leading to significantly reduced susceptibility to antimicrobials. Biofilms are estimated to be involved in 80% of infections in humans and are recognised as major contributors to antibiotic resistance[11]. However, to date antimicrobial regimes for infectious diseases are determined based on the activity against planktonic bacteria and bacterial adaptations such as biofilms and SCVs are still not taken into account to establish adequate therapies.

Standard medical care for CRS is mainly based on oral antibiotics, including penicillins, cephalosporins, macrolides, fluoroquinolones, sulphonamides, tetracyclines, aminoglycosides, monobactams and carbapenems (Figure 13). To increase treatment efficacy antibiotic-combinations can be used, such as amoxicillin/clavulanic acid (beta lactam/beta lactamase inhibitor) and trimethoprim/ sulfamethoxazole (two compounds disrupting the folic acid synthesis at different steps). Last resort antibiotics include polymyxins (e.g. colistin, polymyxin B), glycopeptides (e.g. vancomycin), tigecycline, imipenem/cilastatin, linezolid, advanced-generation

cephalosporins (e.g. ceftaroline, ceftobiprole) and advanced-generation aminoglycosides (e.g. amikacin)[157].

The overuse and misuse of antibiotics together with the bacterial lifestyle in protective biofilms lead to emerging resistance on a global scale[158]. To destroy bacteria in biofilms up to 1000-fold higher drug concentrations are required than for the eradication of planktonic bacteria[17]. These concentrations are difficult to achieve at the site of infection without causing side effects, therefore, antibiotics inherently carry a risk for toxicity[77]. The drug delivery of antibiotics poses challenges for pharmaceutical formulations- compounds need to be able to overcome both hydrophilic and lipophilic barriers, e.g. penetrating through the hydrated biofilm matrix and through the lipophilic cell wall. Drug-drug interactions (such as increased risk of ototoxicity when aminoglycosides and diuretics are taken together), drug-food interactions (such as activity inhibition of tetracyclines by polyvalent cations e.g. contained in dairy products) and drug-host interactions (such as increased hepatic first pass metabolism, drug-degradation in the gastro intestinal tract and increased renal elimination) can further limit the bioavailability of antibiotics and consequently the treatment efficacy. Most antibiotics target pathways of metabolic active bacteria, such as the ribosomal functions, cell wall synthesis and DNA biosynthesis (Figure 13). Hence, slow-growing SCVs and dormant persister cells are hardly affected, resuscitate after antibiotic therapy has terminated and cause a relapse of disease with bacteria of elevated tolerance and resistance[159]. This can lead to a vicious cycle of antibiotic courses and relapsing infections, as seen in the example of chronic rhinosinusitis.
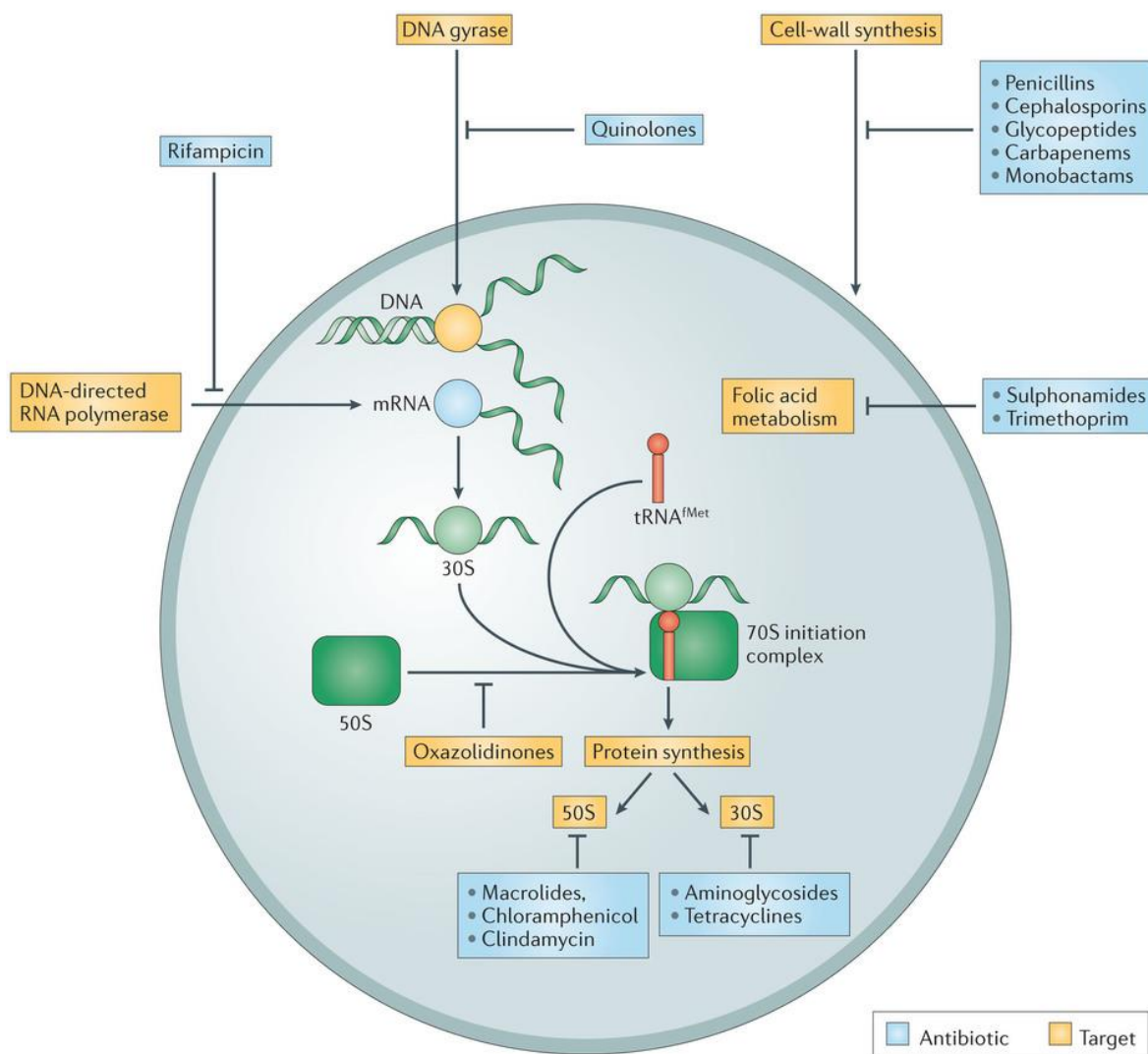
Figure 13. Targets of antibiotics. There are approximately 200 conserved essential proteins in bacteria, but the number of currently exploited targets is very small. The most successful antibiotics hit only three targets or pathways: the ribosome (which consists of 50S and 30S subunits), cell wall synthesis and DNA gyrase or DNA topoisomerase. Reprinted with permission[77,84].

1.8.1   Treatment strategies for chronic rhinosinusitis

Standard medical care for CRS includes nasal irrigation to clear pollutants and thin mucus; oral and topical corticosteroids to reduce inflammation, infiltration and function of eosinophils and neutrophils; as well as culture directed oral antibiotics to fight bacterial infections (Table 3)[144]. Topical antibiotics, non-steroidal anti-inflammatory drugs, leukotriene inhibitors, mucolytics and decongestants can be furthermore used to provide relief, however, the evidence for a beneficial impact on CRS management is limited[160,161]. Endoscopic sinus surgery is frequently an inevitable

intervention for the treatment of CRS aiming to restore sinus ventilation, correct mucosal obstruction, improve mucociliary clearance and rebuild the functional integrity of the sinonasal mucosa. Surgery is commonly followed by long-term antibiotic and corticosteroid therapy. However, therapeutic and surgical interventions have a tremendous impact on health care costs and the patients' quality of life. Antibiotic and corticosteroid treatments are associated with severe side effects and treatment failure due to various factors, such as emerging antibiotic resistant bacteria in biofilms, as well as SCVs[162].

Numerous pathways have been pursued to find effective alternatives to combat biofilms, tackle intracellular pathogens, reduce the persistent inflammation and aid wound healing in the sinuses for improvements in the management of CRS. In addition to standard medical treatments, alternative therapeutic strategies (Table 3), improved surgical techniques, novel medical devices and physical therapies (e.g. therapeutic ultrasound[163] and photodynamic therapy[164]) are emerging. Furthermore, probiotics have been proposed to re-establish a healthy sinus flora and to reduce the prevalence of pathogens. *S. epidermidis* and *Lactobacillus sakei* were reported to diminish the colonisation levels and pathogenicity of *S. aureus*, *Corynebacterium tuberculostearicum* and *P. aeruginosa,* respectively, thereby potentially acting as probiotics promoting a healthy sinus microbiota[165-167].

The pathway from *in vitro* to *in vivo* to clinical pilot studies is long and full of challenges. Although short-term efficacy has been reported for several alternative and multi-pronged strategies, comprehensive clinical evidence is lacking and more research is required.

Table 3. Standard therapeutic strategies (upper part, page 32) and alternative approaches (lower part, page 33) for chronic rhinosinusitis management.

Treatment	Examples	Application	Benefits	Drawbacks	References
Nasal irrigation	Saline	Topical	Improves mucus clearance, ciliary beat activity, clears of allergen, biofilm or inflammatory mediators, protects sinonasal mucosa	Symptomatic relief Saline douches more effective than sprays but low patient compliance	[144,168]
1st generation corticosteroids	Budesonide, Flunisolid, Triamcinolone, Beclomethasone, Dexamethasone	Topical Oral	Immunosuppressive Reduce inflammation, neutrophilic and eosinophilic infiltration and function	Poor biovailability Side effects, e.g. epistaxis, itching, sneezing, dry nose, reduced glucose tolerance, osteoporosis, weight gain	[144,169-171]
2nd generation corticosteroids	Mometasone, Fluticasone, Betamethasone, Ciclesonide	Topical Oral			
Short-term antibiotics	**Penicillins** (Amoxicillin + Clavulanic acid, Penicillin V, Methicillin)	Oral	Broad spectrum activity, Amoxi/Clav = first line CRS treatment	Side effects, e.g. diarrhoea, hypersensitivity, nausea, rash, neurotoxicity, ototoxicity, nephrotoxicity, neutropenia, thrombocytopenia, dermatitis, skin photosensitivity, headache, dizziness, oral and vaginal candidiasis, anaphylactic shock, Allergic reactions Antibiotic resistance	[144,172]
	Tetracyclines (Doxycycline)	Oral	For CRS with polyps, moderately reduces polyp size and symptoms		
	Lincosamides (Clindamycin)	Oral	Activity against anaerobic and some aerobic bacteria, including *S. aureus*		
	Cephalosporins (Cephalexin, Cefuroxime)	Oral	Several generations with broad and narrow spectrum activity		
	Fluoroquinolones (Ciprofloxacin, Levofloxacin)	Oral	Broad spectrum activity		
Long-term antibiotics	**Macrolides** (Clarithromycin, Roxithromycin, Azithromycin, Erythromycin)	Oral	For non eosinophilic CRS Reduce inflammation, fight biofilms, increase inflammatory cell apoptosis	Increased risk of cardiovascular events, brain damage and other side effects Increased risk of interactions Antibiotic resistance	[144,173-176]
	Sulphonamides (Trimethoprim + Sulfamethoxazole)	Oral	Broad spectrum activity, antifungal properties		

Topical antibiotics	Mupirocin **Aminoglycosides** (Tobramycin)	Topical	Lower risk for side effects	Recurring biofilms Antibiotic resistance Scientific evidence lacking, more RCTs required	[177,178]
Non-conventional antibiotic	N,N-dichloro-2,2-dimethyltaurine	Topical	Broad spectrum activity, antiviral and antifungal properties	Potential negative effects on cilia and mucosa RCTs required	[179]
Nasal irrigation	Xylitol/saline	Topical	Clears of allergen, biofilm or inflammatory mediators, improves CRS symptoms	Scientific evidence lacking, more RCTs required	[180,181]
Nasal irrigation	Sodium hypochlorite/saline	Topical	Some activity against biofilms	Scientific evidence lacking	[182]
Nasal irrigation	Surfactants	Topical	Improved drainage, thin mucus Some activity against biofilms, but no eradication	Side effects, e.g. headaches, nasal burning Potential cilia toxicity	[183,184]
Nasal irrigation	Manuka honey	Topical	Antimicrobial/antibiofilm activity of methylglyoxal and other unspecified components	Potential negative effects on cilia and mucosa (dose-dependent) Scientific evidence lacking, RCTs required	[185,186]
Bacteriophage		Topical	Narrow spectrum activity Species-specific targets Harmless to host and commensal bacteria Lower risk for side effects	Potential development of anti-phage antibodies, potential bacterial resistance Scientific evidence lacking, more RCTs required	[187,188]
Surgical gels	Chitosan-dextran gel	Topical	Improved wound healing, prevents adhesions and scarring by promoting homoeostasis	No antibacterial effect	[189,190]

Further approaches include antihistamines[191], mucolytics[192], nasal decongestants[193], leukotriene antagonists[194], monoclonal antibodies[195-197], phytotherapy[198], antimicrobial peptides[199], lipid- or surfactant-based carriers (liposomes, quatsomes)[200,201], nitric oxide[202,203] - Scientific evidence lacking, more RCTs required

RCTs = randomised clinical trials

1.8.2   The antibiotic dilemma

When the Scot Alexander Fleming accidentally discovered the first antibiotic, penicillin, in 1928 he was unaware of the global implications of his finding[204]. Nine years later, the German Ernst Boris Chain identified the chemical composition and developed a method to produce penicillin for therapeutic courses. In collaboration with the Australian Howard Walter Florey penicillin's therapeutic effects were confirmed and Florey started the first ever clinical trial of penicillin in 1941. The significant drug discovery and drug development by Fleming, Chain and Florey revolutionised medical therapy and resulted in a shared Nobel Prize in Physiology or Medicine in 1945. This pioneering work inspired scientists to search for further antibiotics and initiated the "Golden Age of Antibiotic Discovery" in the 1940s to 1960s. However, soon after the discovery of antibiotics, bacterial resistance inevitably emerged (Figure 14). Worsening the situation, antibiotic discovery has decelerated since the 1960s. Neither broad spectrum antibiotics nor narrow spectrum antibiotics against Gram-negative bacteria have been discovered in the last 50 years. Moreover, for half a century only one new antibiotic class (i.e. lipopeptides: daptomycin) with a different mechanism of action received approval for clinical applications[205]. Attempts of researchers and the pharmaceutical industry to discover novel compounds or develop synthetic antibiotics by high-tech approaches such as genomics, combinatorial chemistry, high-throughput screening and rational drug design mainly failed[77]. Whilst the spread of multidrug resistance is increasing exponentially (the first pathogen resistant to all antibiotics has been isolated in 2015), the pace of research and development of new antibiotic treatments has dwindled[206]. Novel antibiotics are urgently needed, but there are currently very few promising products in the antibiotic development pipeline. Teixobactin is one of the few examples of a new class antibiotic that has been recently discovered from uncultured bacteria with a novel device, the iChip[207]. Being highly active against Gram-positive bacteria, including multidrug resistant strains, and without detectable resistance in *S. aureus* and *M. tuberculosis*, teixobactin holds promise as a lead compound to be validated in clinical trials[208]. However, it will take years until pilot studies in humans will commence. Research and development costs of new compounds are generally very high and the pathway to reach administrative approval

and commence market sale is a long and rocky road. Pharmaceutical companies are faced with technical and economic challenges- the replacement of historically used and generally accepted broad-spectrum antibiotics with new compounds of similar properties is extremely difficult. In addition, a lack of profitability diminishes investments in antibiotic discovery[209]. Particularly for new antibiotics such as teixobactin, the investment return is low as the prescription of these new compounds is restricted (short-term therapy and last resort treatments) in order to prevent rapid emergence of resistance.

Antibiotic discovery

Antibiotic resistance

1920s

Sulphonamids: Prontosil (1932)	1936
Penicillins: Penicillin G (1928)	1938

1930s

1942 — Prontosil
1945 — Penicillin G
1946 — Streptomycin

Aminoglycosides: Streptomycin (1943)	1946
Chloramphenicol (1946)	1948
Neomycin (1949)	
Macrolides: Erythromycin (1948)	1951 / 1952
Tetracyclines: Chlortetracycline (1944)	1958 / 1959
Glycopeptides: Vancomycin (1953)	
Rifamycins: Rifampicin (1957)	1964 / 1968
Polymyxins: Colistin (1949)	
Cephalosporins: Cephalotin (1948)	
Quinolones: Nalidixic acid (1962) Ciprofloxacin (1961)	

1940s

1950 — Chloramphenicol / Chlortetracycline
1955 — Erythromycin

1950s

1960 — Vancomycin
1962 — Rifampicin
1963 — Nalidixic acid / Neomycin
1964 — Streptogramin B / Cephalotin
1968 — Ciprofloxacin

1960s

1970s

1977 — Fidaxomicin

1980s

1987 — Daptomycin

Streptogramins: Streptogramin B (1963)	1998
Oxazolidinones: Linezolid (1955)	2000
Lipopeptides: Daptomycin (1986)	2003
Cephalosporins: Ceftaroline (2003)	2010
Fidaxomicin (1948), *C. difficile* specific	2011
Diarylquinolines: Bedaquiline (1997)	2012
ADEP4 (2013) Teixobactin (2015)	?

1990s

2000s
2001 — Linezolid
2006 — Bedaquiline

2010s
2011 — Colistin
2014 — Ceftaroline
2015 — **1st strain resistant to all antibiotics**

Figure 14. Chronological order of antibiotics (year of discovery), clinical approval (underlined years, left) and emergence of resistance (underlined years, right).

As a result the Antimicrobial Resistance Global Report on Surveillance by the WHO predicts that 10 million people will die annually by 2050 due to the implications of antimicrobial resistance[158]. The Review on Antimicrobial Resistance (United Kingdom) estimates associated global costs of 100 trillion USD[206]. The WHO is calling on governments and the pharmaceutical industry to foster antibiotic stewardship and to prioritise drug discovery and drug development. New treatment strategies are urgently needed and several platforms have been proposed[77,210].

- Prodrugs (molecules that are inactive until they penetrate into bacterial cells where they are activated by bacteria-specific enzymes; this increases specificity of the compound and reduces side effects)

- Species-specific compounds (high-throughput screening will rapidly identify species-specific compounds against pathogens, whole-genome sequencing of resistant strains can determine unique targets and novel diagnostic tools based on molecular techniques will make treatments practical)

- Drug discovery from uncultured microorganisms (new cultivation and domestication methods will lead to drug discovery from unexplored sources)

- Rapid dereplication with genomics and transcriptomics (modern techniques to identify targets and to produce new antibiotic derivatives)

- Rules of penetration (validating compounds for their ability to overcome hydrophilic and lipophilic barriers)

- High-throughput screening and anti-infective libraries (structure-function analysis of compounds in libraries to identify candidates with desired properties)

- Silent operons (development of approaches to activate silent operons, units of genomic DNA that are not expressed *in vitro*, could lead to the discovery of new compounds)

- Rational design (drug discovery according to active target sites; by applying the rules of penetration new lead compounds will be optimised and the spectrum of existing narrow-spectrum antibiotics can be extended)

Reviving old successful antibiotic discovery platforms (prodrugs, species-specific compounds, uncultured microorganisms), developing new discovery platforms (high-throughput screening and anti-infective libraries, silent operons, rational design) and combining them with new tools (genomics and transcriptomics, rules of penetration) will help identify lead compounds. Importantly, combination therapies of compounds that hit multiple targets should be considered as an imperative strategy to minimise the risk for antimicrobial resistance[77]. Moreover, innovative approaches need to be able to destroy bacteria in biofilms including their SCVs and persister cells.

1.9   Innovative antibiofilm strategies

While the discovery of antibiotics such as penicillin revolutionised modern medicine, the emergence and spread of antibiotic resistance limits the prospect for many devastating diseases. Additional challenges for novel antimicrobial therapies are overcoming the bacterial protection in biofilms, as well as targeting intracellular bacteria without causing adverse effects. As an example, treatment of *S. aureus* infections has become increasingly difficult because of biofilm-specific resistance and tolerance mechanisms, combined with the emergence of methicillin resistant, vancomycin intermediate resistance and vancomycin resistant *S. aureus* strains (MRSA, VISA and VRSA respectively)[211,212]. Therefore, alternative antibiofilm strategies are urgently required.

The following sections will highlight a number of such alternative strategies against clinically relevant biofilms in general and against *S. aureus* in particular.

A first alternative approach is targeting the bacterial communication system. QS is a process by which bacteria produce and detect signal molecules in a cell density dependent way, and as QS plays an important role in bacterial biofilm formation and resistance, QS inhibitors (QSIs) are promising antibiofilm agents, either alone or as potentiators of conventional antibiotics[67-69]. In the second section, the potential of 'repurposing' approaches, in which libraries of known and approved drugs are screened to identify novel compounds with antibiofilm activity and/or potentiating activity towards antibiotics will be discussed[213-215]. Following this, a brief overview of

other approaches will be described. Ultimately, this will prelude the experimental chapters of this thesis. In chapter 2 colloidal silver nanoparticles as topical treatment for biofilm-related infections will be explored. Based on their small size and physico-chemical properties, silver nanoparticles have potential for a broad antimicrobial application or as beneficial adjuvants in antibiotic therapy to increase the bacterial susceptibility[216]. Moreover, the use of gallium-based therapeutics to combat biofilm infections will be highlighted in chapter 3. The similarity between gallium and iron allows using a "Trojan Horse" strategy to disturb iron metabolism, making gallium containing compounds interesting novel antimicrobial agents[217,218].

1.10 The use of quorum sensing inhibitors to tackle *S. aureus* biofilms

As far back as 1998, Davies *et al.* showed that QS in *P. aeruginosa* is important for biofilm formation, as a QS defective mutant formed flat and undifferentiated biofilms, in contrast with the wild-type[31]. In 2005, it was shown that if QS was blocked in *P. aeruginosa* (either by knocking out the relevant genes or by using QSI), biofilms formed by this organisms became more sensitive to tobramycin and H_2O_2[219]. It was later shown that QSI can 'potentiate' the activity of antibiotics against various bacterial biofilms, in different model systems[68,220].

Hamamelitannin (HAM) is a QSI able to potentiate the activity of vancomycin against *S. aureus* biofilms[68]. HAM targets the *S. aureus* TraP QS system and its effect on biofilm susceptibility is (at least partially) due to an effect on the cell wall thickness and release of eDNA[221]. While treatment of *S. aureus* biofilms with vancomycin typically results in thickening of the cell wall and release of eDNA, these defence mechanisms are down-regulated upon addition of HAM. At the molecular level, this can be explained by the differential expression of genes involved in biosynthesis of peptidoglycan and peptidoglycan precursors (including genes involved in synthesis of L-lysine and glucosamine-6-phosphate) and regulators of autolysis, like *lytS*[221].

Despite its activity, HAM is not an ideal drug-candidate, as it demonstrates several undesirable properties, including a high number of hydroxyl functions leading to high polarity, an aromatic

hydroxyl function making the molecule oxidation- and glucuronidation-sensitive and metabolically unstable ester linkers (Figure 15)[222]. To obtain more active derivatives with more drug-like properties, an extensive structure–activity relationship study was set up and several compounds with high *in vitro* and *in vivo* activity were identified (Figure 15)[222,223]. Several of these highly active compounds showed excellent metabolic stability and lacked toxicity in MRC-5 lung fibroblast cells, making them prime candidates for testing in more advanced models[222].

Hamamelitannin

Property	HAM
tPSA	244
cLogP	- 0.47
H-bond donors	9
H-bond acceptors	14
EC_{50}	145.5 µM

EC_{50} = 96.97 µM

EC_{50} = 0.3890 µM
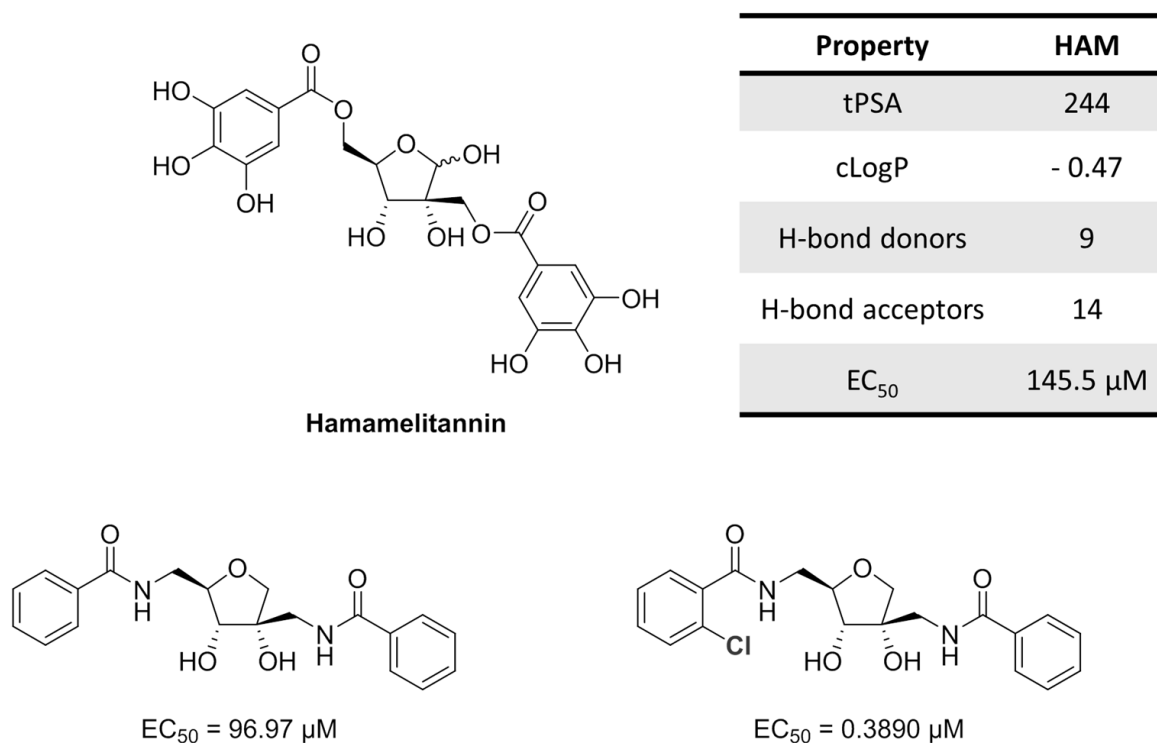
Figure 15. Chemical structure of HAM (top) and two more active derivatives. Table shows some key properties of HAM. The EC_{50} values shown are the concentrations needed to double the effect of vancomycin in vitro.

1.11 Repurposing as a novel approach to find compounds active against *S. aureus* biofilms

1.11.1 What is drug repurposing?

The use of drugs (either drug candidates, abandoned drugs, approved drugs or withdrawn drugs) to treat a disease for which they were initially not developed for is called drug repurposing or repositioning[213-215]. The main advantages of drug repurposing over *de novo* drug development are reduced time and costs in the research and development process, as knowledge concerning safety and pharmacology are available for the repurposing candidates[224]. For lack of a better name, drugs in use for non-bacteriological indications but with antibacterial activity are in literature often called 'non-antibiotic drugs' or 'non-antibiotics'[225]. These compounds might possess a direct antibacterial activity and/or enhance the activity of existing antibiotics by increasing the susceptibility of the bacteria towards the antibiotics, e.g. by controlling efflux pumps. In addition, they might also affect the pathogenicity of bacteria (virulence inhibitors)[226] or interfere with the host resulting in an improved pathogen clearance[227,228]. Several drug classes (e.g. antihistamines, local anaesthetics, anti-hypertensive drugs, tranquilisers, statins and anti-inflammatory drugs) are known to possess antibacterial activity, although they were not developed to treat bacterial infections[226,229-231]. In the following paragraphs, the activity of some of these non-antibiotics against *S. aureus* biofilms will be discussed.

1.11.2 Terfenadine

Jacobs *et al*. screened the Prestwick Chemical Library for antimicrobial agents active against planktonic *S. aureus*; to this end they developed an adenylate kinase assay that identifies compounds that disrupt cellular integrity[232]. Following their initial screen, they evaluated the activity of one hit compound, i.e. the antihistamine terfenadine, for activity against biofilms formed by *S. aureus* UAMS1 (an osteomyelitis clinical isolate). Treatment with terfenadine at 10× MIC resulted in a 2.7-fold increase in adenylate kinase release, corresponding to a 1.1 $\log_{10}$ reduction in

biofilm cell viability. However, treating *S. aureus* infected *Galleria mellonella* larvae with terfenadine did not increase the latter's survival[232]. More recently, 84 terfenadine-based analogues were synthesised and evaluated for activity towards *S. aureus* planktonic cells. Two compounds had lower MIC in comparison with terfenadine, also against other Gram-positive pathogens but their antibiofilm activity has not yet been evaluated[233].

1.11.3 Antibacterial and antibiofilm activity of niclosamide and analogues

In another comprehensive screenings using the same Prestwick Chemical Library, activity was evaluated by measuring inhibition of growth of planktonic *S. aureus* TCH1516; the screen resulted in the identification of 104 hits, most of them belonging to the group of antimicrobials and antiseptics. However, 18 non-antibiotic drugs were also identified and 9 of these hit compounds were evaluated for activity against *S. aureus* biofilms[234]. Three of these showed modest activity, including the anthelmintic niclosamide that caused a reduction in the number of culturable *S. aureus* cells of 1–2 $\log_{10}$ [234]. Rajamuthiah *et al*. investigated the activity of niclosamide and another salicylanilide anthelmintic drug, oxyclozanide against planktonic grown ESKAPE pathogens[235]. MICs against multiple *S. aureus* isolates ranged between 0.0625 and 0.5 µg/ml for niclosamide and between 0.5 and 2 µg/ml for oxyclozanide. These two compounds were also found to prolong survival of *Caenorhabditis elegans* (*in vivo* nematode model) infected with *S. aureus* MW2, with a similar effect as treatment with vancomycin. A third salicylanilide anthelmintic drug closantel was identified as a hit compound in a screen of the Biomol 4 compound library (containing 640 FDA-approved drugs) using a *C. elegans*–*S. aureus* infection assay[236]. In this assay, *C. elegans* was infected with *S. aureus* MW2 BAA-1707 and treated with the library compounds in 384-well MTPs for 5 days. Next, Sytox Orange, a dye that stains death larvae, was added to the wells and an automated microscope was used the next day to generate both transmitted light and fluorescent images enabling the calculation of the number of surviving *C. elegans*. Using this approach, almost all antibiotics present in the library were identified as hits, as well as ten anticancer drugs, an

antiviral drug, an antifungal, an antiarthritic drug, a non-steroidal estrogen and closantel. Although closantel turned out to have low MICs against several antibiotic-resistant *S. aureus* strains, the number of *S. aureus* cells in the infection assay using *C. elegans* was not reduced upon closantel exposure. It is thus possible that closantel targets bacterial virulence rather than survival, and/or has a direct effect on *C. elegans*[236].

1.11.4 Antibacterial and antibiofilm activity of 5-fluorouracil and analogues

5-fluorouracil is an antimetabolite widely used in treatment of cancers[237]. Activity against biofilms was reported in 1992, as sub-MIC levels of 5-fluorouracil diminished biofilm formation of *S. epidermidis*[237,238]. Later, carmofur (1-hexylcarbamoyl-5-fluorouracil) was identified as a hit in the primary screen against planktonic cells of *S. aureus* and showed antibiofilm activity in a secondary screen[234]. Recently, 5-fluorouracil and 5-fluoro-2'-deoxyfluridine, another fluoropyrimidine, were identified in a screen against planktonic *S. aureus* USA300 and activity of 5-fluoro-2'-deoxyfluridine was confirmed in a septicemic MRSA mice infection model with concentrations much lower than the concentrations therapeutically used for cancer treatment, and thus with reduced toxicity[239]. The usefulness of 5-fluorouracil has been demonstrated in human clinical trials in which central venous catheters externally coated with 5-fluorouracil scored better in preventing catheter colonisation than the control catheters coated with silver sulfadiazine or chlorhexidine[237].

1.11.5 Statins

Statins have been described for antibacterial effects by several research groups[231]. Simvastatin at 1/16× MIC up to 4× MIC (62.5 µg/ml) significantly inhibited biofilm formation and at 4×MIC it significantly reduced the number of CFU/ml in mature biofilms of *S. aureus* ATCC 29213[240,241]. Simvastatin was found to be able to disrupt *S. aureus* and *S. epidermidis* biofilms and its potency was higher than that of linezolid or vancomycin: at 2× and 4× MIC of simvastatin, the biofilm mass

(as measured by Crystal Violet staining) was reduced by 40%, while 64× and 128× MIC of linezolid or vancomycin reduced biofilm mass by only 10%[242].

1.11.6 Repurposing candidates with potentiator activity

The combination of antibiotics with non-antibiotic drugs as potentiators could be a valuable approach to overcome antibacterial drug resistance[243-245]. Combination therapy might result in a broader spectrum of drug activity, synergy, a more rapid effect and the use of reduced drug concentrations[246]. In a screening with 1059 previously approved drugs against planktonic *P. aeruginosa* PAO1, *E. coli* BW25113 and *S. aureus* ATCC 29213 in the presence of minocycline, 6, 41 and 35 hits respectively, were identified. These hits were non-antibiotic drugs that synergised with minocycline but had never been used clinically to treat bacterial infections[247]. Disulfiram was one of the hits against *S. aureus*: alone, disulfiram has only weak antibacterial activity but it improved the activity of minocycline in a synergistic way against several MRSA strains, including MRSA USA300.

Ooi *et al*. evaluated the antibiofilm activity of 15 redox-active compounds that have been safely used in humans for several applications (healthcare, cosmetics and consumption) and of which antibacterial activity had been described previously, but not against *S. aureus* planktonic cells and biofilms[248]. All compounds tested were active against planktonic cells (MICs between 0.25 and 128 mg/l) and seven compounds (i.e. AO2246, bakuchiol, benzoyl peroxide, carnosic acid, celastrol, nordihydroguaiaretic acid and totarol) were able to eradicate established biofilms of *S. aureus* SH1000 at concentrations <256 mg/l[248]. Moreover, celastrol and nordihydroguaiaretic acid synergised the activity of gentamicin against *S. aureus* SH1000 biofilms[248]. As both compounds did not cause irritation in a human living skin equivalent model, they might be valuable potentiators to treat superficial skin infections caused by *S. aureus* biofilms[248].

Van den Driessche *et al*. recently screened the NIH Clinical Collection 1&2 against *S. aureus* Mu50 biofilms, formed in 96-well microtiter plates[249]. The screening was performed in the presence of

vancomycin and resulted in the identification of 25 hit compounds that potentiated the activity of the antibiotic. Among these hits, the disinfectants triclosan and hexachlorophene, the antiviral drug efavirenz and the antifungal imidazole drugs miconazole, econazole and oxiconazole were identified. Antifungal imidazoles are known for activity against planktonic *S. aureus* for decades and perform activity by membrane damage and binding to flavohaemoglobins[250,251]. In addition, two anthracyclines, three selective estrogen receptor modulators, flutamide, oxymetholone, amiodarone, carvedilol, honokiol, loxoprofen, MK 886, 5-nonyloxytryptamide and ethacrynic acid were identified as well in the screen. Antibacterial activity of some of these hits had been reported before, e.g. for honokiol[252,253], MK-886 and 5-nonyloxytryptamine[239], but for most of the hits there were no previous indications for potential use as an anti-infective. Also four antipsychotic phenothiazine drugs (fluphenazine, perphenazine, thioridazine and trifluoperazine) and the antidepressant sertraline were hits. Anti-staphylococcal activity had been reported before for these compounds, both *in vitro*[254] and *in vivo*[255,256], but activity against biofilm was not investigated before. Van den Driessche *et al*. showed that thioridazine enhances the activity of tobramycin, flucloxacillin and linezolid against *in vitro* grown *S. aureus* Mu50 biofilms (>1 $\log_{10}$ additional reduction in CFU/biofilm)[249]. Unfortunately, they were unable to confirm this activity in a biofilm model for chronic wounds.

1.12 Other approaches

Other approaches to tackle microbial biofilms include (i) bacterial attachment inhibitors, (ii) matrix degrading agents, (iii) bacterial communication antagonists, (iv) antimicrobial peptides, (v) bacteriophages and bacteriophage-derived enzymes, and (vi) nanoparticles and other drug delivery systems.

(i) The inhibition of bacterial attachment to epithelial or biomaterial surfaces can obstruct biofilm formation. Pilicides and curlicides were reported to impede the biosynthesis, export and assembly of pili and curli of specific microorganisms, thereby hindering bacterial surface adhesion[257,258].

Biomaterials, such as orthopaedic prostheses, catheters and other medical devices can be coated or impregnated with antimicrobial compounds, such as silver, organoselenium, nitric oxide or cationic peptides to prevent bacterial attachment and biofilm formation[259].

(ii) The biofilm matrix poses another target for antibiofilm strategies. Several compounds are known to disrupt the matrix, causing partial dispersal of the biofilm, subsequently facilitating an increased susceptibility to antimicrobials[260-262]. Enzymes like DNases or glycoside hydrolases like $PelA_h$ and dispersin B can degrade matrix-forming polymers[263-266]. In addition, specific small molecules and other compounds like ebselen and nitric oxide have been identified to inhibit c-di-GMP synthesis and signalling, thereby catalysing matrix breakdown and dispersion[267-269].

(iii) Jamming the bacterial communication is yet another strategy to disrupt biofilms. In addition to HAM, many other QSIs have been identified to date that prevent a coordinated bacterial response[67], including natural derived extracts from a plethora of plants (e.g. ginger, cinnamon, curcumin, garlic, horseradish, cloves, coffee, vanilla, guava)[93,270].

(iv) Moreover, antimicrobial peptides, such as defensins, cathelicidins, histatins and peptide-mimics are emerging as alternative antibiofilm approaches[199,271,272]. Based on their positive charge and their amphipathic nature (i.e. their molecular structure features both hydrophilic and hydrophobic parts), antimicrobial peptides can interfere with negatively charged components of biofilms and can disrupt the hydrophobic fatty acid domains of bacterial membranes, thereby destabilising the membrane and inducing cell lysis[273].

(v) Further approaches against biofilms are the use of bacteriophages[188,274,275] and bacteriophage-derived enzymes[276]. Phages are viruses that specifically target bacteria without harming the human body. They specifically infect the target species, replicate and induce lethal damage. After cell death and lysis of bacteria, phages are released and can infect further bacteria. Phage therapy has been known for over 90 years, however, it was abandoned in the western world due to a lack of scientific evidence for their therapeutic efficacy and the predominance of antibiotics as effective therapies. Owing to the emergence and spread of antibiotic resistance, interest in phage therapy has

increased worldwide in the last decades. Bacteriophages and their enzymes are now being investigated as alternative treatments for multidrug resistant- and biofilm-related infections with more scientific evidence through clinical trials arising.

(vi) Apart from that, nanoparticles and other drug delivery systems to eradicate and/or prevent biofilm formation have been proposed, comprising of various materials, including metals, lipids, polymers and surfactants[201,277,278]. The use of silver nanoparticles will be described in the next chapter.

It is apparent that more and more alternative approaches are emerging. While the efficacy of some of these approaches have been validated *in vitro* and *in vivo*, their true value will crystallise once these innovations are translated into therapeutics for the utilisation in everyday practice.

Chapter 2.

Publication: "Taking the silver bullet- colloidal silver particles for the topical treatment of biofilm-related infections"

Statement of authorship

Title of Paper	Taking the silver bullet- colloidal silver particles for the topical treatment of biofilm-related infections
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Richter K, Facal P, Thomas N, Vandecandelaere I, Ramezanpour M, Cooksley C, Prestidge CA, Coenye T, Wormald P-J, Vreugde S. Taking the silver bullet- colloidal silver particles for the topical treatment of biofilm-related infections. ACS Applied Materials & Interfaces. Online first 9 June 2017, DOI: 10.1021/acsami.7b03672

Principal Author

Name of Principal Author (Candidate)	Katharina Richter		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	7/4/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

    i.   the candidate's stated contribution to the publication is accurate (as detailed above);

    ii.   permission is granted for the candidate in include the publication in the thesis; and

    iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Conception of the project, critically revising the article		
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Contribution to the Paper	Conception and design of the project, critically revising the article		
Signature		Date	10/4/2017

2.1 Publication title page

Taking the Silver Bullet Colloidal Silver Particles for the Topical Treatment of Biofilm-Related Infections

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ⓈSupportingInformation

ABSTRACT: Biofilms are aggregates of bacteria residing in a self-assembled matrix, which protects these sessile cells against external stress, including antibiotic therapies. In light of emerging multidrug-resistant bacteria, alternative strategies to antibiotics are emerging. The present study evaluated the activity of colloidal silver nanoparticles (AgNPs) of different shapes against biofilms formed by *Staphylococcus aureus* (SA), methicillin-resistant SA (MRSA), and *Pseudomonas aeruginosa* (PA). Colloidal quasi-spherical, cubic, and star-shaped AgNPs were synthesized, and their cytotoxicity on macrophages (THP-1) and bronchial epithelial cells (Nuli-1) was analyzed by the lactate dehydrogenase assay. The antibiofilm activity was assessed in vitro by the resazurin assay and in an in vivo infection model in *Caenorhabditis elegans*. Cubic and star-shaped AgNPs induced cytotoxicity, while quasi-spherical AgNPs were not toxic. Quasi-spherical AgNPs showed substantial antibiofilm activity in vitro with 96% (± 2%), 97% (± 1%), and 98% (± 1%) biofilm killing of SA, MRSA, and PA, respectively, while significantly reducing mortality of infected nematodes. The in vivo antibiofilm activity was linked to the accumulation of AgNPs in the intestinal tract of *C. elegans* as observed by 3D X-ray tomography. Quasi-spherical AgNPs were physically stable in suspension for over 6 months with no observed loss in antibiofilm activity. While toxicity and stability limited the utilization of cubic and star-shaped AgNPs, quasi-spherical AgNPs could be rapidly synthesized, were stable and nontoxic, and showed substantial in vitro and in vivo activity against clinically relevant biofilms. Quasi-spherical AgNPs hold potential as pharmacotherapy, for example, as topical treatment for biofilm-related infections.

KEYWORDS: *Staphylococcus aureus, MRSA, Pseudomonas aeruginosa*, biofilms, chemotherapy, silver, nanoparticles
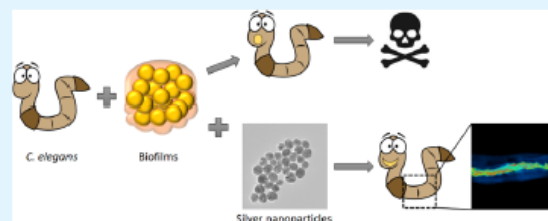
■ INTRODUCTION

According to the National Institutes of Health, bacterial biofilms are responsible for approximately 80% of microbial infections in the human body.[1] Bacterial biofilms consist of aggregates of bacteria in a self-assembled, protective matrix. Cells in a biofilm show reduced susceptibility toward antibiotics, can establish multidrug resistance, and can evade the immune response.[2−4] This poses a challenge to the medical community worldwide and is associated with tremendous health care costs, persistence of disease, low quality of life, and poor clinical outcomes.[5,6] Treatment strategies not based on traditional antibiotics are under investigation as alternatives to fight bacterial biofilms.[7−11]

Various approaches have been proposed, including the use of ionic or colloidal silver.[12] Silver-coated catheters and implants, silver-containing dental resin composites, silver-based disinfectants, or silver-releasing dressings and wound care products have successfully found their way into clinical practice.[13,14] However, most studies are based on the effect of silver against planktonic (nonsurface attached) bacteria, while the interactions with and efficacy against bacterial biofilms are infrequently reported and poorly understood. There is significant potential to expand the utilization of silver-based

A

2.2   Article

Taking the silver bullet- colloidal silver particles as topical treatment for biofilm-related infections[279]

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2.3   Abstract

Biofilms are aggregates of bacteria residing in a self-assembled matrix, which protects these sessile cells against external stress, including antibiotic therapies. In light of emerging multidrug resistant bacteria, alternative strategies to antibiotics are emerging. The present study evaluated the activity of colloidal silver nanoparticles (AgNPs) of different shapes against biofilms formed by *Staphylococcus aureus* (SA), methicillin-resistant SA (MRSA), and *Pseudomonas aeruginosa* (PA).

Colloidal quasi-spherical, cubic, and star-shaped AgNPs were synthesised, and their cytotoxicity on macrophages (THP-1) and bronchial epithelial cells (Nuli-1) was analysed by the lactate dehydrogenase assay. The antibiofilm activity was assessed *in vitro* by the resazurin assay and in an *in vivo* infection model in *Caenorhabditis elegans*.

Cubic and star-shaped AgNPs induced cytotoxicity, while quasi-spherical AgNPs were not toxic. Quasi-spherical AgNPs showed substantial antibiofilm activity *in vitro* with 96% (±2%), 97% (±1%), and 98% (±1%) biofilm killing of SA, MRSA, and PA, respectively, while significantly reducing mortality of infected nematodes. The *in vivo* antibiofilm activity was linked to the accumulation of AgNPs in the intestinal tract of *C. elegans* as observed by 3D X-ray tomography. Quasi-spherical AgNPs were physically stable in suspension for over 6 months with no observed loss in antibiofilm activity.

While toxicity and stability limited the utilisation of cubic and star-shaped AgNPs, quasi-spherical AgNPs could be rapidly synthesised, were stable and nontoxic, and showed substantial *in vitro* and *in vivo* activity against clinically relevant biofilms. Quasi-spherical AgNPs hold potential as pharmacotherapy, for example, as topical treatment for biofilm-related infections.

2.4   Graphical abstract
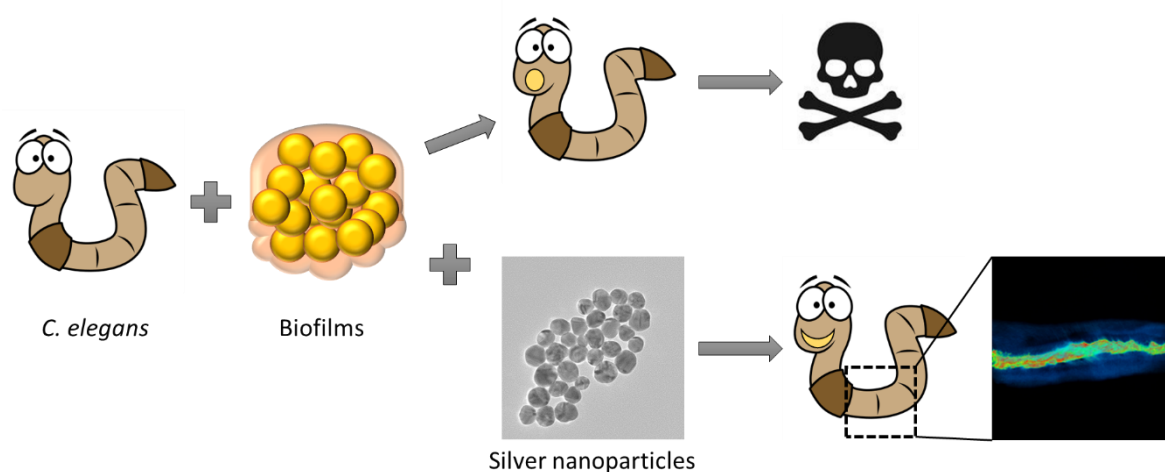
2.5   Introduction

According to the National Institutes of Health, bacterial biofilms are responsible for approximately 80% of microbial infections in the human body[11]. Bacterial biofilms consist of aggregates of bacteria in a self-assembled, protective matrix. Cells in a biofilm show reduced susceptibility towards antibiotics, can establish multidrug resistance, and can evade the immune response[39,72,73]. This poses a challenge to the medical community worldwide and is associated with tremendous health care costs, persistence of disease, low quality-of-life and poor clinical outcomes[280,281]. Treatment strategies not based on traditional antibiotics are under investigation as alternatives to fight bacterial biofilms[1,277,282-284].

Various approaches have been proposed, including the use of ionic or colloidal silver[285]. Silver-coated catheters and implants, silver-containing dental resin composites, silver-based disinfectants

or silver-releasing dressings and wound care products have successfully found their way into clinical practice[286,287]. However, most studies are based on the effect of silver against planktonic (non-surface attached) bacteria, while the interactions with and efficacy against bacterial biofilms are infrequently reported and poorly understood. There is significant potential to expand the utilisation of silver-based therapeutics as alternatives to antibiotics or as adjuvants for the treatment of multidrug resistant bacteria and biofilm-associated infections.

In recent years, interest in silver nanoparticles (AgNPs) in the biomedical field has increased because of their promising antimicrobial properties. These properties are determined by various parameters such as the size and shape of AgNPs[288]. Various protocols for the synthesis of AgNPs have been described comprising simple to very complex reactions utilising various chemicals that influence the physico-chemical properties of the resulting AgNPs. Nanoparticle size smaller than 130 nm was shown to facilitate penetration through the biofilm matrix promoting antimicrobial effects[289]. Small particle size furthermore increases the antimicrobial activity against Gram-negative bacteria[290]. Particle shape is also an important parameter. While literature suggests the greatest antibacterial activity of triangular AgNPs against planktonic *Escherichia coli*[288], the time and chemicals required for their synthesis and purification limit future application in clinical practice. Consequently, for the successful use of stable and nontoxic AgNPs as antimicrobial agents, the particle morphology needs to be controlled using a simple, rapid and cost effective preparation method. Moreover, it is crucial to assess the activity of AgNPs not only against planktonic but also against biofilm-associated bacteria, as the latter are known to require up to 1000-fold higher drug concentrations compared with free-living bacteria[15]. However, these high concentrations are difficult to achieve by oral drug intake without causing severe systemic side effects such as argyria[291]. Depending on the location of the infection, topical treatments offer the opportunity to deliver compounds directly to the infected site, while increasing treatment efficacy and reducing the risk of systemic side effects.

This study evaluated AgNPs of different shapes for the topical treatment of *Staphylococcus aureus*, methicillin-resistant SA (MRSA), and *Pseudomonas aeruginosa* related biofilm infections. These biofilms play a significant role in clinical settings and are associated with a plethora of antibiotic-refractory infectious diseases, such as chronic wounds and chronic rhinosinusitis. The ultimate goal was to determine AgNPs that are economical to produce and that are stable, nontoxic, and equipped with strong antibiofilm activity against *S. aureus*, MRSA, and *P. aeruginosa* suitable for a clinical application.

2.6   Materials and methods

2.6.1   Chemicals

Silver nitrate (AgNO$_3$, 99.9999% trace metals basis), silver trifluoroacetate (CF$_3$COOAg, ≥99.99% trace metals basis), hydroxylamine solution (HA, 50 wt % in H$_2$O), polyvinylpyrrolidone (PVP, average Mw ~55,000), sodium citrate tribasic dehydrate (CIT, ≥99.0%), sodium hydrosulfide hydrate (NaSH) were purchased from Sigma-Aldrich (Steinheim, Germany). L-ascorbic acid (AA) was obtained from BDH Chemicals (Kilsyth, Australia). Ethylene glycol (EG), sodium hydroxide (NaOH) and potassium iodide (KI) were acquired from Chem-Supply (Gillman, Australia). Hydrochloric acid (HCl) was purchased from Scharlau-Chemicals (Barcelona, Spain). Water used in all experiments was prepared in a three-stage Milli-Q Plus 185 purification system (Merck Millipore, Darmstadt, Germany).

2.6.2   Synthesis of colloidal silver nanoparticles

For this study, quasi-spherical, cubic, and star-shaped AgNPs were prepared utilising three simple, robust, and fast protocols that could be adopted for in-house preparation or industrial scale up.

Quasi-spherical AgNPs

The synthesis of quasi-spherical AgNPs was performed as a 5-times scale up of an established protocol described by Li *et al.*[292] (experimental details in Supplementary data S 1). Briefly, 237.5 ml MQ water was boiled for at least 5 minutes in a 500 ml two neck round flask coupled to a coil condenser while stirring at 1500 rpm under reflux. In a separate vial a pre-mixture of 6.25 ml MQ water, 1.25 ml CIT (1% wt), 1.25 ml AgNO$_3$ (1% wt) and 50 μl KI (300 μM) was prepared under stirring at room temperature. Following 4 minutes incubation of the premixture, 250 μl AA (0.1 M) was injected to the boiling water. The injection of the silver premixture followed one minute later ensuring a total incubation time of the premixture for precisely 5 minutes. The colour of the reaction solution quickly changed from colourless to yellow and finally slightly orange. To warrant the formation of quasi-spherical AgNPs, the solution was further boiled for 1 hour under reflux and stirring at 1500 rpm. The quasi-spherical AgNP dispersion was cooled down to room temperature prior to characterisation.

Cubic AgNPs

Following the procedure by Zhang *et al.*[293] (experimental details in Supplementary data S 2), 100 ml EG in a 250 ml round bottom flask was heated (oil bath of 150°C) and stirred. First, 1.2 ml NaSH (3 mM) was injected, 2 minutes later 10 ml HCl (3 mM) was added followed by the addition of 25 ml PVP (20 mg/ml). After 2 more minutes, 8 ml CF$_3$COOAg (282 mM) was added resulting in a colour change of the reaction solution from transparent to grey to slightly yellow within 1 minute, evidencing the formation of Ag seeds. The reaction was allowed to proceed for more than one hour to obtain 50 nm edge, cubic AgNPs. The size of the cubic AgNPs was controlled by monitoring the position of the main localised surface plasmon resonance peak (LSPR) determined through UV-Vis absorption spectra measurements. In particular, for 50 nm edge, cubic AgNPs the LSPR must be around 450 nm if compared to the calibration curve for wavelength *versus* edge length. Once the desired size was achieved, the reaction solution was quenched by placing the reaction flask in an

ice-water bath. Samples were collected and purified by centrifugation, firstly with acetone to remove remaining precursors and EG, and finally four times with MQ water to remove excess PVP.

Star-shaped AgNPs

Suspensions of AgNP stars were prepared by chemical reduction of Ag$^+$ in two steps, first using neutral HA and second using CIT as reducing agents[294] (experimental details in Supplementary data S 3). In brief, 500 µl HA (0.06 M) was mixed with 500 µl NaOH (0.05 M). Afterwards, 9 ml AgNO$_3$ (0.001 M) was added dropwise to the first solution under agitation, facilitating a colour change to brown. After 5 minutes, 100 µl CIT (1%, w/v) was added to the mixture. The final dark grey suspension was shaken for 15 minutes. To guarantee the formation of AgNP stars, the mixture was equilibrated for a minimum of 48 hours.

2.6.3 Characterisation of AgNPs

Ultraviolet-visible (UV-Vis) spectroscopy

The absorbance spectra of AgNPs were recorded with a UV-2600 spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan) directly after the particle synthesis and monthly up to 6 months of storage (protected from light at 4°C) for stability measurements.

Dynamic light scattering (DLS)

The size of AgNPs was analysed by DLS on a Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, United Kingdom) at room temperature. The measurements were conducted at a scattering angle of 173° at 25°C using a helium-neon laser with a wavelength of 633 nm. The particle size (reported as the z-average) and zeta potential of AgNPs were measured.

Transmission electron microscopy (TEM)

TEM imaging of AgNPs was performed on a Tecnai G2 Spirit Transmission Electron Microscope (FEI, Hillsboro, OR, USA) operating at an acceleration voltage of 120 kV, equipped with a FEG LaB6 emitter and BioTWIN lens design. Imaging was undertaken via an in-column Olympus-SIS Veleta CCD camera. Five microliters of each sample was dropped onto a piece of ultrathin Formvar-coated 200-mesh copper grid (ProSciTech, Townsville, Australia) and left to dry in air prior to image acquisition.

2.6.4 Bacterial strains and cell lines

Staphylococcus aureus ATCC 25923 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), methicillin-resistant *Staphylococcus aureus* (clinical isolate collected from a chronic rhinosinusitis patient, which was approved by the human ethics committee at the Queen Elizabeth Hospital, Woodville, Australia) was obtained from Adelaide Pathology Partners (Mile End, Australia) and *Pseudomonas aeruginosa* PA01 was received from the School of Molecular Medical Sciences, University of Nottingham (Nottingham, United Kingdom). The MRSA clinical isolate expressed resistance to penicillin, oxacillin, amoxicillin/clavulanic acid, cephalexin and erythromycin.

Cell lines were purchased from ATCC and included Nuli-1 cells (human bronchial epithelial cells) and THP-1 cells (human monocytic cells).

2.6.5 Cytotoxicity studies

Human cell culture

Nuli-1 cells were grown in serum-free bronchial epithelial cell growth medium (Lonza, Mount Waverley, Australia) that included growth factors, cytokines and supplements. THP-1 cells were cultured in RPMI medium. Cells were maintained in a fully humidified incubator with 5% CO_2 at

37°C, prior to cytotoxicity studies. Cell lines were exposed to AgNPs (0.03 mg/ml Ag, corresponding to 30 ppm Ag) for 1 hour, followed by determination of lactate dehydrogenase (LDH) with a cytotoxicity detection kit (Promega, Madison, WI, USA). Briefly, 50 µl of the supernatant from each well was mixed with 50 µl of LDH reagent and was incubated for 30 minutes in the dark at room temperature. The optical density (OD) was measured at 490 nm on a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany). Cell culture studies were performed as 3 independent experiments with 3 wells per treatment.

2.6.6   Antibiofilm activity studies

In vitro biofilm assay

Single colonies of bacteria suspended in 0.9% saline were adjusted to 1.0 ± 0.1 McFarland units (approximately 3×10^8 CFU/ml), and were diluted 1:15 in broth medium. For the resazurin assay (Life Technologies, Scoresby, Australia), black 96-well microtiter plates (Costar, Corning Incorporated, Corning, NY, USA) were inoculated with 150 µl of the diluted bacterial suspension and were incubated at 37°C for 48 hours on a rotating platform (3D Gyratory Mixer, Ratek Instruments, Boronia, Australia) at 70 rpm. Biofilms were rinsed with phosphate buffered saline (PBS), followed by exposure to AgNPs (0.03 mg/ml Ag, corresponding to 30 ppm Ag) for 1 hour at 37°C on a rotating platform. After a second washing step to remove excess AgNPs, bacterial viability was assessed by the resazurin assay[201,295]. Briefly, 200 µl of a freshly prepared 10% resazurin dilution in broth medium was added to each well and was incubated, protected from light, for up to 7 hours at 37°C on a rotating platform. The fluorescence was measured hourly on a FLUOstar OPTIMA plate reader at $\lambda_{excitation}$ = 530 nm/$\lambda_{emission}$ = 590 nm. Maximum fluorescence was typically reached after 4 hours for both *S. aureus* and MRSA, and 6 hours for *P. aeruginosa*. Antimicrobial activity of AgNPs was quantified according to Equation 1:

$$\% \ \mathrm{BK} = \frac{F_C - F_T}{F_C} \times 100\% \qquad (1)$$

Antimicrobial activity of AgNPs is expressed as the percentage of biofilm killing (% BK), where F_C is the fluorescence of the untreated control biofilms (100% bacterial growth) and F_T is the fluorescence observed in the treated biofilms. Both F_C and F_T were corrected for background fluorescence (sterile medium). Viability studies were performed as three independent experiments with 6 wells per treatment.

In vivo biofilm assay

An infection assay in *Caenorhabditis elegans* AU37 (*glp-4*; *sek-1*) was carried out as previously described[68]. Synchronised nematodes (L4 stage) were suspended in OGM medium (95% M9 buffer, 5% brain heart infusion broth, 10 µg/ml cholesterol) and were placed in a 96-well plate, containing at least 20 worms per well. An overnight culture of bacteria was centrifuged, was resuspended in OGM medium and was adjusted to $2x10^9$ CFU/ml. Nematodes were infected with 25 µl of the bacterial suspension and were exposed to 25 µl of quasi-spherical AgNPs. Controls included uninfected nematodes in OGM medium and infected nematodes without a treatment. During an incubation of 72 hours at 25°C, the number of viable and dead nematodes was counted every 24 hours. Thereafter, the bacterial load per worm was determined. Briefly, nematodes were collected, rinsed with M9 buffer containing 1 mM sodium azide, washed in PBS and counted. The worms were mechanically disrupted by vortexing in microtubes with 1.0 mm silicon carbide beads for 10 minutes (Biospec Products, Bartlesville, OK, USA). The supernatant of the resulting suspension was used for serial dilutions and for plating on agar plates (tryptone soya agar with 7.5% NaCl for *S. aureus* and MRSA; Pseudomonas isolation agar for *P. aeruginosa*) before counting CFU.

X-ray computed tomography of C. elegans

Nematodes were analysed by X-ray computed tomography to elucidate the *in vivo* fate of AgNPs. Nematodes (L4 stage) were exposed to AgNPs for 24 hours as described above. Following a washing

step in M9 medium containing 1 mM sodium azide, the worms were fixed in a mixture of 2%

glutaraldehyde/4% paraformaldehyde for 1 hour. The fixed nematodes were washed and then

stained for 36 hours with Lugol's iodine (aqueous solution containing 0.66% iodine and 1.32%

potassium iodide, Australian Biostain Pty Ltd, Traralgon, Australia) to enhance contrast. The

subsequent dehydration of the worms was accomplished by an increasing ethanol series (25%, 50%,

75%, 95%, 100%, 15 minutes each). Individual nematodes were air-dried and mounted on a pin with

epoxy. X-ray CT images were acquired with a Xradia UltraXRM L200 nanoCT microscope (Carl Zeiss,

Oberkochen, Germany) using an X-ray energy of 40 keV at large field of view absorption (LFOV-ABS)

and an exposure time of 60 seconds. Images were reconstructed by the manufacturer's software

(Xradia TXM 3-VIEW. Version 1.1.6).

2.6.7   Statistical analysis

All experiments were conducted at least in triplicate and are presented as mean ± standard

deviation (SD) unless stated differently. *In vitro* results were analysed using one-way analysis of

variance (ANOVA) following Dunnett's test, and for *in vivo* results two-way ANOVA following

Tukey's test was applied (GraphPad Prism version 7.00, GraphPad Software, La Jolla, CAL, USA).

Statistical significance was assessed at the 95% confidence level.

2.7   Results and discussion

2.7.1   Characterisation of AgNPs

UV-Vis spectra, DLS measurements and TEM images showed quasi-spherical AgNPs of

approximately 40 nm in diameter (derived from DLS and TEM analysis) and cubic AgNPs of

approximately 70 nm (derived from TEM images), both with a narrow size distribution and AgNP

stars of approximately 140 nm (derived from TEM images) with a broad size distribution (Figure 16

and Supplementary data S 4). The zeta potential of all AgNP types investigated in this study was

negative (-37 mV, -9 mV and -25 mV). After 1 month of storage at 4°C protected from light, agglomerates were visually observed for both cubic and star-shaped AgNPs, indicating their limited stability. In contrast, quasi-spherical AgNPs showed no agglomeration and consistent UV-Vis spectra even after 6 months of storage.
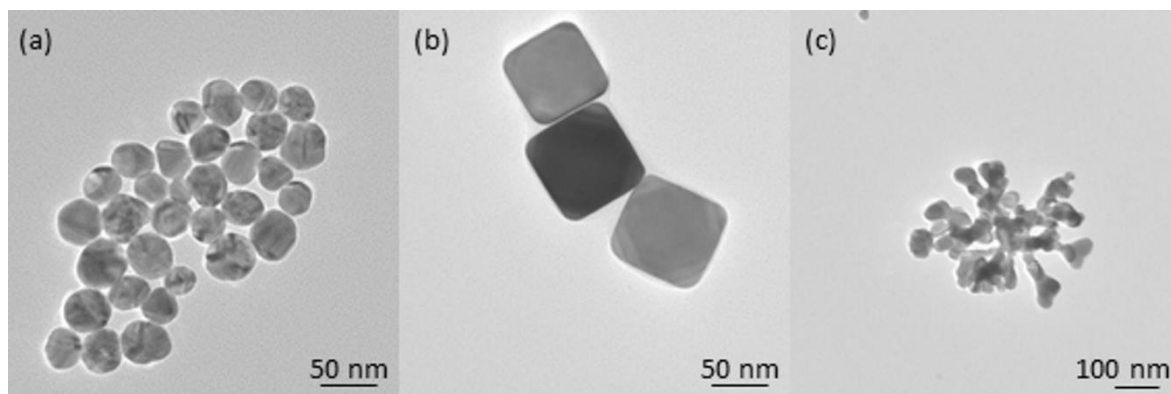
Figure 16. Transmission electron microscopy images of (a) quasi-spherical, (b) cubic, and (c) star-shaped silver nanoparticles.

The suspension stability of AgNPs is influenced by various parameters, such as the synthesis and the chemicals employed, surface area, zeta potential, number of particles, temperature and light exposure[296]. It appears that the different surface chemistries and the larger surface areas of AgNP cubes and stars relative to quasi-spheres resulted in an unfavourable higher energy state of the particles, ultimately promoting particle agglomeration. The agglomeration of AgNP cubes and stars might also be facilitated by their increased contact areas between particles. Furthermore, a negative zeta potential causes electrostatic repulsion preventing particle agglomeration. Hence, the prolonged suspension stability of quasi-spherical AgNPs can be attributed to the higher zeta potential compared to AgNP cubes and stars. As formulation stability is an essential prerequisite for clinical applications, quasi-spherical AgNPs appeared to be the most promising candidates of all prepared particles evaluated in this study.

2.7.2 Cytotoxicity studies

The cytotoxic effect of AgNPs was determined by the LDH assay evaluating the survival of human bronchial epithelial cells (Nuli-1) and macrophages (THP-1) (Figure 17). Following 1 hour exposure to quasi-spherical AgNPs, no toxic effects were observed in both cell lines (80-100% cell viability compared to untreated control). In contrast, cubic AgNPs demonstrated substantial toxicity in Nuli-1 cells resulting in approximately 63% ± 5% cell viability. On the basis of this result, no further cell studies were carried out with cubic AgNPs. Star-shaped AgNPs showed moderate toxicity in Nuli-1 cells (75% ± 4% cell viability), but toxicity was more pronounced in THP-1 cells (52% ± 7% cell viability). Macrophages are the first cells to interact with AgNPs in the human body and are known to readily ingest large amounts of foreign material[297], which can explain the higher toxicity rate in the THP-1 cells. Apart from the cell type, the toxicity of AgNPs also depends on other factors, such as the exposure time, temperature, silver concentration, particle surface charge, surface decorations or the size and shape of AgNPs (which are defined through the AgNP synthesis) and the subsequent reactivity[296,298]. Particle size-dependent cytotoxicity has been described in the literature, for example, by George *et al*.[299] The authors showed that Ag nanospheres of 10 nm induced elevated toxicity in cells compared to Ag nanospheres of 40 nm. It was observed that the smaller the AgNPs, the higher the particle reactivity, and subsequently, the higher the toxic effects. However, the particle shape also directly impacts cytotoxicity. It was reported that cubic AgNPs feature surface defects that increased hazardous effects in cell lines[299]. While Ag nanospheres show relatively few surface defects, other AgNP shapes, such as cubic and star-shaped AgNPs, are known to possess more surface defects facilitating elevated particle reactivity, disruption of biomolecules, and membranolytic effects when in contact with cells[299,300]. Because of the destabilisation of cell membranes, AgNPs with surface defects subsequently induce intracellular oxidative stress, thereby causing reactive oxygen species-mediated cytotoxicity. This can explain the pronounced toxicity of AgNP cubes and stars observed in this study. However, the precise toxicity mechanisms, the role of Ag ion shedding, and the fate of AgNPs were reported to be particle dependent and require further elucidation[301]. Surface coatings have potential to reduce the cytotoxic effects as reported

elsewhere[299], however, this was outside the scope of this study. The toxicity can furthermore depend on the chemicals present in the AgNP dispersion, which in turn depends on the particle synthesis. While AgNP quasi-spheres were produced with exclusively nontoxic agents, chemicals (such as ethylene glycol) required for the synthesis of AgNP cubes and stars could potentially be harmful to cells[302]. To remove those chemicals, dialysis of AgNP cubes and stars was carried out over 5 days. However, the resulting purified AgNPs agglomerated within one week as the stabilising chemicals were removed (data not shown). These results indicated that AgNP cubes and stars are unlikely to be good candidates for clinical applications because of limited stability, toxicity and time-consuming purification processes.
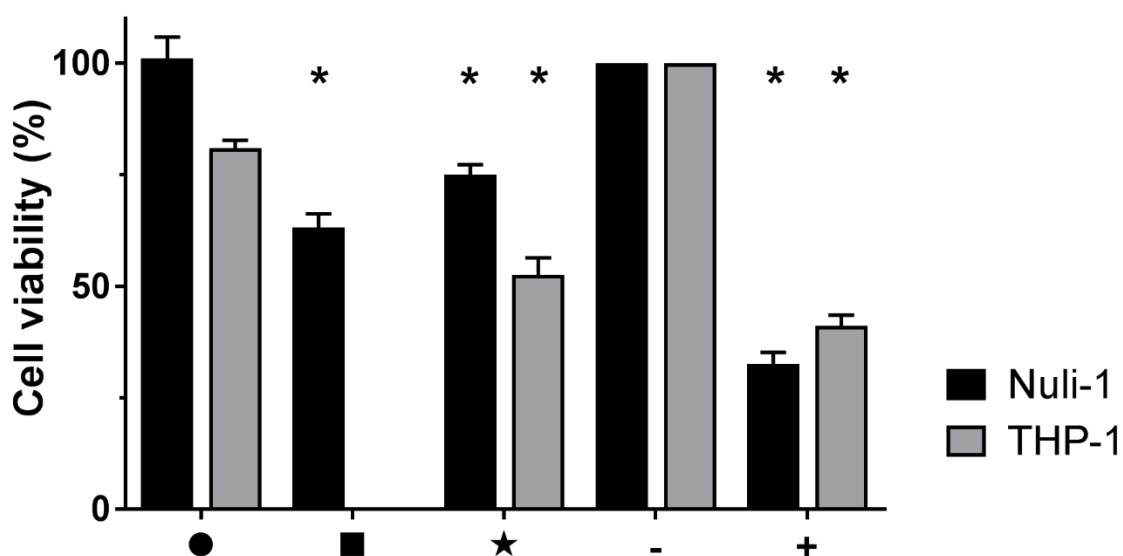
Figure 17. Cell viability (%) of Nuli-1 (black) and THP-1 cells (grey) after 1 hour exposure to quasi-spherical (●), cubic (■) and star-shaped (*) silver nanoparticles, compared to negative (-, untreated) and positive (+, Triton X-100) controls. Data represent the mean ± SD of 3 biological replicates. Statistical comparison to untreated control. * p<0.05

2.7.3 Antibiofilm activity studies

In vitro biofilm assay

The antibiofilm activity of AgNPs was determined *in vitro* by the resazurin viability assay against *S. aureus* biofilms (Figure 18a)[303]. While quasi-spherical AgNPs showed 96% ± 2% biofilm killing (BK), cubic and star-shaped AgNPs showed a substantially lower activity with 75% ± 1% and 79% ± 4% BK, respectively. The moderate antibiofilm activity of AgNP cubes and stars might be due to their larger particle size (70 nm and 140 nm, respectively, Figure 16). Forier *et al*. found that the particle

size was an important parameter contributing to the antibiofilm activity of nanoparticles[289]. The authors reported a threshold of approximately 100-130 nm for latex nanoparticles and liposomes above which particles were not able to penetrate through the biofilm matrix mesh and water channels. This is in line with another study observing enhanced antibiofilm activity for small silica nanoparticles (15 nm) loaded with nitric oxide compared with corresponding larger particles (50 nm and 150 nm)[304]. Moreover, the literature described size-dependent antibacterial effects of AgNPs with an inverse relationship between bactericidal properties and particle size[290]. A reduction in particle size was associated with increased particle reactivity and subsequently elevated antibacterial effects[299]. This suggests that cubic and star-shaped AgNPs in the current study might be too large to penetrate the biofilm matrix and, hence, exhibit only moderate antibiofilm activity, while AgNP quasi-spheres were small enough (40 nm, Figure 16) to reach the bacteria exhibiting enhanced bactericidal effects. In contrast to previous reports, which described no antibacterial activity of AgNP spheres and cubes against *S. aureus* ATCC 25923[298], the present study demonstrated substantial antibiofilm effects of AgNPs quasi-spheres and cubes. The AgNPs used by Holmes *et al.* were similar to the ones utilised in this study in regards to particle shape, size, and zeta potential. The prominent difference in antibacterial activity is thought to rely on the different particle preparation that impacts particle reactivity. In particular, Holmes *et al.* used AgNPs coated with poly-vinylpyrrolidone (PVP)[298] which is known to facilitate particle stability by steric hindrance. While PVP contributes to reduced particle agglomeration, it can also reduce the contact of particles and bacteria leading to diminished antibacterial effects. The absence of this coating could explain the increased reactivity of AgNP quasi-spheres and cubes observed in the present study.

In addition, the antibacterial activity of AgNPs is influenced by various other parameters, such as the exposure time, silver concentration, particle surface charge, surface decorations, or the shape of AgNPs. The latter is defined through, the particle synthesis and is linked to surface defects that facilitate the reactivity of AgNPs[299].

On the basis of the limited antibiofilm activity of cubic and star-shaped AgNPs, the observed moderate cytotoxicity, and the stability concerns of both particle types, cubic and star-shaped AgNPs were not included in further biofilm studies.

Quasi-spherical AgNPs showed substantial antibiofilm activity against both MRSA and *P. aeruginosa* biofilms with 97% ± 1% BK and 98% ± 1% BK, respectively (Figure 18b and c). The particles maintained their antibiofilm activity even after 6 months of storage in the dark at 4°C (results not shown).
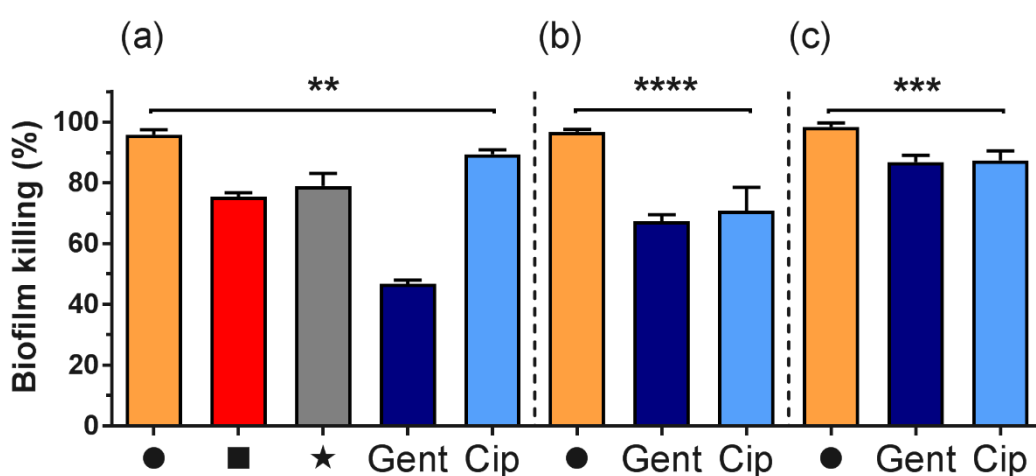
Figure 18. Biofilm killing (%) of (a) S. aureus, (b) MRSA and (c) P. aeruginosa biofilms after exposure to quasi-spherical (●, orange), cubic (■, red) and star-shaped (, grey) silver nanoparticles. Antibiotic controls included 100 µg/ml gentamicin (Gent, dark blue) and 5 µg/ml ciprofloxacin (Cip, light blue). Data represent the mean ± SD of 3 biological replicates. Statistical comparison to Cip.   ** p<0.01   *** p<0.001   **** p<0.0001*

In vivo biofilm assay

The *in vivo* antibiofilm activity of quasi-spherical AgNPs was assessed in an infection model in the round worm *C. elegans*. This is an established model for biofilm infections and for the *in vivo* determination of antibiofilm activity and toxicity of compounds[305,306]. A vast number of worms can be used for studies improving statistical significance, while the approval of an ethics committee is not required. Moreover, low-maintenance costs and ease of handling make *C. elegans* an attractive *in vivo* model[305]. The lifespan of infected but untreated worms is strain- and species-dependent as bacteria can form biofilms, produce virulence factors and excrete diffusible toxins over time that

are nematocidal[305]. When infected with *S. aureus,* 72% ± 3% worm survival was observed after 3 days (Figure 19a). This is considered to be caused by *S. aureus* exotoxin production which is known to diminish the lifespan of worms[306]. The survival rate was significantly increased when infected worms were exposed to quasi-spherical AgNPs (89% ± 4% survival, $p<0.0001$) with a survival rate that was comparable to uninfected worms (Figure 19a). Similarly, the survival rate in MRSA infected worms was significantly higher after AgNP treatment (86% ± 3% survival after 3 days, $p<0.01$) compared to untreated, infected worms (73% ± 2% survival after 3 days) and was comparable to uninfected worms (Figure 19b). Uninfected worms that were exposed to AgNPs showed a similar survival rate to uninfected, untreated worms (data not shown) indicating no toxicity of AgNPs to worms.

Consistent with the increased worm survival, AgNPs actively interfered with *S. aureus* and MRSA reducing the biofilm- and toxin-mediated worm killing. This was further confirmed by a significantly decreased number of *S. aureus* CFU recovered after treatment ($4.5 \pm 1.0 \times 10^3$ CFU per worm *versus* $3.3 \pm 0.6 \times 10^4$ CFU per worm in the absence of treatment, $p<0.05$, Figure 20a). Also for MRSA infected worms, a reduced microbial load was observed after treatment ($3.9 \pm 0.3 \times 10^3$ CFU per worm *versus* $4.7 \pm 2.0 \times 10^4$ CFU per worm in the absence of treatment, Figure 20b). The bacterial load of uninfected worms was determined as a control. No CFU per worm was recovered, indicating that the reduction of CFU can be entirely attributed to the antibacterial effect of AgNPs.

In *P. aeruginosa* infected worms, the survival rate was only 23% ± 4% after 3 days (Figure 19c). *P. aeruginosa* is known to produce hydrogen cyanide inducing an elevated paralytic worm killing[307,308], which could explain the low survival number in this study. The AgNP treatment significantly increased the survival of *P. aeruginosa* infected worms to 86% ± 2% after 3 days ($p<0.0001$), which is comparable to untreated worms. In line with this, the microbial load recovered after treatment was significantly reduced ($5.4 \pm 6.1 \times 10^3$ CFU per worm *versus* $1.1 \pm 2.3 \times 10^6$ CFU per worm in the absence of treatment, $p<0.05$, Figure 20c).

The disposition of AgNPs in nematodes was investigated by X-ray tomography (Figure 21). The observed accumulation of AgNPs in the intestinal tract suggested that AgNPs were taken up by the nematodes during feeding. Moreover, elevated concentrations of AgNPs in the gut could explain antimicrobial effects exerted within the worms consistent with the reduced CFU after AgNP treatment.
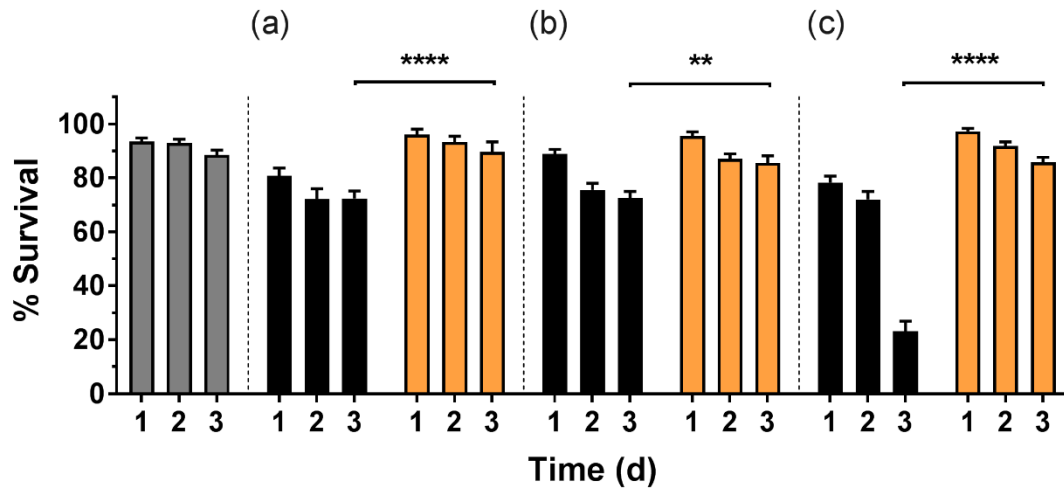
*Figure 19. C. elegans survival (%) over 3 days of uninfected worms (grey bars); worms infected (black bars) with (a) S. aureus, (b) MRSA, or (c) P. aeruginosa; and infected worms treated with quasi-spherical silver nanoparticles (orange bars). Data represent the mean ± SEM of at least 12 biological replicates.   ** p<0.01   **** p<0.0001*
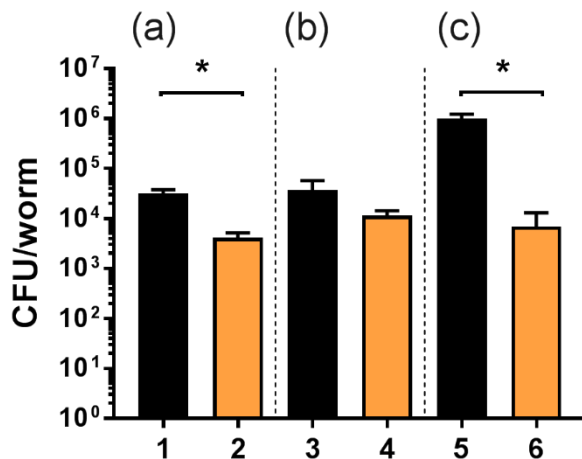
*Figure 20. Colony forming units (CFU) per C. elegans worm after 3 days of infection (black bars: 1, 3, 5) with (a) S. aureus, (b) MRSA, or (c) P. aeruginosa and treatment with quasi-spherical silver nanoparticles (orange bars: 2, 4, 6). Data represent the mean ± SEM of at least 12 biological replicates.   * p<0.05*
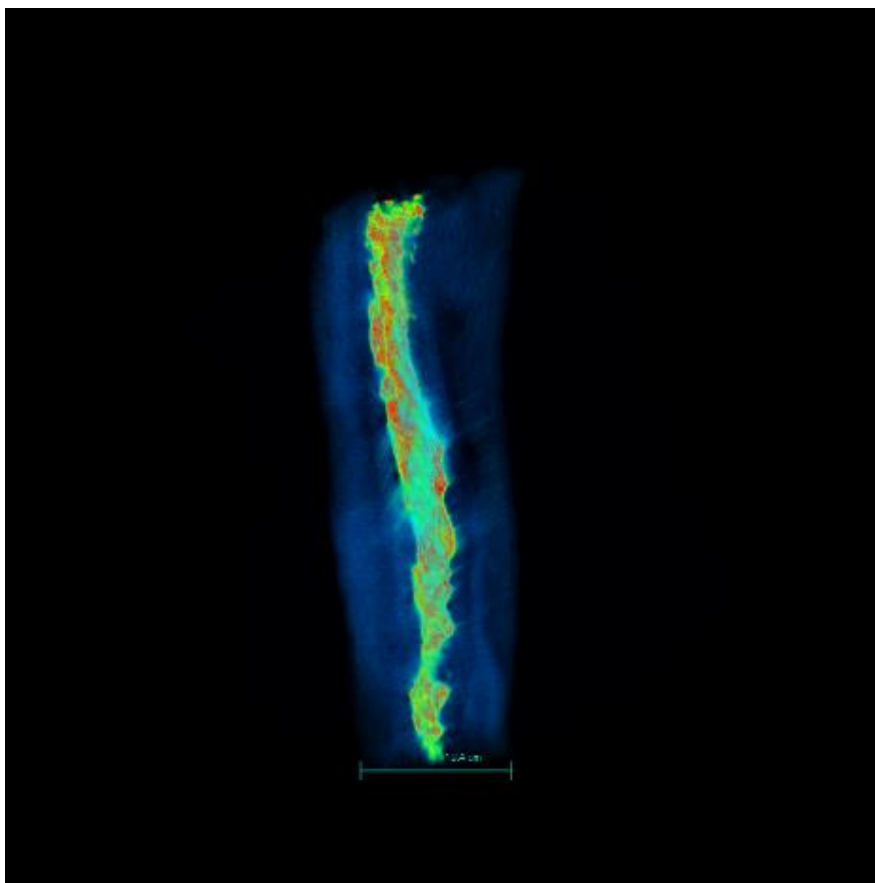
Figure 21. X-ray tomography of C. elegans (blue) after exposure to silver nanoparticles, scale bar is 19.4 μm. Colours reflect various intensities of AgNPs (green = high density of AgNPs, orange = low density of AgNPs, blue = iodine staining, absence of AgNPs).

While the antimicrobial properties of silver-based medicines have been known since ancient times[309], the utilisation of silver as therapeutic agent in modern medicine needs to be thoroughly evaluated to ensure therapeutic activity, safety and feasibility for clinical practice. To minimise the risk of systemic side effects such as argyria, topical treatments represent an interesting alternative route to oral drug delivery.

The antibiofilm activity of AgNPs relies on different mechanisms of action. First, AgNPs can interfere with the bacterial cell membrane, altering its function, blocking the energy transfer and inhibiting vital bacterial enzymes[310]. Second, after penetration into bacteria, AgNPs can inhibit the function of bacterial proteins, disturbing respiration and other cellular pathways such as translation and transcription[290]. Furthermore, AgNPs can react with sulphur and phosphorous groups, for example, when binding to DNA, thereby inhibiting bacterial growth[290]. On the basis of the redox potential of

AgNPs, silver ions can be released that produce toxic oxygen radicals causing additional damage to bacteria[311]. Ultimately, the broad antibacterial activity is based on the combined effect of both AgNPs and silver ions[312].

According to the literature, the shape of AgNPs can influence the antibacterial effect. Triangular AgNPs were shown to be more active against *E. coli* than AgNP spheres, rods or silver ions[288]. However, the time-consuming synthesis, the potentially toxic chemicals involved and the subsequent time-consuming purification of triangular AgNPs limit their clinical application.

While surface modifications using surfactants, polymers, polysaccharides, or other molecules have been suggested to enhance the antimicrobial activity of AgNPs[313-317], these can also reduce the antibacterial activity, for example, by steric hindrance, preventing particle adhesion to bacteria[298,311]. This suggests that surface coatings need to be carefully selected. Any surface modifications require additional steps potentially increasing the manufacturing time, synthesis complexity, and costs. The focus of this study was to establish a simple, robust, fast, and low-cost production of colloidal AgNPs for clinical use as a topical treatment. The current data indicated that quasi-spherical AgNPs show elevated antibiofilm activity without being toxic to human cell lines. Applied, for example, as a nasal rinse for chronic rhinosinusitis patients, quasi-spherical AgNPs could represent a promising alternative treatment strategy to antibiotics or a beneficial adjuvant[216] in antibiotic therapy to increase bacterial susceptibility.

2.8 Conclusion

The synthesis, characterisation, cytotoxicity and antibiofilm activity of quasi-spherical, cubic and star-shaped AgNPs were successfully described and evaluated in the context of utilisation for clinical practice as a topical treatment. While cytotoxicity and short-term stability limit the use of both cubic and star-shaped AgNPs, quasi-spherical AgNPs were simple, fast and cost-effective to produce, were not toxic to two cell lines and showed substantial antibiofilm activity against clinically relevant biofilms even after 6 months of storage. A dispersion containing quasi-spherical

AgNPs has potential to be used as a topical treatment for biofilm-related infections, for example, as a nasal rinse for chronic rhinosinusitis patients.

2.9 Acknowledgements

2.10 Funding information

2.11 Disclosures

None to declare.

2.12 Supplementary data

S 1. Experimental details for the synthesis of quasi-spherical silver nanoparticles.

		Final concentration
MQ water	237.5 ml	
Ascorbic acid 0.1 M	250 µl	
MQ water	6.25 ml	
Sodium citrate 1% wt.	1.25 ml	
AgNO₃ 1% wt.	1.25 ml	0.03 mg/ml Ag
KI 300 µM	50 µl	
TOTAL VOLUME	246.55 ml	

S 2. Experimental details for the synthesis of cubic silver nanoparticles.

		Final concentration
NaSH 3 mM in EG	1.2 ml	
HCl 3 mM in EG	10 ml	
PVP 20 mg/ml in EG	25 ml	
CF₃COOAg 282 mM in EG	8 ml	1.6 mg/ml Ag
EG	100 ml	
TOTAL VOLUME	144.2 ml	

S 3. Experimental details for the synthesis of star-shaped silver nanoparticles.

		Final concentration
Hydroxylamine (50% w/w in water) 60 mM	500 µl	
NaOH 50 mM	500 µl	
AgNO₃ 1 mM	9 ml	0.09 mg/ml Ag
Sodium citrate 1% wt.	100 µl	
TOTAL VOLUME	10.1 ml	
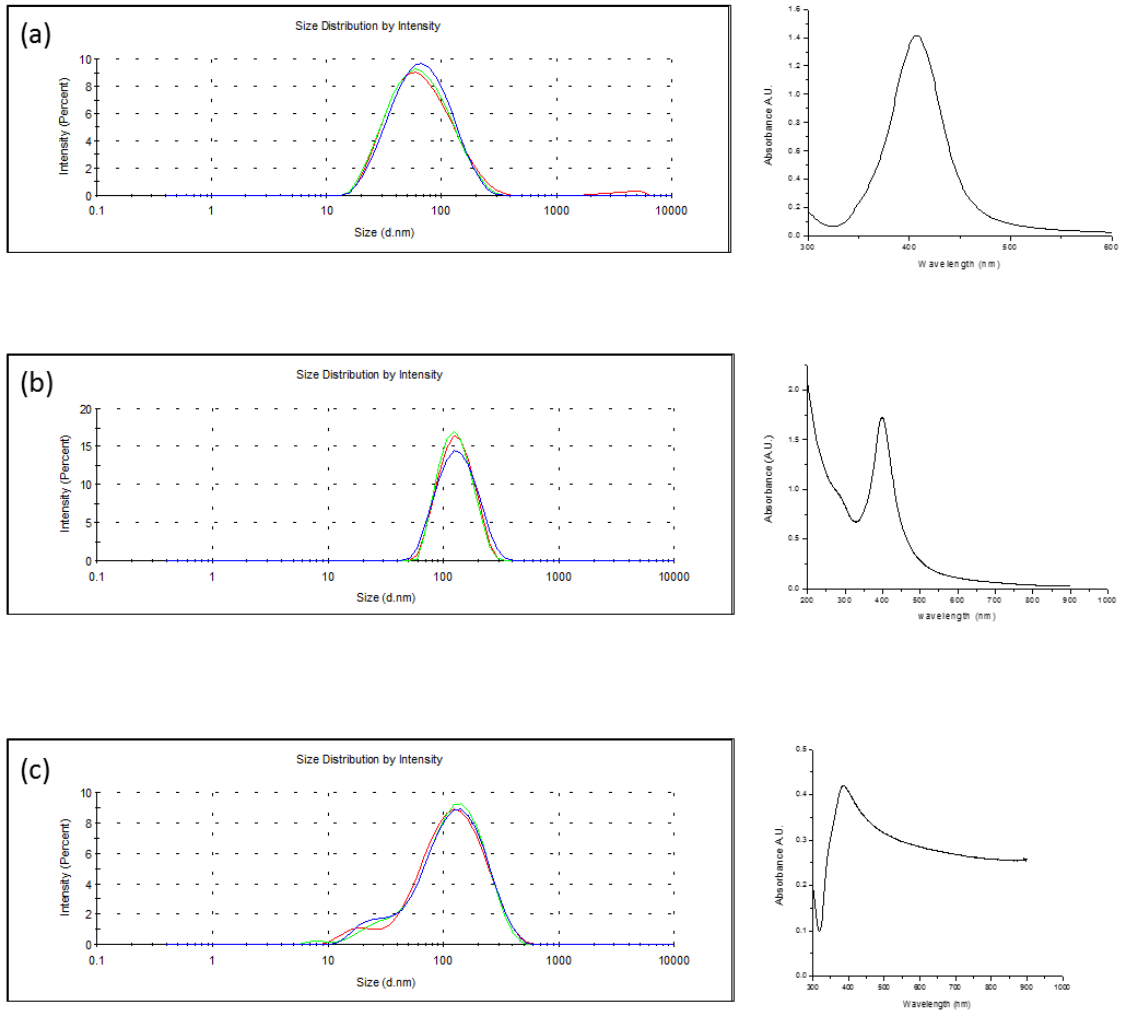
S 4. DLS (left) and UV-Vis (right) measurements of (a) quasi-spherical, (b) cubic, and (c) star-shaped silver nanoparticles.

Chapter 3.

The use of gallium based therapeutics against bacterial biofilms

3.1 Rationale

The underlying rationale of gallium-based therapeutics lies in disrupting bacterial iron metabolism. By following a "Trojan Horse" strategy, gallium compounds target bacterial iron acquisition systems for intracellular uptake and subsequently interfere with essential cellular processes to exhibit antibacterial and antibiofilm activity.

3.2 Iron metabolism

Iron plays a crucial role for bacterial growth, survival and pathogenesis; it is an important redox catalyst for various cellular processes and many metabolic enzymes and receptors rely on iron, including respiratory proteins that utilise iron for ATP production. Furthermore, iron is essential for DNA synthesis, respiration, protection against reactive oxygen species (ROS) and for biofilm formation[218,318], which depends on iron levels higher than needed for vegetative growth[319]. However, iron homoeostasis is a critical key factor of both host and bacteria, as the redox potential of iron poses a toxicity risk. Iron acts as a catalyst of the Fenton reaction that generates toxic hydroxyl radicals causing lipids, DNA and protein damage in cells[218]. Therefore, iron levels and distribution must be controlled.

As virtually all human pathogens rely on iron, the human body established iron-withholding methods as defence mechanism. Nutritional immunity is a process by the innate immune system limiting free iron levels in the host. The majority, i.e. 80% of human iron is complexed intracellularly to haem[45,320,321] (iron propoporphyrin IX) as part of haemoglobin inside erythrocytes, or stored in cells as ferritin. Iron can also be extracellularly bound to the protein transferrin, incorporated into redox co-factors of metalloenzymes or bound to the protein lactoferrin on mucosal surfaces[218].

3.3   Iron acquisition and homoeostasis by pathogens

To overcome the iron-withholding mechanisms, bacteria established various systems to sequester iron from the host and their direct environment. These strategies are fundamental for bacterial virulence and vary according to the host niche, the microbe's preference for an intracellular or extracellular lifestyle and the preference for specific iron sources. Sources include free iron, iron chelates with host compounds or iron complexed to haem/haemoglobin[218]. Bacteria can switch between multiple iron acquisition systems or utilise them simultaneously to sequester sufficient amounts of iron. These systems include

(i)   Siderophore production. Bacteria release molecules that bind iron with high affinity in the host and bring them into the microbe via special energy-dependent membrane receptors. Thereafter, siderophore-iron can be released by enzymatic degradation or reduction. Over 500 siderophores have been discovered to date for a wide range of Gram-positive and Gram-negative bacteria, including *S. aureus, P. aeruginosa, Escherichia coli, Mycobacterium tuberculosis, Legionella pneumophila, Bacillus anthracis*, among others[217,218].

(ii)   Xenosiderophore uptake. Bacteria utilise iron from siderophores not produced by the microbe, e.g. desferoxamine B, a siderophore from *Streptomyces pilosus*. This is known for *S. aureus, Vibrio vulnificus, Yersinia enterocolitica*, among others[322].

(iii)   Stealth siderophore production. The innate immune system can capture bacterial siderophores by siderocalin (an "anti-siderophore") as a counteracting defence during infections. Bacteria evolved to produce structural modified siderophores that are not recognised by siderocalin, thereby circumventing the immune defence. This is known for *B. anthracis, Salmonella typhimurium*, among others[323,324].

(iv)   Haem acquisition systems. Bacteria liberate haem from haemoglobin/erythrocytes, bind haem to cell surface receptors and carry it across the cell wall and membrane(s) via transport proteins into the cytoplasm. This can also be achieved by haemophores

(haem-chelating molecules, similar to siderophores) that bind haem extracellularly with high affinity to deliver it into the cytoplasm. Once inside bacteria, haem can be cleaved by oxygenases to release free iron or utilised as intact molecule acting as cofactor for cytochromes, haem-containing enzymes like catalases and other haemoproteins. This is known for a wide range of Gram-positive and Gram-negative bacteria, including *S. aureus, S. epidermidis, Haemophilus influenzae, B. anthracis, P. aeruginosa, M. tuberculosis*, among others[325].

(v)   Transferrin or lactoferrin receptors. Bacteria can directly obtain iron from the host by liberating iron from transferrin or lactoferrin before intracellular uptake. This is known for *Neisseria meningitidis, M. tuberculosis*, among others[326].

(vi)   Reductive iron transporters, which is primarily seen in pathogenic fungi[327].

It is known that pathogenic fungi, such as *Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus*, rely on iron and evolved iron acquisition systems similar to those found in bacteria. Pathogenic fungi are able to produce siderophores, sequester haem and use reductive uptake to acquire iron. For the latter, fungi are equipped with enzymes on their surface that reduce ferric to ferrous iron, thereby removing host chelating molecules to enable the passage into fungi via iron transporters. In some fungi, these transporters can also utilise ferritin as iron source. Iron homoeostasis facilitates germination, resistance to oxidative stress and virulence of pathogenic fungi. Moreover, some human parasites require iron for pathogenesis and virulence, like human hookworms that cause iron deficiency anaemia, or plasmodium species that cause malaria[328,329].
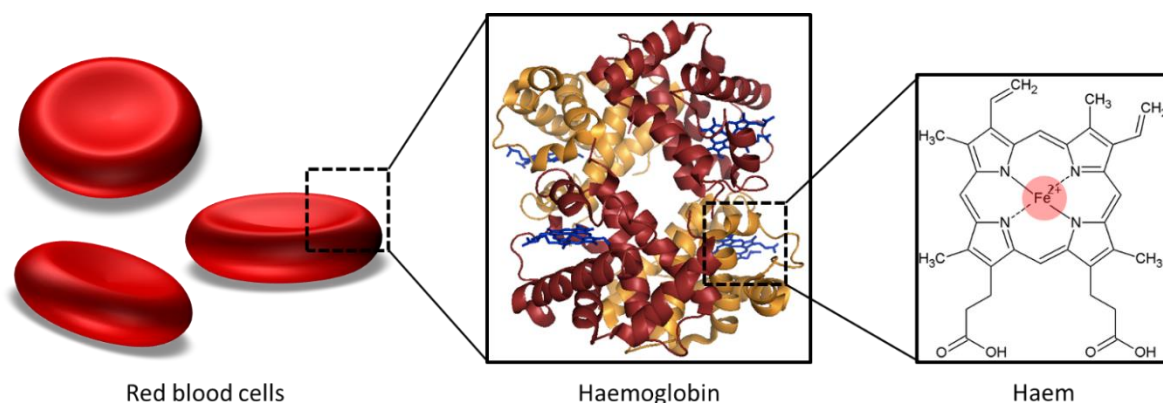
Figure 22. Red blood cells contain haemoglobin which comprises of alpha and beta chains (yellow and red) surrounding 4 haem molecules (blue).

Most pathogens acquire the majority of iron through haem (iron protoporphyrin IX), which is the most abundant iron source in the human body as part of haemoglobin inside erythrocytes (Figure 22)[45,320,321]. One red blood cell contains 280 million haemoglobin molecules, each holding 4 haem molecules, providing over 1 billion iron atoms[330]. During an infection large amounts of haem accumulate at the infection site due to the lysis of erythrocytes, thereby, iron becomes available for pathogens. Haem is the preferred iron source for *S. aureus* that is equipped with various systems, such as the iron-regulated surface determinant system, to sequester iron/haem from the host[321,331]. Haem acquisition systems are also known for other species, such as the Has and Phu haem uptake systems in *P. aeruginosa*[332]. However, bacterial iron uptake, storage and efflux need to be coordinated to facilitate homoeostasis and prevent toxicity.

Once inside bacteria, iron is liberated from haem to be utilised for cellular processes or stored to foster bacterial survival and virulence, while excess iron/haem and their toxic metabolites are removed via efflux pumps[218,331]. There are three types of iron storage proteins in bacteria, i.e. ferritin, bacterioferritin and Dps proteins. While the latter contribute to bacterial virulence, the first and second promote growth in times of iron deficiency, neutralise redox radicals and foster bacterial survival[218]. When the intracellular iron storage capacity is reached, iron/iron-complexes and their toxic metabolites are eliminated via efflux systems like ABC transporters, as seen in the

haem-regulated transporter HrtAB system of *S. aureus*[333]. Thereby bacteria master to regulate iron acquisition, distribution and detoxification for a survival advantage.

3.4 Bacterial iron metabolism as therapeutic target

As virtually all pathogens rely on iron for growth and virulence, the bacterial iron metabolism represents a target for medical intervention strategies. Antimicrobials can interact with the iron metabolism in several ways, including the inhibition of the siderophore biosynthesis, e.g. by para-aminosalicylic acid which is used for the treatment of tuberculosis[218]. Another way to interfere with iron metabolism is by utilising sideromycins, which are synthetic siderophores covalently bound to antibiotics. Thereby MICs can be reduced and the activity of antibiotics, such as beta-lactams and fluoroquinolones potentiated. This approach was shown to be active e.g. against *P. aeruginosa in vitro* and *in vivo*[334]. Apart from that, "Trojan Horse" compounds that utilise bacterial iron uptake systems to sneak into bacteria and inhibit essential cellular iron-dependent pathways are a further alternative strategy. Various therapeutic approaches, including the utilisation of iron mimicking drugs, such as gallium compounds[335,336], and haem analogues, like gallium-protoporphyrin[303,337], are under investigation with promising results *in vitro* and *in vivo*.

3.5 Gallium

Gallium is the element most similar to iron in regards to chemical behaviour. Similarities can be seen in the ionic radius, electronegativity, ionisation potential and electron affinity that affect chemical bond formation[338]. Therefore, gallium can be utilised as iron analogue, being able to occupy iron binding sites on receptors and proteins, thereby disrupting iron-dependent processes (Figure 23). By following a "Trojan Horse" strategy, gallium containing compounds exploit bacterial iron acquisition systems for internal uptake or penetrate through the bacterial cell wall[217,218]. On a

cellular level, gallium competes with iron and interferes with its absorption, metabolism and activity, thereby disrupting vital iron-dependent processes[217].
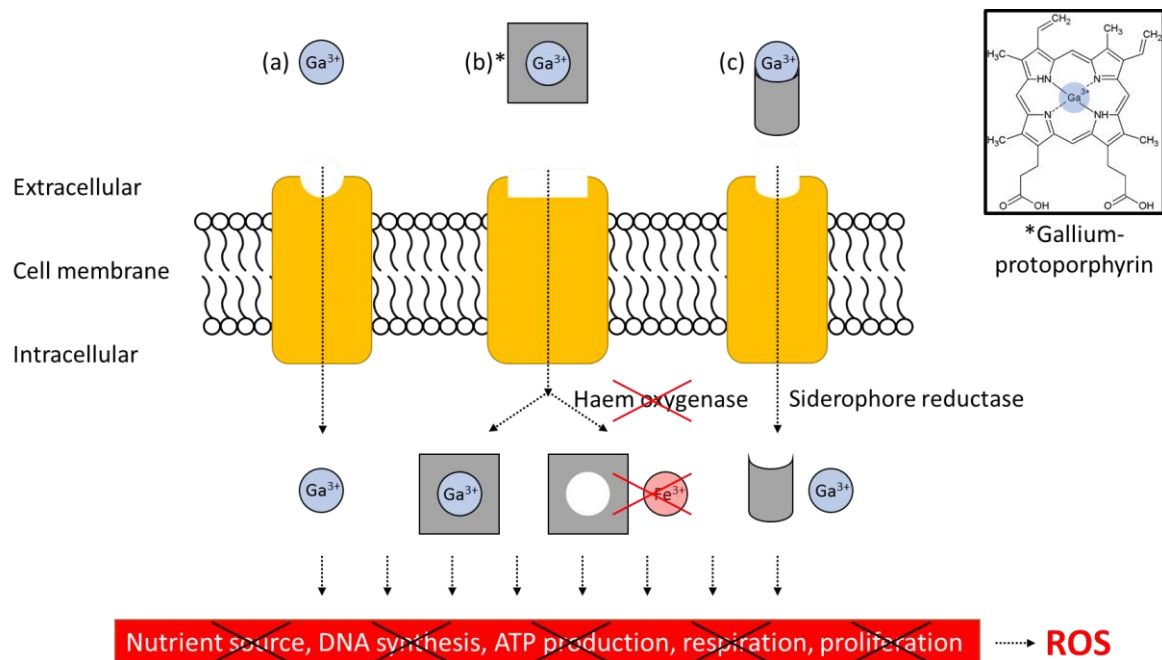
Figure 23. Gallium as iron analogue and gallium-protoporphyrin as haem analogue can be taken up by bacterial iron transporters (yellow) as (a) free gallium, (b) gallium-protoporphyrin and (c) siderophore-gallium. Inside bacteria gallium inhibits vital cellular pathways, ultimately leading to the generation of reactive oxygen species (ROS) that induce cell death.

Biological systems may not be able to separate gallium from iron. Most importantly gallium only exists as Ga^{+3} ion, thus, in contrast to iron (which exists as Fe^{+2} and Fe^{+3}) gallium is unable to transfer electrons and cannot induce redox reactions. Therefore, gallium cannot be utilised as enzyme-cofactor, impeding respiration, DNA synthesis and bacterial proliferation, and generating ROS[339,340]. Gallium is able to block iron receptors and inhibit biological utilisation of Fe^{+2}/Fe^{+3} ions which are toxic if not bound to proteins or small molecules[341].

3.6   Resistance

Resistance against antimicrobials relies on various drug- and microbe-dependent mechanisms, including target modification, drug inactivation, restricted drug penetration and increased efflux (as discussed in chapter 1).

Development of resistance against gallium compounds is expected to be low as they utilise a different mode of action. Based on selective pressure if bacteria down-regulate receptors to reduce the binding and uptake of gallium, the uptake of iron would simultaneously diminished, being counterproductive for survival[342,343].

3.7   Gallium therapeutics

Gallium can be delivered as simple salt or complex/conjugate with other molecules. Substantial antimicrobial effects were shown against various pathogens *in vitro* and *in vivo*, however, the antibiofilm activity appears to be species and strain dependent and constrained by iron levels[335,344-348].

3.7.1   Gallium salts

Gallium nitrate [$Ga(NO_3)_3$] is a salt whose pharmacokinetic profile and low-to-moderate toxicity are already known in humans[349]. $Ga(NO_3)_3$ is mainly excreted by the kidneys and can potentially cause nephrotoxicity; however, in a clinical phase I trial this was dependent on the dose, treatment duration and the way $Ga(NO_3)_3$ was delivered[349]. It was shown that renal toxicity can be reduced by a low drug dosage and longer infusion times. Apart from that, $Ga(NO_3)_3$ was shown to exhibit antibiofilm activity against *S. aureus*[350]. It was reported that it is antibacterial even against stationary phase bacteria, which are usually found in the centre of biofilms and which frequently show reduced susceptibility to antibiotics[335]. $Ga(NO_3)_3$ furthermore inhibited growth in planktonic and biofilm bacteria of *P. aeruginosa in vitro*, as well as *in vivo* in murine models of acute lethal

pneumonia and chronic airway biofilm infections[335]. In contrast, $Ga(NO_3)_3$ failed to exhibit substantial *in vitro* antibiofilm activity in clinical *Burkholderia cepacia* complex isolates from cystic fibrosis patients[344]. The antibiofilm properties of $Ga(NO_3)_3$ were strain and species dependent and when grown with iron levels similar to cystic fibrosis conditions *B. cepacia* complex isolates were resistant to $Ga(NO_3)_3$.

Another salt, gallium maltolate was reported to exhibit antimicrobial activity in a mouse model for burn wound infections, showing higher efficacy than $Ga(NO_3)_3$ and preventing the systemic spreading of *P. aeruginosa*. In the same model, a substantially reduced wound colonisation of *S. aureus* and *A. baumanii* was also demonstrated[336].

While the *in vitro* and *in vivo* antimicrobial activity of gallium salts are interesting to note, their administration is challenging. After oral intake, gallium forms poorly soluble precipitates in the gastrointestinal tract limiting the bioavailability and antimicrobial activity in humans[338].

3.7.2   Gallium complexes

Complexes like gallium citrate offer improved stability as gallium ions are chelated, thereby hydrolysis under physiological pH can be prevented[351]. Several *in vitro* studies showed broad antibacterial and antibiofilm activity of gallium citrate against Gram-positive and Gram-negative bacteria, as well as low drug resistance[217,346]. Gallium citrate was also shown to impair biofilm formation of a multidrug resistant strain of *Klebsiella pneumoniae* on wound dressings and soft tissue *in vivo*, thereby improving wound healing[347].

Siderophore-gallium complexes are another strategy to introduce gallium to bacteria. Siderophores are small molecules released by bacteria under iron limitation to sequester iron outside bacteria for internal uptake. Staphyloferrin A, a siderophore produced by *S. aureus*, was synthesised and loaded with gallium. This complex, however, failed to exhibit antimicrobial effects against MRSA[217]. Siderophores of other bacteria including *B. cepacia*, *P. aeruginosa* and *Mycobacterium tuberculosis*

were synthesised and complexed to gallium. Despite antibacterial effects *in vitro*, the synthetic gallium-siderophores did not exceed the activity of simpler gallium complexes like gallium citrate and studies about the activity against biofilms are lacking[217,352].

Gallium complexed to the xenosiderophore/iron chelator desferoxamine (produced by *Streptomyces pilosus*) was shown to kill stationary phase *P. aeruginosa* and mature biofilms *in vitro*[345]. However, other studies demonstrated higher uptake of gallium citrate by *P. aeruginosa*, resulting in more pronounced antibacterial effects than gallium-desferoxamine[346].
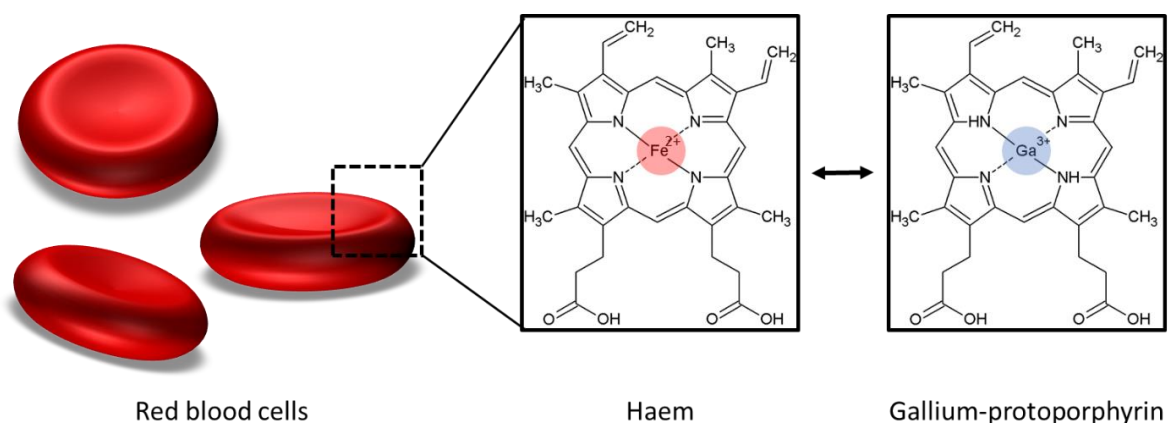
Figure 24. Structural similarities between haem and gallium–protoporphyrin that is able to mimic haem as iron source.

Other gallium complexes with antimicrobial properties include synthetic haem analogues. These compounds show structural three-dimensional similarity to haem, comprising of the protoporphyrin ring and a metal central ion, therefore known as non-iron metalloporphyrins[353-355]. The potency of metalloporphyrins is determined by the metal ion and by the presence of haem acquisition systems in pathogens. Being most similar to haem, gallium-protoporphyrin IX (GaPP, Figure 24) demonstrated strong activity against a plethora of microbes, including MRSA[337,356,357]. *S. aureus* is known to favour haem as preferred nutrient source[321] and is equipped with more active haem uptake systems than siderophore-based systems, facilitating the use of haem analogues like GaPP[358]. Furthermore, GaPP showed substantial growth inhibition of several Gram-positive and

Gram-negative pathogens, including *P. aeruginosa, A. baumanii,* multidrug resistant strains of *Mycobacterium abscessus* and even the malaria parasite *Plasmodium falciparum*[337,356,357]. It was observed that GaPP exhibits stronger antimicrobial and antibiofilm activity than other gallium complexes and gallium salts *in vitro* and *in vivo*, even against highly virulent strains with established $Ga(NO_3)_3$ tolerance[348,356]. However, GaPP's low water solubility and modest dose-depending toxicity pose challenges for its applicability as therapeutic drug[337,359].

There is potential for the development of synthetic gallium complexes with improved aqueous solubility and potency, and for incorporation of gallium into smart drug delivery systems that overcome solubility and toxicity concerns.

3.8 Pharmaceutical formulations

Although gallium compounds show antimicrobial and antibiofilm activity against various bacteria, they have not found their way into clinical practice. There are two FDA approved gallium formulations on the market that are used as diagnostic agents in cancer therapy; these are the radioactive labelled gallium injections (i) gallium citrate Ga 67, FDA approved since 1976, and (ii) gallium dotatate Ga 68, FDA approved since 2016. The formulation Ganite (a gallium nitrate-citrate injection for cancer-related hypercalcaemia, FDA approved from 2003 to 2014) showed antimicrobial activity *in vitro*, however, this effect appeared species and strain dependent. Concentrations higher than the recommended dose would be required for a broad antibiofilm effect, raising toxicity concerns[359].

There is one gallium citrate formulation in the drug development pipeline, trade named Panaecin by Aridis Pharmaceuticals. Clinical phase I studies commenced in November 2011 in the USA and indicated promising treatment efficacy in cystic fibrosis-associated respiratory tract infections after intravenous administration of gallium citrate. In January 2017, Aridis announced to progress into clinical phase IIa studies.

In light of emerging antibiotic resistance the utilisation of gallium compounds may be an interesting approach for future therapies. So far, gallium citrate appeared to be a promising candidate, however, intravenous injection is the only application route to date. Other delivery options such as an inhalation for cystic fibrosis would benefit the applicability of the treatment.

3.9   Combination therapies with gallium

A multi-pronged approach using compounds with different modes of action offers the potential of additive or synergistic antimicrobial effects, while possibly reducing the risk for emerging resistance compared with monotherapy. Certain gallium compounds have the ability to potentiate antibiotics and combining antimicrobial compounds with gallium may lead to promising antibiofilm strategies.

As an example, liposomes containing gallium and gentamicin showed significant antibiofilm activity against *P. aeruginosa in vitro*,[360] indicating gallium's potential to augment antimicrobial effects of antibiotics.

Desferoxamine-gallium (DFO-Ga) applied together with gentamicin (Gent) showed a synergistic effect in the antibiofilm activity against *P. aeruginosa in vitro* against reference strains and clinical isolates from wounds and cystic fibrosis sputum, as well as in an *in vivo* model of eye infections[345]. In a rabbit model of keratitis DFO-Ga-Gent reduced the severity of the *P. aeruginosa* infection and accelerated wound healing.

Another interesting approach is to combine multiple compounds that disrupt bacterial iron metabolism at different levels. The following chapters will highlight the multi-pronged strategy of the iron chelator deferiprone and the haem analogue gallium-protoporphyrin and explore their antibiofilm activity against clinically relevant pathogens and SCVs.

3a. Publication: "Mind "De GaPP": *in vitro* efficacy of deferiprone and gallium-protoporphyrin against *Staphylococcus aureus* biofilms"

Statement of authorship

Title of Paper	Mind "De GaPP": *in vitro* efficacy of deferiprone and gallium-protoporphyrin against *Staphylococcus aureus* biofilms
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Richter K, Ramezanpour M, Thomas N, Prestidge CA, Wormald P-J, Vreugde S. Mind "De GaPP": in vitro efficacy of deferiprone and gallium-protoporphyrin against Staphylococcus aureus biofilms. International Forum of Allergy and Rhinology. 2016;6(7):737-43.

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Name of Principal Author (Candidate)	Katharina Richter		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	7/4/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

    i.   the candidate's stated contribution to the publication is accurate (as detailed above);

    ii.   permission is granted for the candidate in include the publication in the thesis; and

    iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Acquisition of data, analysis and interpretation of data		
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Signature		Date	7/4/2017

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Contribution to the Paper	Critically revising the article		
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Contribution to the Paper	Conception of the project, critically revising the article		
Signature		Date	10/4/2017

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Conception and design of the project, critically revising the article		
Signature		Date	10/4/2017

3a.1 Publication title page

ORIGINAL ARTICLE

Mind "De GaPP": in vitro efficacy of deferiprone and gallium-protoporphyrin against *Staphylococcus aureus* biofilms

Katharina Richter, MSc[1], Mahnaz Ramezanpour, PhD[1], Nicky Thomas, PhD[1,2], Clive A. Prestidge, PhD[2],
Peter-John Wormald, MD[1] and Sarah Vreugde, MD, PhD[1]

Background: Biofilms are clusters of bacteria embedded in a protective matrix that frequently cause failure of medical treatments and increase the risk of recurrent infections. In particular, *Staphylococcus aureus* biofilms are associated with a series of chronic and nosocomial infections that are increasingly resistant to antibiotics. This study proposes a novel intervention strategy targeting the essential iron metabolism for bacterial growth, survival and pathogenesis using the compounds deferiprone (Def) and gallium-protoporphyrin (GaPP).

Methods: *S. aureus* biofilms were challenged with Def/GaPP as single and dual treatments. In vitro anti-biofilm efficacy was assessed by the AlamarBlue viability assay and confocal microscopy. In vitro cytotoxicity of the treatments was examined by the lactate dehydrogenase assay on mouse fibroblast (L929) and human bronchial epithelial cells (Nuli-1).

Results: Def (20 mM) and GaPP (200 μg/mL) monotherapy for 2 hours showed 35% and 74% biofilm removal, respectively, whereas simultaneous Def/GaPP administration showed 55% biofilm removal. In contrast, the consecutive treatment (2 hours Def followed by 2 hours GaPP) achieved 95% biofilm removal. Cytotoxicity studies indicated no cell hazard in all treatments.

Conclusion: This study demonstrated the in vitro efficacy of a novel treatment combination against *S. aureus* biofilms targeting the bacterial iron metabolism. The consecutive Def/GaPP treatment showed significantly enhanced biofilm efficacy than the individual compounds, while being not toxic to 2 cell lines. This novel treatment combination is a promising approach to combat *S. aureus*-associated biofilm infections having high potential for future clinical application. © 2016 ARS-AAOA, LLC.

Key Words:
biofilms; deferiprone; gallium-protoporphyrin IX; iron metabolism; *S. aureus*

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Approximately 99% of bacteria reside in biofilms, which are clusters of bacterial cells, embedded in a self-produced matrix of extracellular polymeric substances.[1–3] The biofilm state is advantageous for bacterial survival because it acts as a protective shield, enabling the bacteria to adapt to hostile environmental conditions, evade the immune system, and ultimately to establish resistance against antimicrobials.[1–10] Bacteria residing in biofilms require up to 1000-fold higher concentrations of antimicrobial treatments than their planktonic (free-floating) counterparts.[2] Therefore, bacterial biofilms represent 1 of the biggest challenges for the medical community. Clinically relevant biofilms are associated with severe and recalcitrant diseases,

3a.2 Article

Mind "De GaPP": *in vitro* efficacy of deferiprone and gallium-protoporphyrin against *Staphylococcus aureus* biofilm[303]

Katharina Richter[1], Mahnaz Ramezanpour[1], Nicky Thomas[3,4], Clive A. Prestidge[3,6], Peter-John Wormald[1], Sarah Vreugde[1]

3a.3 Abstract

Background: Biofilms are clusters of bacteria embedded in a protective matrix that frequently cause failure of medical treatments and increase the risk of recurrent infections. In particular, *Staphylococcus aureus* biofilms are associated with a series of chronic and nosocomial infections that are increasingly resistant to antibiotics. This study proposes a novel intervention strategy targeting the essential iron metabolism for bacterial growth, survival and pathogenesis using the compounds deferiprone (Def) and gallium-protoporphyrin (GaPP).

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Conclusion: This study demonstrated the *in vitro* efficacy of a novel treatment combination against *S. aureus* biofilms targeting the bacterial iron metabolism. The consecutive Def-GaPP treatment showed significantly enhanced biofilm efficacy than the individual compounds, while being not

toxic to 2 cell lines. This novel treatment combination is a promising approach to combat *S. aureus*-associated biofilm infections having high potential for future clinical application.

3a.4 Introduction

Approximately 99% of bacteria reside in biofilms, which are clusters of bacterial cells, embedded in a self-produced matrix of extracellular polymeric substances[16,19,361]. The biofilm state is advantageous for bacterial survival because it acts as a protective shield, enabling the bacteria to adapt to hostile environmental conditions, evade the immune system, and ultimately to establish resistance against antimicrobials[13,14,16,18-21,39,43,361]. Bacteria residing in biofilms require up to 1000-fold higher concentrations of antimicrobial treatments than their planktonic (free-floating) counterparts[19]. Therefore, bacterial biofilms represent one of the biggest challenges for the medical community. Clinically relevant biofilms are associated with severe and recalcitrant diseases, including endocarditis, osteomyelitis, chronic wounds, and chronic rhinosinusitis[140,362-364]. Within this context, *Staphylococcus aureus* represents one of the most notorious bacteria causing superficial, invasive, chronic and nosocomial infections[16,43,365].

Iron is crucial for bacterial growth, survival, and pathogenesis[319,366]; hence, the iron metabolism represents a potential target for novel intervention strategies. In *S. aureus* iron also plays a major role in cellular processes such as DNA synthesis, energy generation, as a cofactor for multiple bacterial enzymes, and as a protection against reactive oxygen species (ROS)[45,46]. Because 99.9% of host iron is intracellularly bound, thus limiting its availability, *S. aureus* has established robust mechanisms to sequester iron from its host[45,320,331]. Haem represents the most abundant iron source within the human body (as iron protoporphyrin IX) and is the preferred iron source for *S. aureus*[321]. The lysis of erythrocytes during an infection triggers the local accumulation of haem; hence, iron becomes available as a nutrient source[367].

Deferiprone (Def) is an iron chelator approved by the U.S. Food and Drug Administration for the treatment of thalassemia major[368]. Def is capable of chelating free iron at the ratio 3:1, hence

90

balancing an iron overload in the blood. Moreover, Def can capture iron from the environment around bacteria, causing a depletion of iron as a nutrient source[369-373].

Gallium-protoporphyrin IX (GaPP) belongs to the family of non-iron metalloporphyrins and has antibacterial properties[337]. The compound shows structural similarity to haem due to the shared tetrapyrrole backbone structure; therefore, GaPP can mimic haem as a preferred iron source of bacteria[337]. Once inside the bacterial cell, non-iron metalloporphyrins preserve their structure and show antibacterial effects by interfering with essential cellular pathways in the cytoplasm and in the plasma membrane[45]. It was shown that GaPP is the most potent non-iron metalloporphyrin against several planktonic bacteria[337]; however, the knowledge of how GaPP affects bacterial biofilms is far from complete and potential treatment combinations with other compounds have not been investigated yet.

In this study, the *in vitro* efficacy of a novel treatment combination of Def and GaPP against *S. aureus* biofilms was evaluated by targeting the bacterial iron metabolism. The treatment combination was hypothesised to be superior to the treatment with the individual compounds. Various concentrations of single compounds as well as different concentrations and incubation times of the dual treatment were assessed. Furthermore, *in vitro* toxicity studies with two cell lines were carried out.

3a.5 Materials and methods

3a.5.1 Antibiofilm efficacy studies

Culture conditions and biofilm formation

Single colonies of *S. aureus* ATCC 25923 (clinical isolate; American Type Culture Collection (ATCC), Manassas, VA, USA) were immersed in 0.9% saline (Sigma Aldrich, Steinheim, Germany), adjusted to 1.0 ± 0.1 McFarland units (3×10^8 colony forming units/ml), and diluted 1:15 in nutrient broth (Oxoid, Basingstoke, United Kingdom). Then 150 µl of the diluted bacterial suspension was added

to each well of a 96-well microtiter plate (Costar; Corning Incorporated, Corning, NY, USA) and incubated at 37°C for 72 hours on a rotating platform (3D Gyratory Mixer; Ratek Instruments, Boronia, Australia) at 70 rpm. After 20 hours incubation medium was removed and fresh nutrient broth added.

Biofilm treatment

Biofilms were washed with phosphate buffered saline (PBS; Sigma Aldrich) to remove planktonic cells, followed by exposure to (1) deferiprone (3-hydroxy-1,2-dimethylpyridin-4(1*H*)-one; Sigma Aldrich), (2) gallium-protoporphyrin IX (Frontier Scientific, Logan, UT, USA), (3) a concurrent combination of both compounds; and (4) a consecutive combination of both compounds. Def was dissolved in water and GaPP was dissolved in an aqueous 0.03% solution of Tween 80 (Sigma Aldrich); i.e. above the critical micelle concentration of Tween 80 (0.015%). After 2 hours treatment incubation at 37°C on a rotating platform, a second washing step followed to remove the excess treatments. Controls included wells with the bacterial suspension in broth (i.e. 100% bacterial growth, negative control), wells with pure broth (i.e. 0% bacterial growth, positive control), and wells with the bacterial suspension in broth and Tween 80 as a control for GaPP dilutions. All experiments were performed as 3 independent experiments with at least 4 wells per treatment.

Viability assessment

Bacterial viability was assessed on the washed biofilm using the AlamarBlue cell viability assay[201,295]. After drying, 200 µl of a freshly prepared 10% AlamarBlue dilution (Life Technologies, Scoresby, Australia) in nutrient broth was added to each well. Plates were incubated protected from light at 37°C on a rotating platform for up to 7 hours. The fluorescence was measured hourly on a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany). The method was set at bottom reading

and fluorescence was measured at $\lambda_{excitation}$ = 530 nm/$\lambda_{emission}$ = 590 nm. Maximum intensities were typically reached after 6 hours incubation and used for quantification according to Equation 1:

$$\% \, \mathrm{BK} = \frac{F_C - F_T}{F_C} \times 100\% \qquad (1)$$

Bacterial viability was determined as the percentage of biofilm killing (% BK), where F_C represents the fluorescence intensity of the controls (i.e. 100% bacterial growth) and F_T indicates the maximum intensity of the treatments. Both F_C and F_T were corrected by the intensity of background (i.e. 0% bacterial growth).

Confocal microscopy

S. aureus biofilms were grown and treated on Falcon Culture Slides (In Vitro Technologies, Noble Park, Australia) at the same conditions as previously stated. Treated biofilms were washed twice followed by fixation with 5% glutaraldehyde (Sigma Aldrich) for 30 minutes at room temperature. LIVE/DEAD BacLight staining (SYTO 9/propidium iodide; Life Technologies) was incubated on biofilms for 15 minutes in the dark prior to analysis by confocal laser scanning microscopy (LSM 710; Carl Zeiss, Jena, Germany) using a 40×/0.6 objective. The excitation/emission wavelengths of the BacLight staining were 485/530 nm and 485/630 nm, respectively.

3a.5.2 Minimal inhibitory concentration

Minimal inhibitory concentration (MIC) values were assessed for Def and GaPP against planktonic *S. aureus* using standard methods[374]. Treatment concentrations ranged from 0.1 to 20 mM Def and 0.1 to 25 μg/ml GaPP. The MIC was determined as the lowest drug concentration preventing bacterial growth.

3a.5.3 Cytotoxicity studies

Cell culture

L929 cells (mouse fibroblast cell line) and Nuli-1 cells (human airway epithelial cell line) were obtained from ATCC. The Nuli-1 cell line was cultured in serum-free Bronchial Epithelial Cell Growth Medium (Lonza, Mount Waverley, Australia) containing growth factors, cytokines, and supplements. L929 cells were grown in Dulbecco's Modified Eagle Medium (Sigma Aldrich) supplemented with glutamine and 10% foetal bovine serum. Cells were maintained in a fully humidified incubator with 5% CO_2 at 37°C.

Cell viability test

The cytotoxicity of Def and GaPP were determined using a lactate dehydrogenase (LDH) bioassay kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). Briefly, cells were seeded at 1×10^4 in 100 µl culture medium per well in 96-well flat-bottom plates and incubated for 24 hours (37°C, 5% CO_2) to allow attachment. To assess the toxicity of the dual treatment, cells were first exposed to 100 µl of Def (20 mM) for 2 hours, followed by a washing step and treatment with 100 µl GaPP (100, 200, 300, 400, and 500 µg/ml) for 2 hours. In addition, cells were separately treated with either Def or GaPP for 2 hours. Negative controls included untreated cells and positive controls included cells exposed to Triton X-100 (Sigma Aldrich). The OD was measured at 490 nm. All experiments were performed as 3 independent experiments with at least 4 wells per treatment.

3a.5.4 Statistics and software

Results were statistically analysed using one-way analysis of variance (ANOVA) and unpaired t test (GraphPad Prism version 6.00 for Windows; GraphPad Software, La Jolla, CAL, USA) and statistical significance was assessed at the 95% confidence interval.

3a.6 Results

3a.6.1 Antibiofilm efficacy studies

Single treatments

S. aureus biofilms were treated for 2 hours with Def ranging from 0.5 to 50 mM. As depicted in Figure 25a, the percentage of biofilm killing significantly increased with higher Def concentrations and plateaued at 34% for concentrations above 20 mM.

S. aureus biofilms were exposed for 2 hours to GaPP ranging from 1 to 200 µg/ml demonstrating significant efficacy in a dose-dependent manner. The highest concentration (200 µg/ml) killed 77% of *S. aureus* biofilms (Figure 25b).

S. aureus biofilms were additionally treated with the individual compounds for 4 hours. The results suggested no significantly different efficacy of the 4 hour treatments compared to the 2 hour treatments (data not shown).
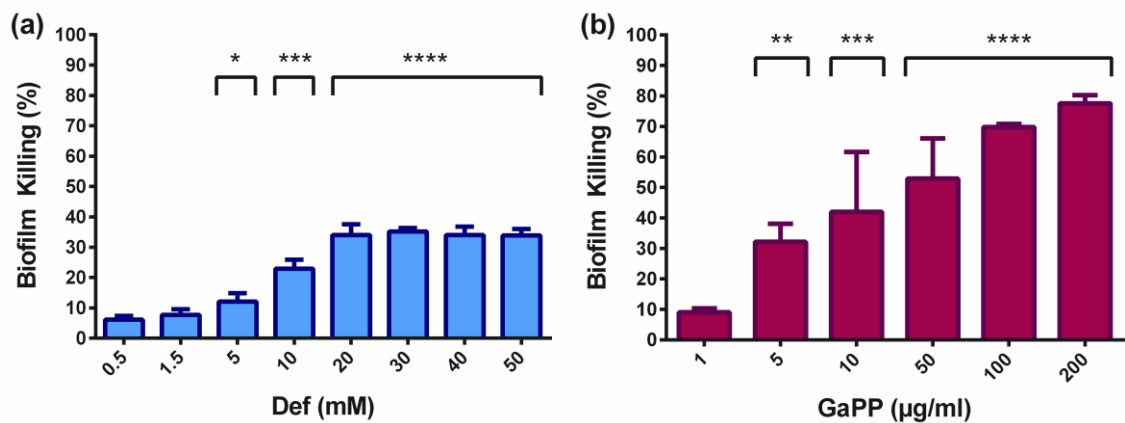
*Figure 25. S. aureus biofilm killing (%) by (a) deferiprone (Def in mM) and (b) gallium-protoporphyrin (GaPP in µg/ml) relative to untreated control. Data are the mean of 3 biological repeats ± SD.   * p<0.05   ** p<0.01   *** p<0.001 **** p<0.0001*

Dual treatments: Def + GaPP

For the evaluation of a treatment combining Def and GaPP, the drug concentrations were chosen in accordance with the most effective single treatment concentrations (i.e. 20 mM Def, 200 µg/ml GaPP). The efficacy of the dual treatment against *S. aureus* biofilms was assessed in two ways.

First, both compounds were applied simultaneously as a concurrent treatment and incubated for 2 hours. The concurrent treatment removed 48% of *S. aureus* biofilms as shown in Figure 26. However, this result was not significantly higher than the single Def treatment, but significantly lower than the single GaPP treatment ($p < 0.05$) and the consecutive, dual treatment ($p < 0.01$).

Second, *S. aureus* biofilms were exposed to a consecutive treatment of 2 hours Def followed by 2 hours GaPP. This consecutive treatment eradicated *S. aureus* biofilms almost completely (94% biofilm killing, Figure 26) and was significantly different from the single treatments of Def ($p < 0.001$) and GaPP ($p < 0.05$) and the concurrent dual treatment ($p < 0.01$).
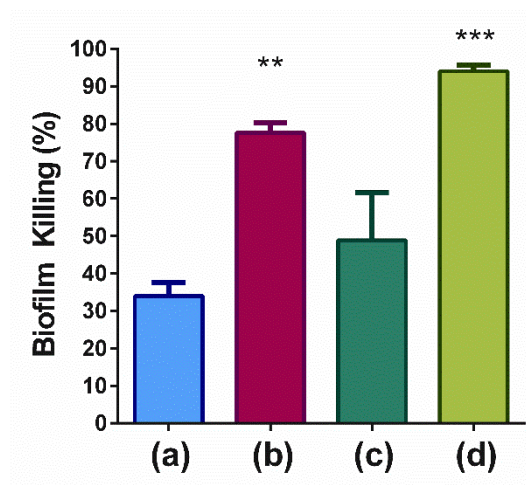
*Figure 26. Gallium-protoporphyrin (GaPP in µg/ml) and dual treatments compared to deferiprone (Def in mM) treatment. (a) Def 20, (b) GaPP 200, (c) concurrent Def 20 + GaPP 200, (d) consecutive Def 20 + GaPP 200. Data are the mean of 3 biological repeats ± SD.   ** p<0.01   *** p<0.001*

Confocal microscopy

S. aureus biofilms were grown and treated on Falcon Culture Slides followed by LIVE/DEAD BacLight staining and visualisation with confocal laser scanning microscopy. Representative images of the different treatments are shown in Figure 27.
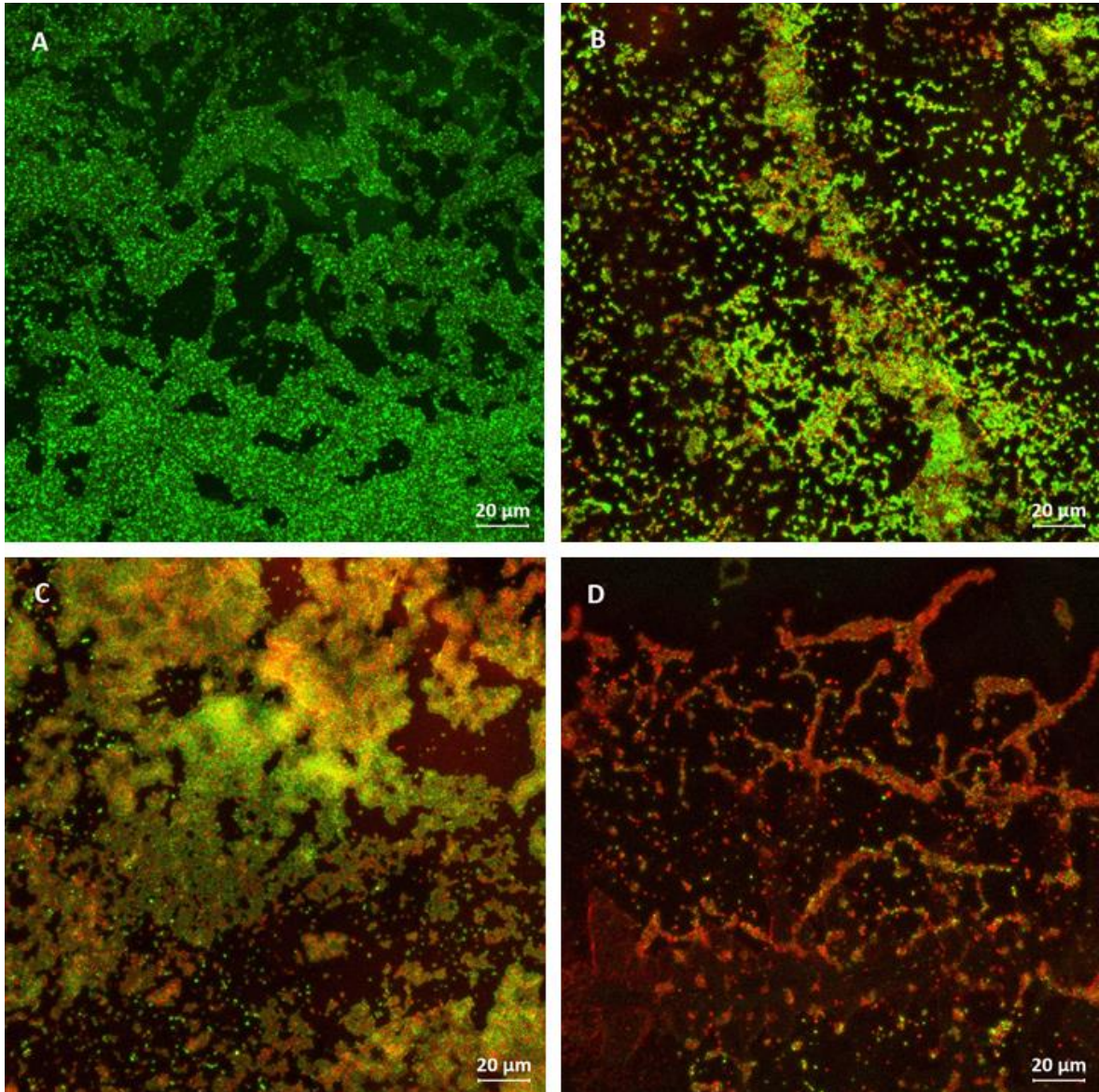
Figure 27. Visualisation of treated S. aureus biofilms using LIVE/DEAD BacLight staining and confocal laser scanning microscopy. (A) untreated control, (B) deferiprone (Def) 20 mM, (C) gallium-protoporphyrin (GaPP) 200 µg/ml, (D) consecutive Def 20 mM + GaPP 200 µg/ml.

Performance of consecutive, dual treatments

S. aureus biofilms were exposed to consecutive treatments of 2 hours Def (0.5 to 20 mM) followed by 2 hours GaPP (1 to 200 µg/ml). In Figure 28 the biofilm killing was compared to the single GaPP treatments represented by the horizontal bar (i.e. biofilm killing of 9% for GaPP 1, 32% for GaPP 5, 41% for GaPP 10, 52% for GaPP 50, 69% for GaPP 100, and 77% for GaPP 200, adapted from Figure 25b). Overall, consecutive treatments exceeded the antibiofilm efficacy of GaPP. In particular when low GaPP concentrations were used, the consecutive treatments appeared to have a synergistic effect (Supplementary data S 5).
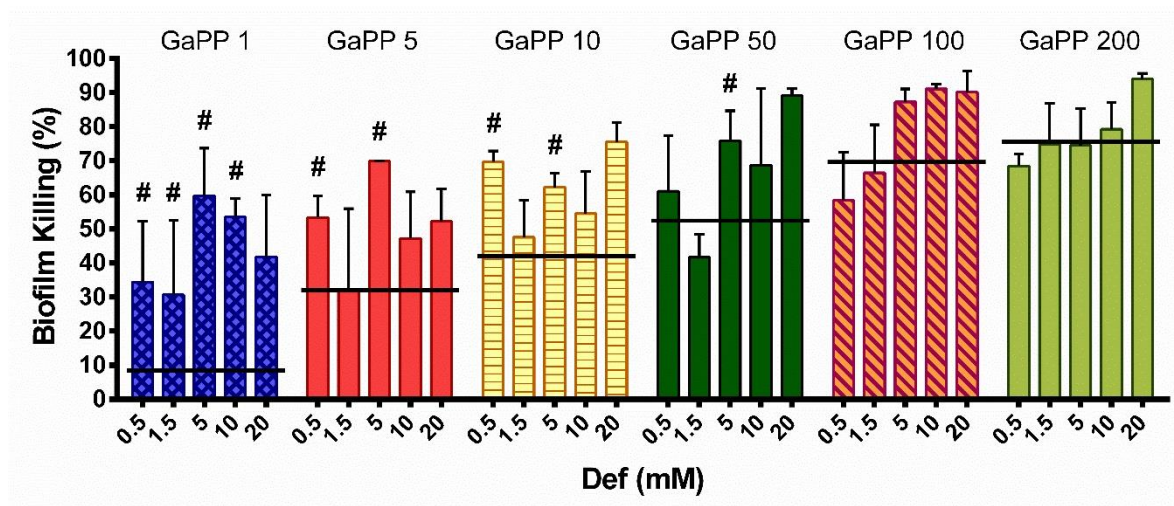
Figure 28. Consecutive treatments of deferiprone (Def in mM) and gallium-protoporphyrin (GaPP in µg/ml) compared to single GaPP treatment (horizontal bars). Data are the mean of 3 biological repeats ± SD.   # potential synergistic effects.

Consecutive, dual treatments with prolonged Def exposure

The influence of prolonged Def incubation during a consecutive treatment was assessed. Eight different treatment combinations were chosen as model treatments. Figure 29 compares the efficacy of the model treatments according to two different Def incubation times (i.e. 2 hours *versus* 8.5 hours) prior to 2 hours GaPP treatment. The prolonged initial Def treatment significantly enhanced the performance against *S. aureus* biofilms (85% biofilm killing) when low Def (up to 1.5 mM) and GaPP (up to 5 µg/ml) concentrations were used.
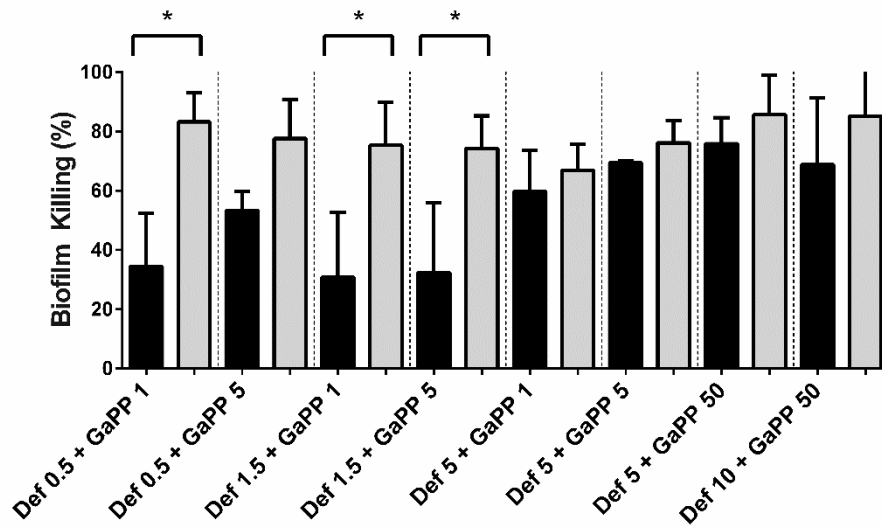
*Figure 29. Comparison of consecutive treatments with different deferiprone (Def in mM) exposure: 2h Def followed by 2h gallium-protoporphyrin (GaPP in µg/ml) (black) versus 8.5h Def followed by 2h GaPP (grey). Data are the mean of 3 biological repeats ± SD.   * p<0.05*

3a.6.2 Minimal inhibitory concentration

The MIC of Def was evaluated at 2 mM (14.4 µg/ml) and the MIC of GaPP was 0.6 µg/ml against planktonic *S. aureus*.

3a.6.3 Cytotoxicity studies

Single treatments

Induction of cell hazard was determined by the LDH assay. Def (20 mM) as a single treatment was tested on L929 and Nuli-1 cell lines. No statistically significant difference was observed. Similarly, single treatment with GaPP had no significant effect on either cell lines at concentrations ranging from 100 to 400 µg/ml. Only 500 µg/ml GaPP induced cell toxicity on both cell lines (data not shown).

Consecutive, dual treatment

L929 cells were not sensitive to any of the tested concentrations in consecutive treatments with 2 hours Def and 2 hours GaPP (Figure 30). In the Nuli-1 cell line, treatment with Def and GaPP reduced viability only at 500 µg/ml GaPP (Figure 30).
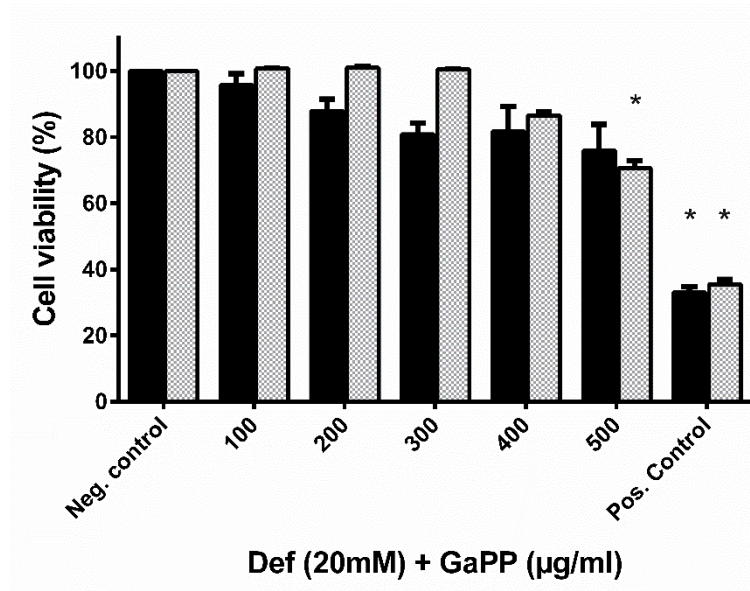
*Figure 30. Cell viability (%) of L929 (black) and Nuli-1 (grey) cells compared to untreated controls after a consecutive deferiprone (Def) and gallium-protoporphyrin (GaPP) treatment. Data are the mean of 3 biological repeats ± SD. * p<0.05*

3a.7 Discussion

In this study a novel treatment combination interfering with the bacterial iron metabolism was examined using Def and GaPP. Both drugs have been assessed individually in the literature[45,319,337,354,371,372]; however, to the best of our knowledge, there has been no report on a treatment combining Def and GaPP.

The treatment with Def and GaPP is based on the rationale to target the bacterial iron metabolism because iron is essential for bacterial growth and pathogenesis, particularly for *S. aureus*[319,366].

In line with previous reports, the individual treatment with Def and GaPP demonstrated a dose-dependent antibiofilm effect; however, none of the individual compounds was able to eradicate *S. aureus* biofilms (Figure 25). The antimicrobial activity of Def is likely due to iron chelation and

concomitant nutrient deprivation, as reported elsewhere[369,373,375]. The antimicrobial activity of GaPP is considered to be due to the interference with cellular pathways inside bacterial cells[45,337,354].

Importantly, the *in vitro* efficacy of a dual, consecutive Def and GaPP treatment revealed superior efficacy, as shown by the almost complete eradication of *S. aureus* biofilms, compared to both the individual compounds and the dual, concurrent treatments (Figure 26). This finding might be explained by mechanistic effects of each individual compound[45,46,321] working synergistically together as illustrated in Figure 31.
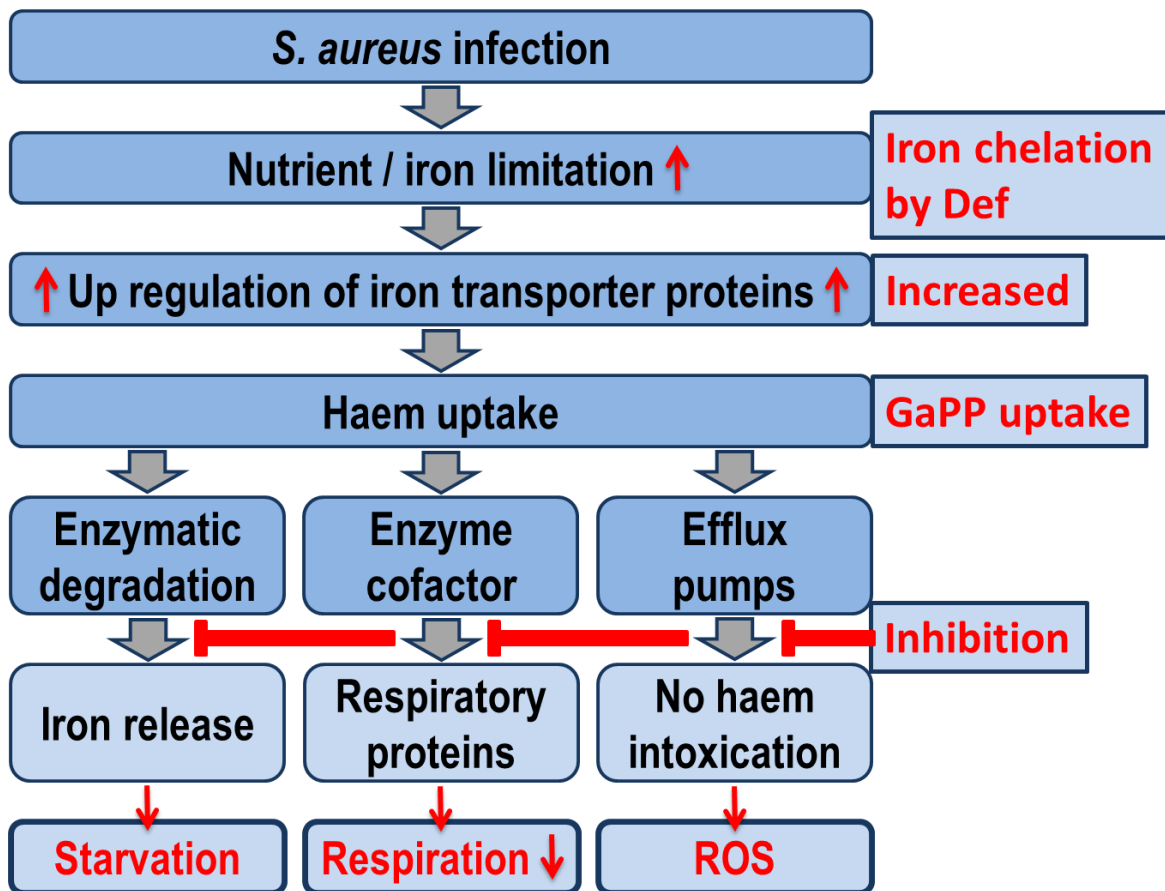
Figure 31. Iron metabolism in S. aureus and interference by deferiprone (Def) and gallium-protoporphyrin (GaPP) treatment (red). Reactive oxygen species (ROS)

Def as an iron chelator can trigger iron deprivation in *S. aureus*. In response, the bacteria upregulate iron transporter proteins in order to sequester iron from any available iron source in the environment[45,46]. When GaPP is subsequently administered the iron-deprived bacteria recognise the haem ring as their preferred iron source[45,355]. Once inside the bacteria GaPP interferes with

essential bacterial pathways leading to starvation, limited respiration, and introduction of ROS, ultimately killing the bacteria[45].

Namely, GaPP lacks the oxidation potential of haem, because the gallium ion only exists in the +3 oxidation state, whereas iron is found as Fe+2 and Fe+3. Consequently, after treatment with GaPP, respiratory proteins including membrane-bound cytochromes are incapable of transferring electrons for adenosine triphosphate production, resulting in limited respiration and contributing to the production of ROS[45]. Furthermore, GaPP cannot be cleaved by bacterial enzymes, hence precluding nutrient/iron release and inducing starvation[45,321]. Moreover, efflux pumps play an important role in the haem homoeostasis of *S. aureus*[45]. Inhibition of the efflux pumps by GaPP could provoke haem accumulation in *S. aureus*, catalysing the generation of toxic oxygen radicals and subsequent DNA and protein damage.

Def and GaPP appeared most effective when the compounds were applied as a consecutive treatment. This is hypothesised to be due to the latency for upregulation of iron transporter proteins after Def treatment. The increased presence of iron transporters would subsequently augment and accelerate GaPP uptake into bacterial cells. In contrast, a concurrent treatment lacks the initial time for upregulation of iron transporter proteins by Def, hence less GaPP could enter bacterial cells. This might explain the lower efficacy of the concurrent treatment compared to the consecutive treatment. Furthermore, an interaction of Def and GaPP during concomitant treatment cannot be ruled out. The planar tetrapyrrole structure of GaPP enables the access of Def from two sides, facilitating Def interaction (chelation) with the central gallium ion, hence decreasing the antimicrobial activity of the entire GaPP molecule.

The time lag required for the upregulation of iron transporters is also supported by the improved efficacy of the Def/GaPP following an extension of the Def incubation time to 8.5 hours while maintaining the consecutive treatment time with GaPP at 2 hours. Despite the lower concentrations of Def (0.5 to 1.5 mM) higher efficacies of the consecutive treatments were observed with

prolonged Def exposure. It appears that upregulation of iron transporters by Def is a fundamental step for a highly efficient consecutive treatment combination with GaPP[46,331].

3a.8 Conclusion

In conclusion, the consecutive treatment of Def and GaPP represents a nontoxic and highly efficient novel treatment strategy to control *S. aureus* biofilms *in vitro*. Further studies are on the way to investigate the *in vitro* efficacy against various other bacteria including clinical isolates, to determine the efficacy and safety *in vivo* and to optimise the treatment for clinical application.

3a.9 Acknowledgements

3a.10 Funding information

3a.11 Disclosures

None to declare.

3a.12 Supplementary data

S 5. S. aureus biofilm killing (BK in %) by single and consecutive treatments of deferiprone (Def in mM) and gallium-protoporphyrin (GaPP in µg/ml). Equation used: BK single Def + BK single GaPP = BK Def-GaPP Theory. The potential effect of the consecutive treatment has been classified as (i) synergistic, if difference is >10%, (ii) additive, if difference is between 10% and -10%, or (iii) negative, if difference is <-10%. Data are the mean of 3 biological repeats.

Consecutive treatment	BK (%) by single		BK (%) by Def + GaPP		Difference (%)	Effect
	Def	GaPP	Theory	Measured		
Def 0.5 + GaPP 1	6.09	9.04	15.13	34.43	19.30	**Synergistic**
Def 0.5 + GaPP 5	6.09	32.18	38.27	53.34	15.07	**Synergistic**
Def 0.5 + GaPP 10	6.09	41.99	48.08	69.65	21.57	**Synergistic**
Def 0.5 + GaPP 50	6.09	52.90	58.99	61.14	2.15	Additive
Def 0.5 + GaPP 100	6.09	69.86	75.95	58.37	-17.58	Negative
Def 0.5 + GaPP 200	6.09	77.61	83.7	68.53	-15.17	Negative
Def 1.5 + GaPP 1	7.65	9.04	16.69	30.69	14.00	**Synergistic**
Def 1.5 + GaPP 5	7.65	32.18	39.83	32.32	-7.51	Additive
Def 1.5 + GaPP 10	7.65	41.99	49.64	47.59	-2.05	Additive
Def 1.5 + GaPP 50	7.65	52.90	60.55	41.74	-18.81	Negative
Def 1.5 + GaPP 100	7.65	69.86	77.51	66.52	-10.99	Negative
Def 1.5 + GaPP 200	7.65	77.61	85.26	74.94	-10.32	Negative
Def 5 + GaPP 1	12.07	9.04	21.11	59.67	38.56	**Synergistic**
Def 5 + GaPP 5	12.07	32.18	44.25	69.86	25.61	**Synergistic**
Def 5 + GaPP 10	12.07	41.99	54.06	62.15	8.09	Additive
Def 5 + GaPP 50	12.07	52.90	64.97	75.77	10.80	**Synergistic**
Def 5 + GaPP 100	12.07	69.86	81.93	87.29	5.36	Additive
Def 5 + GaPP 200	12.07	77.61	89.68	74.50	-15.18	Negative
Def 10 + GaPP 1	22.88	9.04	31.92	53.58	21.66	**Synergistic**
Def 10 + GaPP 5	22.88	32.18	55.06	47.21	-7.85	Additive
Def 10 + GaPP 10	22.88	41.99	64.87	54.46	-10.41	Negative
Def 10 + GaPP 50	22.88	52.90	75.78	68.72	-7.06	Additive
Def 10 + GaPP 100	22.88	69.86	92.74	91.05	-1.69	Additive
Def 10 + GaPP 200	22.88	77.61	> 100	79.25	-20.75	Negative
Def 20 + GaPP 1	34.00	9.04	43.04	41.80	-1.24	Additive
Def 20 + GaPP 5	34.00	32.18	66.18	52.22	-13.96	Negative
Def 20 + GaPP 10	34.00	41.99	75.99	75.64	-0.35	Additive
Def 20 + GaPP 50	34.00	52.90	86.9	89.21	2.31	Additive
Def 20 + GaPP 100	34.00	69.86	> 100	90.24	-9.76	Additive
Def 20 + GaPP 200	34.00	77.61	> 100	94.03	-5.97	Additive

3b. Publication: "A topical hydrogel with deferiprone and gallium-protoporphyrin targets bacterial iron metabolism and has antibiofilm activity"

Statement of authorship

Title of Paper	A topical hydrogel with deferiprone and gallium-protoporphyrin targets bacterial iron metabolism and has antibiofilm activity
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Richter K, Thomas N, Claeys J, McGuane J, Prestidge CA, Coenye T, Wormald P-J, Vreugde S. A topical hydrogel with deferiprone and gallium-protoporphyrin targets bacterial iron metabolism and has antibiofilm activity. Antimicrobial Agents and Chemotherapy. Online first 10 April 2017, DOI: 10.1128/aac.00481-17

Principal Author

Name of Principal Author (Candidate)	Katharina Richter		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	7/4/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	10/4/2017

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Conception and design of the project, critically revising the article		
Signature		Date	10/4/2017

3b.1 Publication title page

A Topical Hydrogel with Deferiprone and Gallium-Protoporphyrin Targets Bacterial Iron Metabolism and Has Antibiofilm Activity

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ABSTRACT Many infectious diseases are associated with multidrug-resistant (MDR) bacteria residing in biofilms that require high antibiotic concentrations. While oral drug delivery is frequently ineffective, topical treatments have the potential to deliver higher drug concentrations to the infection site while reducing systemic side effects. This study determined the antibiofilm activity of a surgical wound gel loaded with the iron chelator deferiprone (Def) and the heme analogue gallium-protoporphyrin (GaPP), alone and in combination with ciprofloxacin. Activity against MDR *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Acinetobacter johnsonii* biofilms was assessed in the colony biofilm and artificial wound model by enumeration of CFU and correlative light/electron microscopy. While *Staphylococcus* biofilms were equally susceptible to GaPP and Def-GaPP gels ($\log_{10}$ reduction of 3.8 and 3.7, respectively), the Def-GaPP combination was crucial for significant activity against *P. aeruginosa* biofilms ($\log_{10}$ reduction of 1.3 for GaPP and 3.3 for Def-GaPP). When Def-GaPP gel was combined with ciprofloxacin, the efficacy exceeded the activity of the individual compounds. Def-GaPP delivered in a surgical wound gel showed significant antibiofilm activity against different MDR strains and could enhance the gel's wound-healing properties. Moreover, Def-GaPP indicated a potentiation of ciprofloxacin. This antibiofilm strategy has potential for clinical utilization as a therapy for topical biofilm-related infections.

KEYWORDS antimicrobial combinations, biofilms, drug delivery, iron metabolism

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Medical treatments for chronic infectious diseases are typically based on oral delivery of high-dose, long-term antibiotic therapies. Despite the risk for emerging antimicrobial resistance and the potential of side effects (e.g., gastrointestinal disorders, neutropenia, nephrotoxicity), there is a lack of suitable alternatives. Depending on the disease nature and localization, topical treatments can deliver high dosages of antimicrobials directly to an infection site while reducing unwanted systemic effects. Higher drug dosages are particularly needed to combat microbial biofilms (1). The ability of bacteria to form biofilms and establish resistance to antibiotics is a major biomedical threat, adding billions of dollars to health care costs worldwide (1–3). Biofilms are responsible for 80% of microbial infections in humans and are a common cause of chronic infections, including chronic wound and chronic sinus infections (4, 5), with increasing tolerance and subtle resistance mechanisms to antibiotic therapies (6–8).

3b.2 Article

A topical hydrogel with deferiprone and gallium-protoporphyrin targets bacterial iron metabolism and has antibiofilm activity[284]

Katharina Richter[1], Nicky Thomas[3,4], Jolien Claeys[5], Jonathan McGuane[1], Clive A. Prestidge[3,6], Tom Coenye[5], Peter-John Wormald[1], Sarah Vreugde[1]

3b.3 Abstract

Many infectious diseases are associated with multidrug resistant (MDR) bacteria residing in biofilms that require high antibiotic concentrations. Whilst oral drug delivery is frequently ineffective, topical treatments have the potential to deliver higher drug concentrations to the infection-site while reducing systemic side-effects.

This study determined the antibiofilm activity of a surgical wound-gel loaded with the iron chelator deferiprone (Def) and the haem analogue gallium-protoporphyrin (GaPP), alone and in combination with ciprofloxacin. The activity against MDR *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Acinetobacter johnsonii* biofilms was assessed in the colony biofilm and artificial wound model by enumeration of colony forming units and correlative light/electron microscopy.

While *Staphylococcus* biofilms were equally susceptible to GaPP and Def-GaPP gel ($\log_{10}$ reduction of 3.8 and 3.7, respectively), the Def-GaPP combination was crucial for a significant activity against *P. aeruginosa* biofilms ($\log_{10}$ reduction of 1.3 for GaPP and 3.3 for Def-GaPP). When Def-GaPP gel was combined with ciprofloxacin, the efficacy exceeded the activity of the individual compounds.

Def-GaPP delivered in a surgical wound-gel showed significant antibiofilm activity against different MDR strains and could enhance the gel's wound healing properties. Moreover, Def-GaPP indicated

a potentiation of ciprofloxacin. This antibiofilm strategy has potential for a clinical utilisation as therapy for topical biofilm-related infections.

3b.4 Introduction

Medical treatments for chronic infectious diseases are typically based on oral delivery of high-dose, long-term antibiotic therapies. Despite the risk for emerging antimicrobial resistance and the potential of side-effects (e.g. gastro-intestinal disorders, neutropenia, nephrotoxicity), there is a lack of suitable alternatives. Depending on the disease nature and localisation, topical treatments can deliver high dosages of antimicrobials directly to an infection-site, while reducing unwanted systemic effects. Higher drug dosages are particularly needed to combat microbial biofilms[376]. The ability of bacteria to form biofilms and establish resistance to antibiotics is a major biomedical threat, adding billions of dollars to health care costs worldwide[280,281,376]. Biofilms are responsible for 80% of microbial infections in humans and are a common cause of chronic infections, including chronic wound and chronic sinus infections[153,155], with increasing tolerance and subtle resistance mechanisms to antibiotic therapies[11,72,73].

Topical delivery of antimicrobials to the nose and paranasal sinuses in nebulisers and irrigations has been reported to be beneficial against biofilm-associated chronic rhinosinusitis[186,377]. Another promising approach is the use of gels that can be directly instilled into the sinuses. Current phase I/II trials of a chitosan-dextran hydrogel demonstrate improved clinical outcomes after sinus surgery[190]. When prepared *in situ*, succinyl-chitosan and dextran-aldehyde form a nontoxic, biocompatible, biodegradable gel that facilitates post-operative wound healing by promoting homoeostasis and preventing adhesions[190,378-381]. The latter is a particularly important post-surgical complication of endoscopic sinus surgery that frequently causes surgical failure[382]. While the benefits of the blank gel have been proven in clinical practice[190], its antimicrobial potential as drug delivery system has not been fully explored. The incorporation of antimicrobials in the gel may

enhance its clinical use and expand its application to other medical conditions, such as biofilm-associated wound infections.

In the present study, an antimicrobial strategy is evaluated using a surgical hydrogel loaded with the iron chelator deferiprone (Def) and the haem analogue gallium-protoporphyrin (GaPP)[1]. Richter *et al.* recently reported on the *in vitro* activity of Def-GaPP against *S. aureus* biofilms by interfering with bacterial iron metabolism[303]. However, studies to date are based on the pure compounds in solution and a translational drug delivery strategy for clinical applications has not yet been investigated.

3b.5 Materials and methods

3b.5.1 Bacterial strains and culture media

S. aureus ATCC 25923, *S. epidermidis* ATCC 12228 and *A. johnsonii* ATCC 17946 were purchased from American Type Culture Collection (Manassas, VA, USA). *P. aeruginosa* PA01 was received from the School of Molecular Medical Sciences, University of Nottingham, United Kingdom. Clinical MRSA and *P. aeruginosa* isolates were obtained from Adelaide Pathology Partners (Mile End, Australia). The specimens were collected from chronic rhinosinusitis and cystic fibrosis patients, respectively, which was approved by the human ethics committee at the Queen Elizabeth Hospital (Woodville, Australia). The MRSA strain showed resistance against penicillin, oxacillin, amoxicillin/clavulanic acid, cephalexin and erythromycin. Nutrient agar/broth was used for *Staphylococcus* species and *A. johnsonii*, while for *Pseudomonas* strains Luria Bertani agar/broth was used.

3b.5.2 Preparation of hydrogels

Hydrogels were prepared as described previously[383] consisting of dextran-aldehyde, succinyl-chitosan and a buffer solution. The gel was loaded with 20 mM of deferiprone (3-hydroxy-1,2-dimethylpyridin-4(1*H*)-one, Sigma, Castle Hill, Australia) and/or gallium-protoporphyrin IX (100 or

500 µg/ml, Frontier Scientific, Logan, UT). Controls included blank gel and the gel loaded with 5 µg/ml of ciprofloxacin (Cip) (i.e. 40 times above the MIC for *S. aureus* ATCC 25923). Cip was chosen as control due to its clinical relevance as broad-spectrum therapy against Gram-positive and Gram-negative bacteria that are e.g. associated with infections of the respiratory tract and skin.

3b.5.3 Determination of drug release kinetics

Ten millilitre of release medium (phosphate buffered saline) was added to 5 ml of gel and incubated at 37°C on a rotating platform (70 rpm) for 20 days. Aliquots of 0.5 ml were taken at specific time points (0.5, 1, 2, 8, 16, 24, 48, 72, 96, 120, 170, 220, 290, 460 hours) and replaced with fresh release medium. The concentrations of Def and GaPP were quantified by UV-Vis spectroscopy (Evolution 201 UV-Vis Spectrophotometer, Thermo Fisher Scientific, Scoresby, Australia) at 280 nm and 405 nm, respectively, by interpolating from a standard curve.

3b.5.4 Determination of the minimal inhibitory concentration

Def and GaPP were solubilised in buffer (used for the hydrogel preparation) to determine the MIC using the colony suspension and broth microdilution method[374]. The concentrations ranged from 0.08-40 mM Def (i.e. 10.8-5568 µg/ml), 0.1-50 µg/ml GaPP and 0.03-16 µg/ml Cip.

3b.5.5 Activity in the agar diffusion model

Bacteria from a freshly streaked out agar plate were immersed in 0.9% saline and adjusted to 7.0 McFarland units. Twenty microlitre of this suspension was suspended in 25 ml of liquid 0.7% agar (50°C) and poured into a Petri dish. After the agar solidified, cavities of 0.9 cm diameter were punched, aspirated and filled with 200 µl of gel. The inhibition diameter was measured after 24 hours incubation at 37°C.

3b.5.6 Activity in the colony biofilm model

Single colonies of bacteria were immersed in 0.9% saline and adjusted to 1.0 McFarland units (approximately 3×10^8 CFU/ml). Following a 1:1000 dilution in broth, 1 µl of the suspension was spotted on a Whatman polycarbonate membrane filter (for MRSA) or cellulose nitrate membrane filter (for all other strains) with a 0.2 µm pore size (GE Healthcare, Little Chalfont, United Kingdom)[384,385]. The filters were placed on agar plates and incubated at 37°C (30°C for *A. johnsonii*) for 24 hours (48 hours for *A. johnsonii* and *S. epidermidis*), before transferring the filters onto AB trace agar (minimal growth agar including 0.5% glucose and 0.5% peptone). Biofilms were exposed to 100 µl gel for up to 5 days at 37°C (30°C for *A. johnsonii*). The filters were transferred onto new AB trace agar after 2.5 days. Finally, bacteria were recovered from the filters in PBS by vortexing (1 min) and sonication (15 min), diluted and plated for CFU counting.

3b.5.7 Biofilm visualisation

Following gel exposure, colony biofilms were fixed in 2.5% glutaraldehyde (ProSciTech, Kirwan, Australia) and incubated with Live/Dead BacLight (Life Technologies, Scoresby, Australia). Biofilms were dehydrated in an ethanol series and cross-sectioned before embedding in paraffin wax. Sections of 3 µm were cut, placed on glass slides, deparaffinised and rehydrated prior to analysis by confocal laser scanning microscopy (LSM 710, Carl Zeiss, Jena, Germany) using a 63x/1.4 oil objective. The excitation/emission wavelengths were 485/530 nm and 485/630 nm.

To correlate confocal microscopy images with scanning electron microscopy images (SEM Gemini 2, Carl Zeiss) using Zeiss' shuttle and find software, additional samples were prepared as above. After deparaffinisation and rehydration, samples were incubated with osmium tetroxide (ProSciTech) followed by dehydration in an ethanol series and hexamethyldisilazane (ProSciTech) incubation. Finally, samples were sputter-coated with 10 nm of gold particles.

3b.5.8 Activity in an artificial wound model

An artificial dermis of hyaluronic acid (1.20-1.80 MDa, Lifecore Biomedical, Chaska, MN, USA) and collagen (Corning Incorporated, Corning, NY, USA) was prepared as previously described[386]. A mixture of lyophilised bovine plasma (Sigma), 19 ml Bolton broth, 1 ml horse blood and 10 IU of heparin was added to the dermis. The dermis was infected with 10 µl of an overnight culture adjusted to 1×10^6 CFU/ml (*S. aureus* ATCC 25923, a clinical MRSA isolate, *P. aeruginosa* PA01). After 24 hours biofilm formation at 37°C, biofilms were exposed to 150 µl of loaded hydrogels (Def, GaPP 500 and Def-GaPP 500 gels) for 24 hours at 37°C. The dermis was washed and placed in 10 ml of 0.9% saline. Biofilms were extracted by vortexing and sonication (alternating cycles of 3 x 30 sec), diluted and plated for CFU counting.

3b.5.9 Statistics and software

All experiments were conducted in triplicate and are presented as mean ± standard deviation (SD). Results were analysed using two-way analysis of variance (ANOVA) with Dunnett's test (GraphPad Prism version 6.00, GraphPad Software, La Jolla, CAL, USA). Statistical significance was assessed at the 95% confidence level.

3b.6 Results

3b.6.1 Drug release

The Def/GaPP concentration in the release medium was expressed as the percentage of the original concentration in the gel. All Def was released from the gel within 48-72 hours, while the release of GaPP gradually increased over time, reaching approximately 20-25% after 460 hours (Figure 32). These release profiles were independent of drug concentrations in the gels (Def 20 mM; GaPP 100 and 500 µg/ml). Interestingly, there was no statistical difference between the release of individual compounds and the release of the corresponding compounds from the combination gel.
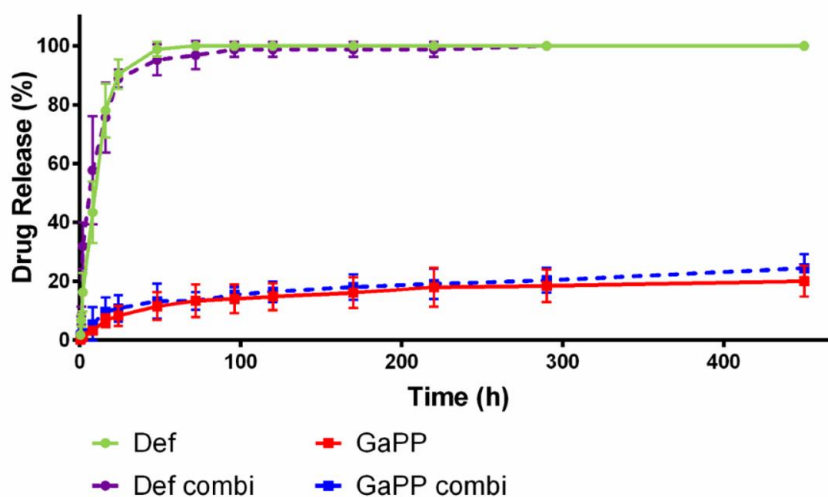
Figure 32. Release profiles of gels loaded with 20 mM deferiprone (Def, green circles), 500 µg/ml gallium-protoporphyrin (GaPP, red squares) or a combination of both (Def combi: purple circles; GaPP combi: blue squares, dotted lines). Data represent the mean ± SD of 3 replicates.

3b6.2   Minimal inhibitory concentration

The MICs against planktonic bacteria ranged from 87 µg/ml (*A. johnsonii*) to 5568 µg/ml (MRSA) for Def, and from <0.1 µg/ml (*S. epidermidis*) to >50 µg/ml (*P. aeruginosa* PA01) for GaPP (Table 4). When used in combination, the MICs for both compounds were typically lower, although the extent of this difference was strain-dependent (Table 4).

Table 4. Minimal inhibitory concentrations of deferiprone (Def), gallium-protoporphyrin (GaPP), the combination of both compounds and ciprofloxacin (Cip).

| Isolate | MIC (µg/ml) of: | | | |
	Def	GaPP	Combination Def-GaPP	Cip
S. aureus ATCC 25923	2784	12.5	696/6.25	0.125
MRSA clinical isolate	5568	50	2784/25	2
S. epidermidis ATCC 12228	696	<0.1	<10.8/<0.1	0.125
P. aeruginosa PA01	174	>50	87/0.78	0.125
P. aeruginosa clinical isolate	348	>50	87/0.78	0.125
A. johnsonii ATCC 17946	87	0.78	87/0.78	0.03

3b6.3   Effect of loaded hydrogels on bacterial biofilms

Agar diffusion model

The growth inhibition of gels was determined in an agar diffusion model (Figure 33) with blank gel as negative control (no growth inhibition) and Cip gel as positive control. The Def gel showed slight growth inhibition, while the GaPP gel showed substantial activity against all bacteria (up to 3.5 $\log_{10}$ reduction) except *P. aeruginosa* (no growth inhibition). When Def-GaPP were combined, the gel showed similar growth inhibition as the GaPP gel against *Staphylococcus* species, slightly higher inhibition against both the clinical *P. aeruginosa* isolate and *A. johnsonii*, and substantially higher inhibition against *P. aeruginosa* PA01 (3.3 $\log_{10}$ reduction, Figure 33).
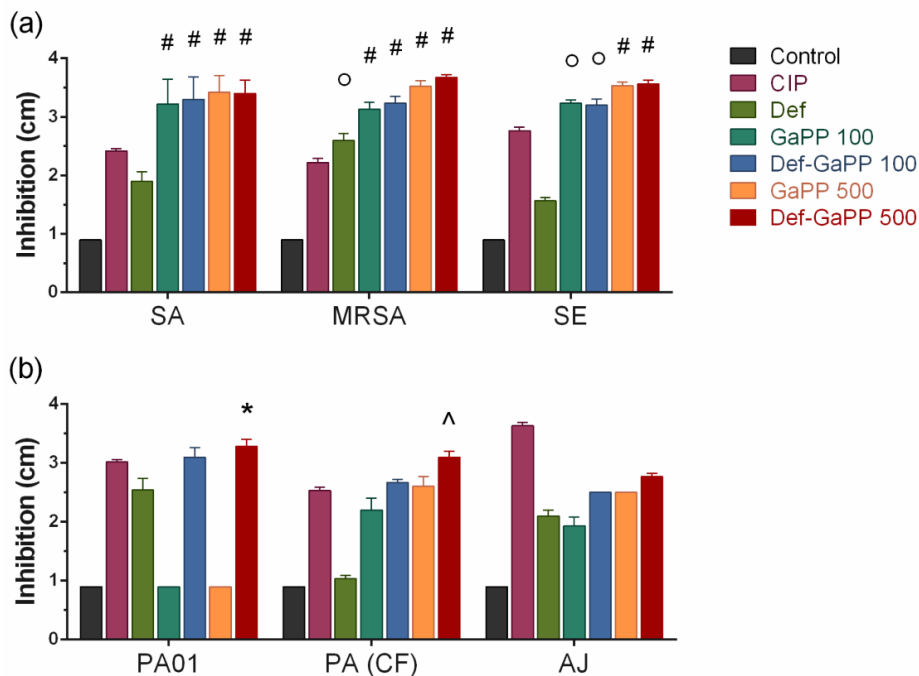
Figure 33. Inhibition zone diameter (cm) of (a) Gram-positive and (b) Gram-negative bacteria after exposure to loaded hydrogels. Strains used include S. aureus ATCC 25923 (SA), a clinical MRSA isolate (MRSA), S. epidermidis ATCC 12228 (SE), P. aeruginosa PA01 (PA01), a clinical P. aeruginosa isolate from a cystic fibrosis patient (PA (CF)) and A. johnsonii ATCC 17946 (AJ). Hydrogels include control: blank gel (black), Cip: ciprofloxacin 5 µg/ml (pink), Def: deferiprone 20 mM (light green), GaPP 100: gallium-protoporphyrin 100 µg/ml (dark green), Def-GaPP 100 (blue), GaPP 500 (orange), Def-GaPP 500 (red). Data represent the mean ± SD of 3 biological replicates. Statistical comparison to ciprofloxacin-loaded gel. * p<0.05   O p<0.01   ^ p<0.001   # p<0.0001

Colony biofilm model

The blank gel showed no activity and the Def gel showed low activity against all biofilms (Figure 34), while the effect of GaPP gel was concentration- and strain-dependent. GaPP in a low concentration

(100 µg/ml) demonstrated substantial activity against *S. epidermidis* biofilms only (4.3 $\log_{10}$ reduction), while at 500 µg/ml GaPP was more active ($\log_{10}$ reduction of 3.8, 1.4 and 4.6 in *S. aureus*, MRSA and *S. epidermidis* biofilms; $\log_{10}$ reduction of 1.3, 2.6 and 1.7 in two *P. aeruginosa* and *A. johnsonii* biofilms). When Def and GaPP 500 were combined, the gel showed similar antibiofilm activity as GaPP 500 against *S. aureus,* MRSA, *S. epidermidis* and *A. johnsonii* biofilms ($\log_{10}$ reduction of 3.8, 1.4, 4.3 and 2.0, respectively). In contrast, in two *P. aeruginosa* biofilms the Def-GaPP 500 combination demonstrated higher activity than the individual compounds ($\log_{10}$ reduction of 3.3 and 3.9). The triple combination of Def, GaPP 100 and Cip in gel exceeded the antibiofilm activity of the individual compounds and Cip alone against all biofilms, except the clinical *P. aeruginosa* isolate. Moreover, the triple combination (with 100 µg/ml GaPP) showed even higher activity against MRSA, *S. epidermidis* and *P. aeruginosa* PA01 biofilms than the most active Def/GaPP gels containing 500 µg/ml GaPP.
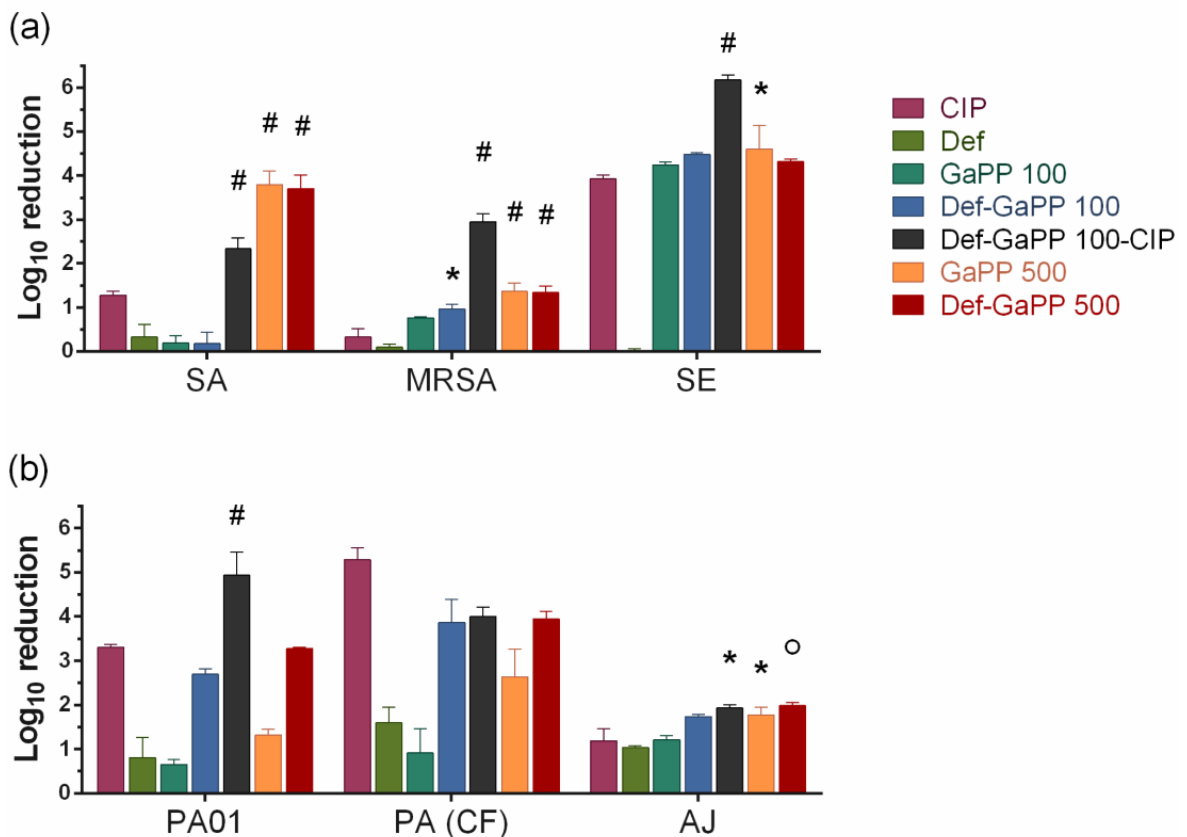
Figure 34. Log_{10} reduction of (a) Gram-positive and (b) Gram-negative colony biofilms after exposure to loaded hydrogels. Strains used include S. aureus ATCC 25923 (SA), a clinical MRSA isolate (MRSA), S. epidermidis ATCC 12228 (SE), P. aeruginosa PA01 (PA01), a clinical P. aeruginosa isolate from a cystic fibrosis patient (PA (CF)) and A. johnsonii ATCC 17946 (AJ). Hydrogels include Cip: ciprofloxacin 5 µg/ml (pink), Def: deferiprone 20 mM (light green), GaPP 100: gallium-protoporphyrin 100 µg/ml (dark green), Def-GaPP 100 (blue), Def-GaPP 100-Cip (black), GaPP 500 (orange), Def-GaPP 500 (red). Data represent the mean ± SD of 3 biological replicates. Statistical comparison to ciprofloxacin-loaded gel. * p<0.05   O p<0.01   # p<0.0001

Macroscopic and microscopic biofilm analysis

The macroscopic analysis of colony biofilms after treatment confirmed the antibiofilm activity of the different gels (Figure 35). While biofilms grew extensively in both the blank gel and Def gel, a species- and strain-dependent antibiofilm effect was observed for the gels loaded with GaPP, Def-GaPP and Cip. Against Gram-positive biofilms both GaPP and Def-GaPP gels inhibited bacterial growth substantially, whereas against *P. aeruginosa* biofilms the presence of Def was crucial for antibiofilm activity.
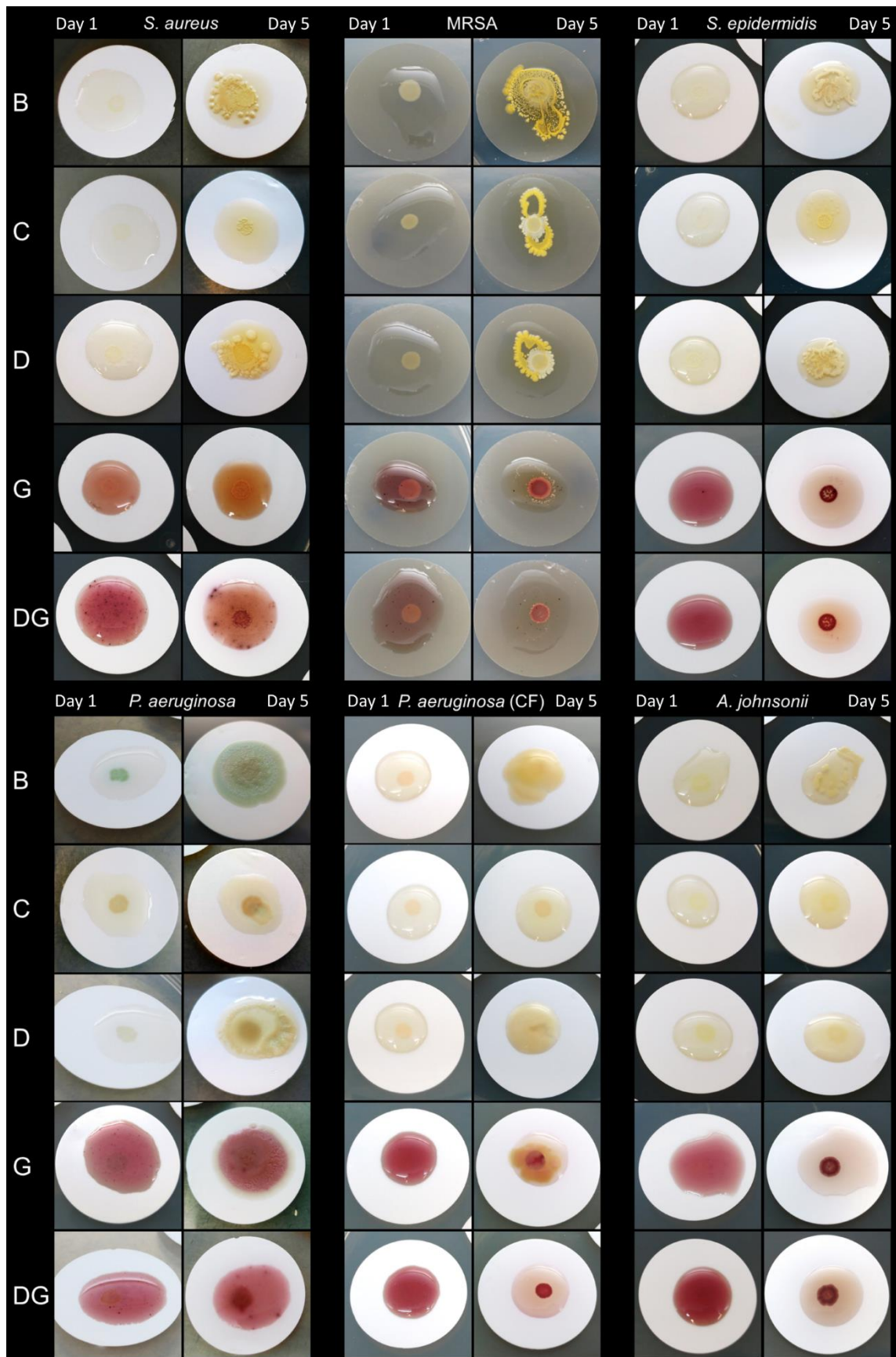
Figure 35. Bacterial biofilm growth over time after initial exposure to loaded hydrogels. Strains used include S. aureus ATCC 25923, a clinical MRSA isolate, S. epidermidis ATCC 12228, P. aeruginosa PA01, a clinical P. aeruginosa isolate from a cystic fibrosis patient and A. johnsonii ATCC 17946. Hydrogels include blank control gel (B), ciprofloxacin 5 µg/ml (C), deferiprone 20 mM (D), gallium-protoporphyrin 500 µg/ml (G), Def-GaPP 500 (DG).

Confocal laser scanning microscopy with Live/Dead staining confirmed the antibiofilm activity of the loaded hydrogels. In Figure 36, a representative cross-section of *S. aureus* colony biofilm after exposure to Def-GaPP 500 gel is shown indicating that the majority of cells were killed as reflected by the red staining (propidium iodide).
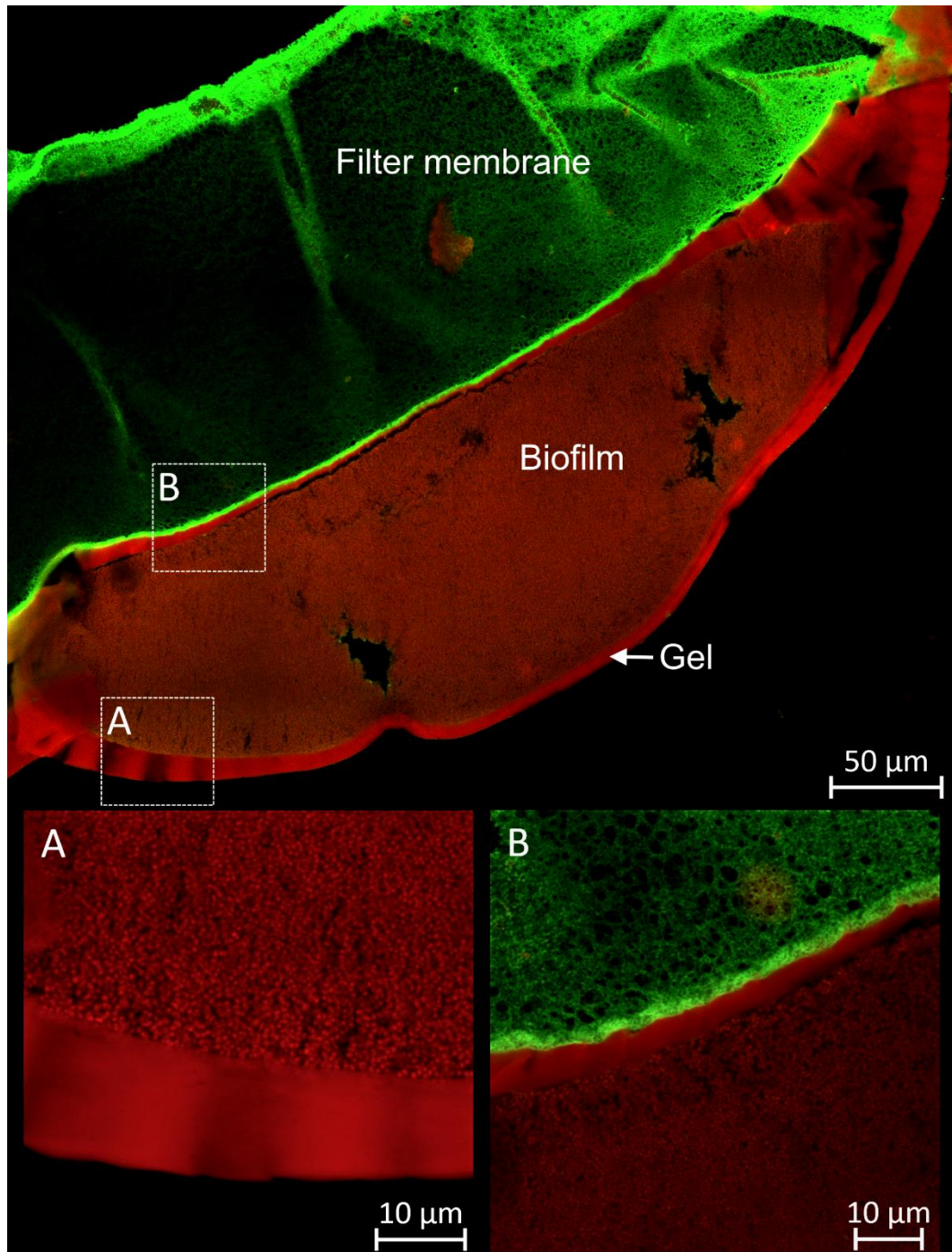
Figure 36. Cross-section of S. aureus colony biofilm after exposure to Def-GaPP 500 gel. Visualisation by confocal laser scanning microscopy after Live/Dead staining. The green autofluorescent filter membrane is visible under the red stained S. aureus biofilm and gel.

119

The antibiofilm activity of hydrogels was further confirmed by a novel platform of correlative light/electron microscopy, which allows the direct overlay of confocal and scanning electron microscopy images. This allowed the Live/Dead visualisation of the specimen being complemented with in-depth, 3-dimensional information. In Figure 37 an example of a colony biofilm cross-section is shown. A thick *S. aureus* biofilm can be seen between the green autofluorescent membrane filter (left) and Def-GaPP 500 gel (right) that completely covered the biofilm surface. The red colour indicates a substantial reduction in live bacterial cells after treatment exposure. Gaps between the membrane filter, biofilm and gel are artefacts of the sample preparation. Grey areas illustrate electron microscopy details not captured by confocal laser scanning microscopy.
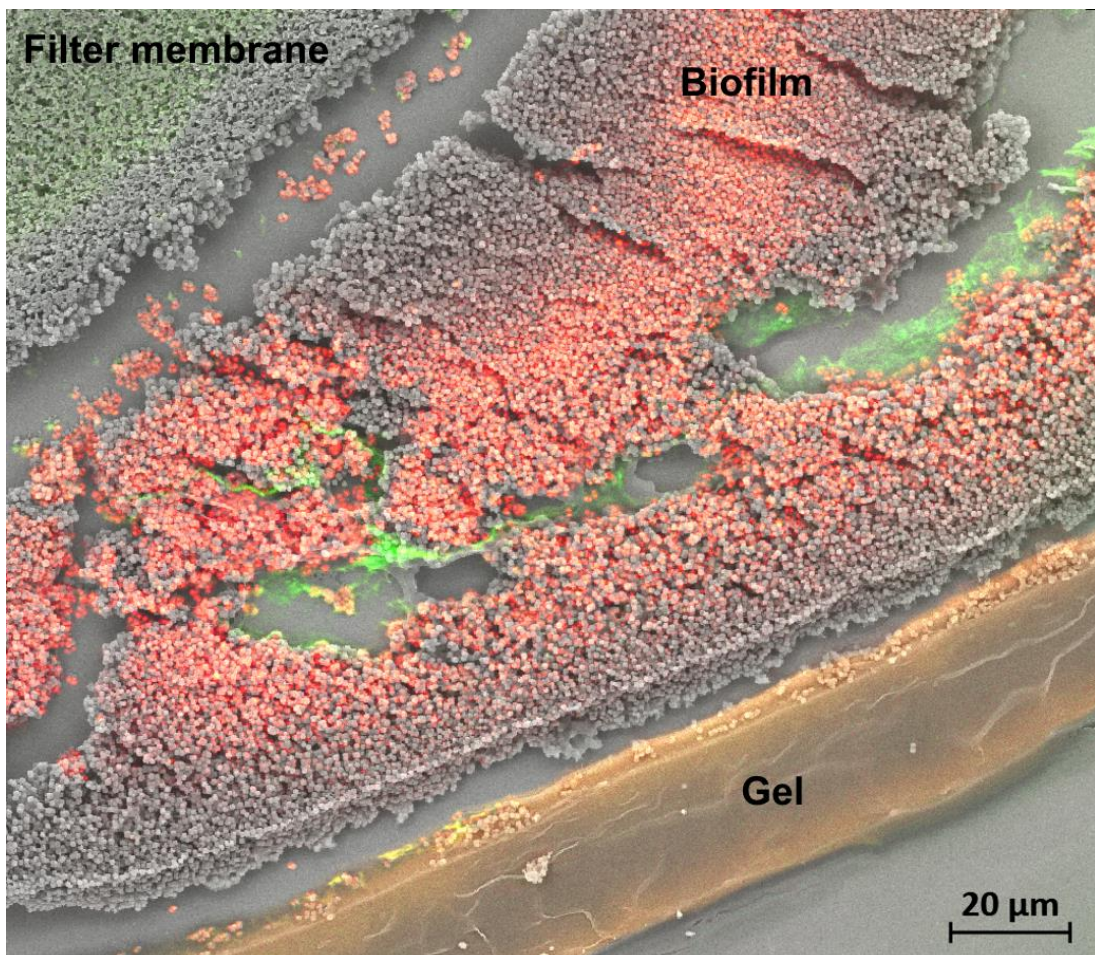
Figure 37. Correlative light/electron microscopy image of S. aureus biofilm exposed to Def-GaPP 500 gel, stained for live/dead cells. Green filter membrane (top left, green autofluorescence), red stained S. aureus biofilm (center) and gel (bottom, yellow) are shown.

Artificial wound model

The antibiofilm activity was also evaluated in an *in vitro* wound model where *S. aureus*, MRSA and

P. aeruginosa biofilms were grown on an artificial dermis and exposed to loaded gels (Figure 38).

The blank gel demonstrated no activity against all biofilms and showed similar biofilm growth as

the untreated control. The Def gel showed up to 0.5 $\log_{10}$ reduction, while the GaPP 500 gel showed

up to 0.2 $\log_{10}$ reduction. The combination of Def-GaPP 500 demonstrated a substantial antibiofilm

activity with a 0.7 $\log_{10}$ reduction against both *S. aureus* and MRSA biofilms, and a 1.9 $\log_{10}$

reduction against *P. aeruginosa* biofilm, thereby exceeding the activity of the individual

compounds.
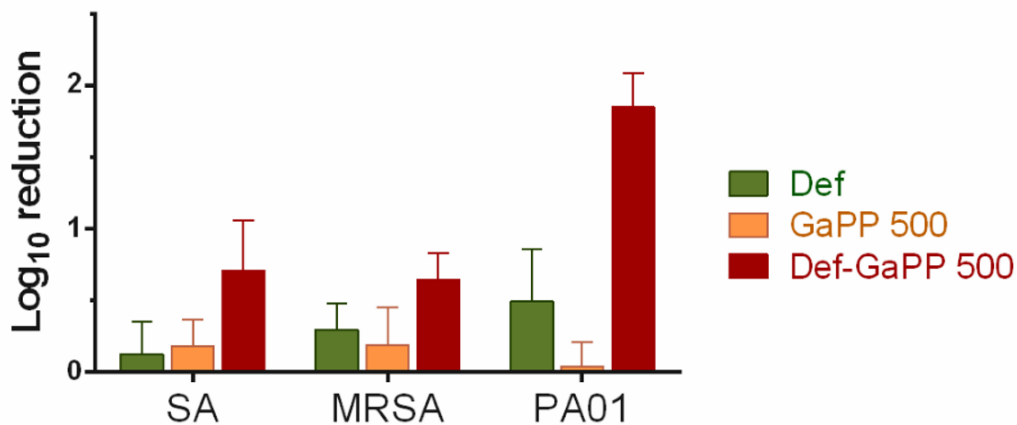
Figure 38. Effects of loaded hydrogels in an artificial wound model. Log$_{10}$ reduction of S. aureus ATCC 25923 (SA), a clinical MRSA isolate (MRSA) and P. aeruginosa PA01 (PA01) after exposure to loaded hydrogels with Def: deferiprone 20 mM (light green), GaPP 500: gallium-protoporphyrin 500 µg/ml (orange) and Def-GaPP 500 (red). Data represent the mean ± SD of 3 biological replicates.

3b.7 Discussion

In the present study the antibiofilm activity of a gel formulation combining the iron chelator Def

and the haem analogue GaPP was investigated. While both compounds have been described

previously as single treatments[45,337,372,387] and in combination[303], the present study is the first to

incorporate Def and GaPP in a clinically-relevant hydrogel, thereby potentially serving as a novel

antimicrobial strategy in the context of topical biofilm-related infections. The surgical hydrogel was

used as a carrier to deliver Def and GaPP to biofilms, thereby complementing the gel's wound healing properties with antimicrobial activity for topical treatment. In order to evaluate the potential of the Def-GaPP gel as an alternative antimicrobial therapy, the drug release kinetics and the antibiofilm activity against multiple Gram-positive and Gram-negative biofilms were determined.

By targeting the bacterial iron metabolism that is vital for growth, survival and virulence of virtually all bacteria[318,319,388], Def induces starvation and upregulation of iron acquisition systems[46] while GaPP exploits the latter. By mimicking haem (i.e. iron-protoporphyrin), the preferred iron source of many bacteria[319,321], GaPP is taken up into bacterial cells where it inhibits essential cellular pathways, disrupts the respiratory chain and induces reactive oxygen species that are toxic to bacteria[354].

In a previous study with the pure compounds[303] the most effective and nontoxic treatment combination was identified to be 20 mM Def and 200 µg/ml GaPP, while GaPP concentrations of 100 µg/ml and lower showed also significant antibiofilm activity. Furthermore, enhanced antimicrobial effects have been described against *S. aureus* biofilms *in vitro* when Def and GaPP as pure compounds in solution were applied consecutively[303]. Hence, to maximise antimicrobial activity, it is important for a carrier material combining both compounds to facilitate a quick release of Def while enabling the sustained release of GaPP. This was accomplished by using a surgical hydrogel that is established in clinical practice to improve wound healing post-sinus surgery as a drug delivery system. The gel was loaded with 20 mM Def, which is a water-soluble drug that was completely released within 48-72 hours, while the low water-solubility of GaPP resulted in a slower, gradual release over time (Figure 32). In our experimental system, the total amount of GaPP released from the hydrogel was limited (approximately 20-25% of the incorporated GaPP was released after 20 days). As previously reported[303] GaPP shows extensive antibiofilm activity at concentrations of 100-200 µg/ml in solution. Considering a release of approximately 20% GaPP (Figure 32), 500 µg/ml GaPP were incorporated in the gel, corresponding to a released GaPP

concentration of 100 µg/ml. For comparative reasons, 100 µg/ml GaPP were included in this study as well, corresponding to a released GaPP concentration of 20 µg/ml. Despite incomplete GaPP release from the gel resulting in up to 100 µg/ml after 20 days in the current study, substantial antibiofilm effects against different strains including clinical MRSA and *P. aeruginosa* isolates were observed. Optimisation of the formulation towards an improved GaPP release might enhance the gel's antimicrobial properties. This could potentially be achieved, for example, by physical drug modifications like particle size reduction, or by chemical gel modifications like incorporation of co-solvents or surfactants to increase the solubility and subsequent release of GaPP[201,389]. However, as the surgical hydrogel dissolves over 2 weeks when applied into the human sinuses post-surgery[190], the release of both compounds is likely to be enhanced in the clinical setting. Further *in vivo* studies are needed to assess the gel's antimicrobial and wound-healing properties.

In contrast to previous reports[383,390], no significant antimicrobial or antibiofilm activity of the blank gel against all tested biofilms were observed in the present study (Figure 33, 34 and 35). This is likely due to the use of different models. The colony biofilm model produces a thick biofilm with a stratified profile (Figure 37). This structure gives rise to pronounced oxygen and nutrient gradients, i.e. aerobic conditions at the air-biofilm interface and micro-aerobic/anaerobic conditions predominating in the biofilm interior[384]. The blank gel interacts with the biofilm by binding to bacterial cell wall proteins[383], while Def-loaded gel additionally chelates/deprives nutrients[369], thereby affecting the biofilm indirectly and causing upregulation of iron acquisition systems[46]. These interactions, however, showed only limited effects on antibiofilm activity in the current study. In contrast, GaPP can enter bacterial cells by exploiting the haem uptake system as bacteria recognise GaPP's tetrapyrrole ring as cue for haem as a favourable iron source[337,355]. Inside bacteria, GaPP exhibits antibacterial activity by disrupting essential cellular pathways[45,354], as (i) GaPP cannot transfer electrons essential for ATP production by respiratory proteins, (ii) bacterial enzymes are not able to cleave GaPP, impeding nutrient/iron release thus inducing starvation, and (iii) efflux pumps crucial for haem homoeostasis are blocked by GaPP[45,321]. These effects limit bacterial respiration, provoke accumulation of redox-active molecules inside bacteria and catalyse the

production of reactive oxygen species that subsequently cause DNA and protein damage. While the current results showed no significant differences in antibiofilm activity between GaPP gel and Def-GaPP gel against *Staphylococcus* species (Figure 33 and 34), the incorporation of Def is crucial for a substantial antibiofilm activity against *P. aeruginosa*. This may be the result of a Def-induced upregulation of iron transporter proteins that augment the uptake and therefore the antibiofilm effect of GaPP in bacteria. Moreover, the combination of Def and GaPP with Cip had a more pronounced antibiofilm effect compared to the individual compounds and the Def-GaPP combination. Whether Def and GaPP also have the ability to increase the susceptibility of biofilms to other antibiotics remains to be investigated.

Consistent with the findings in the colony biofilm model, the Def-GaPP gel exhibited substantial antibiofilm activity in an artificial wound model (Figure 38). However, the absolute reduction in viable bacteria after Def-GaPP exposure was lower than in the colony biofilm model. This can be explained by the nutrient-rich environment in the wound model that included blood as iron source. As bacteria recognise haem, the antibiofilm effect of the haem analogue GaPP was expected to be low. When GaPP was combined with Def, the gel could deprive nutrients from bacteria and deliver GaPP as a "Trojan Horse" for a pronounced antibiofilm activity.

The utilisation of Def is also considered to be beneficial in light of its strong wound healing properties[391]. By scavenging free radicals, Def is known to accelerate wound healing *in vivo*[391]. Moreover, the hydrogel itself shows homoeostatic and anti-scarring properties, facilitating post-operative wound healing while being biocompatible[190]. By combining these properties with wound healing and antimicrobial effects of Def and GaPP, the gel is expected to improve treatment activity of chronic rhinosinusitis and infected wounds due to prolonged compound exposure time and prevention of premature gel clearance at the site of infection. Therefore, this treatment strategy may represent a promising approach for topical applications in clinical practice.

3b.8 Conclusion

In conclusion, the present *in vitro* study revealed that a surgical hydrogel incorporating Def and GaPP was able to release both compounds and showed significant antibiofilm activity against Gram-positive and Gram-negative bacteria. In light of emerging antibiotic resistant pathogens, the proposed strategy targeting bacterial iron metabolism might be a promising non-antibiotic alternative.

3b.9 Acknowledgements

3b.10 Funding information

3b.11 Disclosures

Competing financial interests: PJW holds a patent on the chitosan-dextran hydrogel. PJW and SV

hold a patent on the treatment combination of deferiprone and gallium-protoporphyrin. All other

authors: none to declare.

3c. Publication: "Deferiprone and gallium-protoporphyrin have the capacity to potentiate the activity of antibiotics in *Staphylococcus aureus* small colony variants"

Statement of authorship

Title of Paper	Deferiprone and gallium-protoporphyrin have the capacity to potentiate the activity of antibiotics in *Staphylococcus aureus* small colony variants
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Richter K, Thomas N, Guimin Z, Prestidge CA, Coenye T, Wormald P-J, Vreugde S. Deferiprone and gallium-protoporphyrin have the capacity to potentiate the activity of antibiotics in Staphylococcus aureus small colony variants. Frontiers in Cellular and Infection Microbiology 2017, 7 (280).

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Name of Principal Author (Candidate)	Katharina Richter		
Contribution to the Paper	Conception and design of the project, acquisition of data, analysis and interpretation of data, drafting the article		
Overall percentage (%)	85		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	10/4/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

    i.   the candidate's stated contribution to the publication is accurate (as detailed above);

    ii.   permission is granted for the candidate in include the publication in the thesis; and

    iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	10/4/2017

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Conception and design of the project, critically revising the article		
Signature		Date	10/4/2017

3c.1 Publication title page

Deferiprone and Gallium-Protoporphyrin Have the Capacity to Potentiate the Activity of Antibiotics in *Staphylococcus aureus* Small Colony Variants

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Small colony variants (SCVs) of bacteria like *Staphylococcus aureus* are characterized by a reduced colony size and are linked to increased antibiotic tolerance and resistance. Their altered expression of virulence factors, slow growing properties and their ability to form biofilms make the eradication of SCVs challenging. In the context of biofilm-related infectious diseases involving *S. aureus* SCVs, a therapy targeting bacterial iron metabolism was evaluated. The combination of the iron-chelator deferiprone (Def) and the heme-analog gallium-protoporphyrin (GaPP), in solution and incorporated in a surgical wound gel, was tested for activity against planktonic and sessile SCVs. To this end, the activity of Def-GaPP was assessed against planktonic *S. aureus* SCVs, as well as against *in vitro* and *in vivo* biofilms in the colony biofilm model, an artificial wound model and a *Caenorhabditis elegans* infection model. While Def alone failed to show substantial antibacterial activity, GaPP and the combination of Def-GaPP demonstrated concentration- and strain-dependent antibacterial properties. Specifically, the Def-GaPP combination significantly reduced the bacterial load in an artificial wound model and increased the survival of *S. aureus* SCV infected *C. elegans*. When Def-GaPP were combined with gentamicin or ciprofloxacin, the triple combinations exceeded the antibiofilm activity of the individual compounds in the colony biofilm model. In targeting bacterial iron metabolism, Def-GaPP showed significant activity against planktonic and sessile SCVs. Moreover, Def-GaPP could potentiate the activity of gentamicin and ciprofloxacin. Delivered in a wound healing gel, Def-GaPP showed promise as a new topical strategy against infections with *S. aureus* SCVs.

Keywords: biofilms, small colony variants, *Staphylococcus aureus*, iron, deferiprone, gallium-protoporphyrin, wound models, *Caenorhabditis elegans*

129

3c.2 Article

Deferiprone and gallium-protoporphyrin have the capacity to potentiate the activity of antibiotics in *Staphylococcus aureus* small colony variants[392]

Katharina Richter[1], Nicky Thomas[3,4], Zhang Guimin[7], Clive A. Prestidge[3,6], Tom Coenye[5], Peter-John Wormald[1], Sarah Vreugde[1]

3c.3 Abstract

Small colony variants (SCVs) of bacteria like *Staphylococcus aureus* are characterised by a reduced colony size and are linked to increased antibiotic tolerance and resistance. Their altered expression of virulence factors, slow growing properties and their ability to form biofilms make the eradication of SCVs challenging. In the context of biofilm-related infectious diseases involving *S. aureus* SCVs, a therapy targeting bacterial iron metabolism was evaluated.

The combination of the iron chelator deferiprone (Def) and the haem analogue gallium-protoporphyrin (GaPP), in solution and incorporated in a surgical wound gel, was tested for activity against planktonic and sessile SCVs. To this end, the activity of Def-GaPP was assessed against planktonic *S. aureus* SCVs, as well as against *in vitro* and *in vivo* biofilms in the colony biofilm model, an artificial wound model and a *Caenorhabditis elegans* infection model.

While Def alone failed to show substantial antibacterial activity, GaPP and the combination of Def-GaPP demonstrated concentration- and strain-dependent antibacterial properties. Specifically, the Def-GaPP combination significantly reduced the bacterial load in an artificial wound model and increased the survival of *S. aureus* SCV infected *C. elegans*. When Def-GaPP were combined with gentamicin or ciprofloxacin, the triple combinations exceeded the antibiofilm activity of the individual compounds in the colony biofilm model.

In targeting bacterial iron metabolism, Def-GaPP showed significant activity against planktonic and sessile SCVs. Moreover, Def-GaPP could potentiate the activity of gentamicin and ciprofloxacin. Delivered in a wound healing gel, Def-GaPP showed promise as a new topical strategy against infections with *S. aureus* SCVs.

3c.4 Graphical abstract
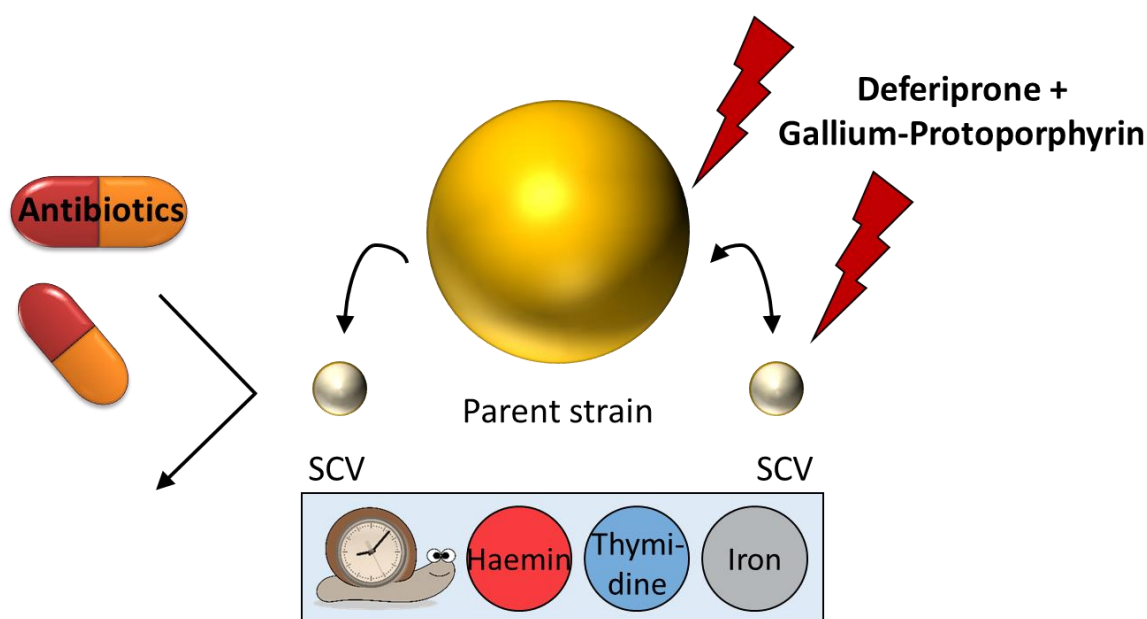
3c.5 Introduction

Small colony variants (SCVs) are naturally occurring bacteria derived from a parent strain characterised by a small colony morphology (approximately 10% the size of the parent strain colony), slow growth rate, altered virulence factors and increased antibiotic tolerance or resistance[37]. The switch to a phenotypic altered strain can be inheritable or transient[116]. *S. aureus* SCVs are frequently non-pigmented, non-haemolytic and dependent on external growth factors like menadione, haemin and thymidine; they are able to survive inside eukaryotic cells, including human macrophages[37,122,393]. Due to their intracellular lifestyle, *S. aureus* SCVs can escape the immune attack and are protected against antibiotics leading to persistence of disease. SCVs are associated

with antibiotic-refractory and recalcitrant infections, such as chronic rhinosinusitis, respiratory tract infections in cystic fibrosis, osteomyelitis, chronic wounds or implant infections[37,106,112]. The recovery of SCVs in routine clinical investigations requires special nutrients and prolonged culture, making SCV isolation and identification difficult[36]. Prolonged treatment regimens with a variety of antibiotics are required to treat SCV-associated infections, often combined with surgical interventions. However, clinical outcomes are frequently unsatisfying due to treatment failure and recurrence of disease[112].

Worsening the situation, SCVs can be induced by medical therapies, e.g. by exposure to antibiotics such as gentamicin, or disinfectants such as triclosan[133,134]. It is furthermore known that sub-therapeutic antibiotic exposure can trigger biofilm formation of *S. aureus* SCVs and their parent strains, further complicating treatment[19,135,136]. Despite the clinical significance there is little knowledge concerning *S. aureus* SCV biofilms and their susceptibility to antibiotics. Innovative treatment approaches, based on compounds with a different mode of action, such as disrupting bacterial iron metabolism[1], may be a strategy worth approaching. *S. aureus* SCVs, like all bacteria, rely on iron for growth and survival[394], hence, the iron metabolism could be an interesting therapeutic target.

In the present study, the antimicrobial activity of a treatment combining the iron chelator deferiprone (Def) and the haem analogue gallium-protoporphyrin (GaPP) was assessed against planktonic and biofilm-associated SCVs.

3c.6 Materials and methods

3c.6.1 Bacterial strains

Bacterial strains were collected from the sinonasal cavities of chronic rhinosinusitis patients. Ethics approval was obtained from The Queen Elizabeth Hospital Human Research Ethics Committee, Woodville, SA, Australia. Strains included one *Staphylococcus aureus* clinical isolate (parent strain

P1), which was prolonged subcultured at MIC or higher concentrations of gentamicin (≥2 µg/ml). This induced small colony variants (SCV1) that featured elevated gentamicin tolerance. Another strain was a clinically isolated *S. aureus* small colony variant (SCV2).

3c.6.2 Characteristics of bacterial strains

Catalase, coagulase and haemolytic activity

Catalase and coagulase activity of bacterial strains were determined by suspending cells in saline in a glass tube. The catalase activity was observed by gas formation following addition of hydrogen peroxide. The coagulase activity was determined by clumping of bacterial cells after addition of plasma. Haemolytic activity was determined by streaking out bacteria on sheep blood agar (Oxoid, Thermo Fisher Scientific, Scoresby, Australia). Following incubation at 37°C for 24 hours the presence of a haemolysis zone was observed.

Auxotrophy determination

The auxotrophy type of bacteria was determined as previously described[395]. Briefly, a bacterial suspension adjusted to 1.0 McFarland units (approximately 3×10^8 CFU/ml) was diluted 1:100 in physiological saline. One hundred µl were spread on chemically defined medium agar[395]. Sterile disks were infiltrated with 10 µl of haemin, menadione and thymidine, respectively, and placed on top of the agar. Plates were incubated for 48 hours at 37°C. According to the growth zones around the disks auxotrophy was determined.

MIC determination

The colony suspension and broth microdilution method[374] were used to determine the MIC of deferiprone (Def, 3-hydroxy-1,2-dimethylpyridin-4(1*H*)-one, Sigma, Castle Hill, Australia) and

gallium-protoporphyrin IX (GaPP, Frontier Scientific, Logan, UT, USA) over 48 hours. The concentrations ranged from 0.08-40 mM Def (i.e. 10.8-5568 µg/ml) and 0.1-50 µg/ml GaPP. In addition, the MICs of ciprofloxacin (Cip), gentamicin (Gent), mupirocin (Mup), doxycycline (Doxy), chloramphenicol (Chlor), cephalexin (Ceph), vancomycin (Van), amoxicillin (Amoxi), and streptomycin (Strep) were determined (concentration range 0.06–32 µg/ml). All compounds were purchased from Sigma unless stated differently.

Bacterial growth

Bacteria were suspended in tryptone soya broth (Oxoid) and adjusted to an OD 600 of 0.01. Bacterial growth was measured in a 96-well plate over 40 hours at 37°C using an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA). Loops were taken after 24 and 40 hours to determine the colony morphology.

3c.6.3 Hydrogel preparation

Hydrogels were prepared by adequate mixing of dextran-aldehyde, succinyl-chitosan and a buffer solution, as previously described[284]. Def (20 mM) and/or GaPP (100 or 500 µg/ml) were incorporated in the gel and compared to blank gel and antibiotic loaded gel including 5 µg/ml Cip and 100 µg/ml Gent. Cip and Gent were chosen due to their clinical relevance as antibiotic therapies for respiratory tract, skin, blood, bone and soft tissue infections. Furthermore, fluoroquinolone antibiotics (such as Cip) were described as being highly effective against SCVs *in vitro* and *in vivo*[112,393].

3c.6.4 *In vitro* activity in the colony biofilm model

A bacterial suspension was prepared in 0.9% saline and adjusted to 1.0 McFarland units (approximately 3×10^8 CFU/ml). After diluting bacteria 1:1000 in tryptone soya broth, 2 µl were

spotted on UV-sterilised Whatman polycarbonate membrane filters (0.2 µm pore size, GE Healthcare, Little Chalfont, United Kingdom) and placed on tryptone soya agar[385]. Following biofilm formation after 24 hours incubation at 37°C, the membrane filters were transferred onto AB trace agar (minimal growth agar including 0.5% glucose and 0.5% peptone). One hundred microliters of freshly prepared hydrogel was placed on biofilms and incubated for 2.5 days at 37°C. The filter membranes were then aseptically transferred to new AB trace agar plates and incubated for 2.5 days at 37◦C. Finally, the filters were collected in PBS to extract bacteria by vortexing (1 min) and sonication (15 min), prior to serial dilutions and plating on tryptone soya agar for CFU counting and colony morphology determination after 3 days incubation at 37°C.

The antibiofilm effect of drug loaded gels was rated using the Bliss Independence Model[396,397]. The synergy of treatment combinations (Def-GaPP100, Def-GaPP100-Cip, Def-GaPP100-Gent, Def-GaPP500) was calculated according to Equation 2, with values above zero corresponding to synergistic effects.

$$S = \left(\frac{a}{MG}\right) * \left(\frac{b}{MG}\right) - \left(\frac{ab}{MG}\right) \tag{2}$$

S = synergistic effect, a = Log_{10} of biofilm after exposure to compound a, b = Log_{10} of biofilm after exposure to compound b, ab = Log_{10} of biofilm after exposure to treatment combination ab, MG = Log_{10} of untreated biofilm (maximum growth)

3c.6.5 *In vitro* activity in an artificial wound model

Hyaluronic acid (1.20-1.80 MDa, Lifecore Biomedical, Chaska, MN, USA) and collagen (Corning Incorporated, Corning, NY, USA) were used to prepare an artificial dermis[386], which was immersed in lyophilised bovine plasma, 19 ml Bolton broth (Oxoid), 1 ml horse blood and 10 IU of heparin. Subsequently, 10 µl of an overnight culture adjusted to $1x10^6$ CFU/ml was spotted on top of the dermis and incubated for 24 hours at 37°C. The formed biofilms were exposed to 150 µl gel (blank gel, Def, GaPP 500, Def-GaPP500 and Cip gels) for 24 hours at 37°C. Following a washing step, the

dermis was immersed in 10 ml of 0.9% saline to recover the bacteria by vortexing and sonication (alternating cycles of 3 x 30 sec), prior to serial dilutions and plating for CFU counting. In addition, the colony morphology was determined.

3c.6.6   *In vivo* activity in a *C. elegans* infection model

Synchronised nematodes, *Caenorhabditis elegans* AU37 (*glp-4*; *sek-1*), were grown to L4 stage, suspended in OGM medium (95% M9 buffer, 5% brain heart infusion broth, 10 µg/ml cholesterol) and added into 96-well plates with at least 20 worms per well[68]. Nematodes were infected with 25 µl of an overnight culture adjusted to $2x10^9$ CFU/ml in OGM medium and exposed to 25 µl of treatment (Def 20 mM, GaPP 500 µg/ml or a combination of both). Uninfected nematodes in OGM medium as well as infected but untreated nematodes were used as controls. The number of viable and dead nematodes was assessed every 24 hours over 3 days incubation at 25°C. Subsequently, nematodes were first washed in M9 buffer containing 1 mM sodium azide, then washed in PBS prior to counting. The nematodes were mechanically disrupted by vortexing the worms in microtubes with 1.0 mm silicon carbide beads for 10 minutes (BioSpec Products, Bartlesville, OK, USA). Serial dilutions of the supernatants were plated (tryptone soya agar with 7.5% NaCl) for CFU counting and colony morphology determination.

3c.6.7   Statistics and software

All experiments were conducted in triplicate and are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Results were analysed using two-way analysis of variance with Dunnett's test (GraphPad Prism version 7.02, GraphPad Software, La Jolla, CAL, USA). Statistical significance was assessed at the 95% confidence level.

3c.7 Results

3c.7.1 Characteristics of bacterial strains

The colony morphology of bacterial strains is shown in Figure 39 and the growth curves are displayed in Figure 40. A summary of bacterial characteristics is shown in Table 5.
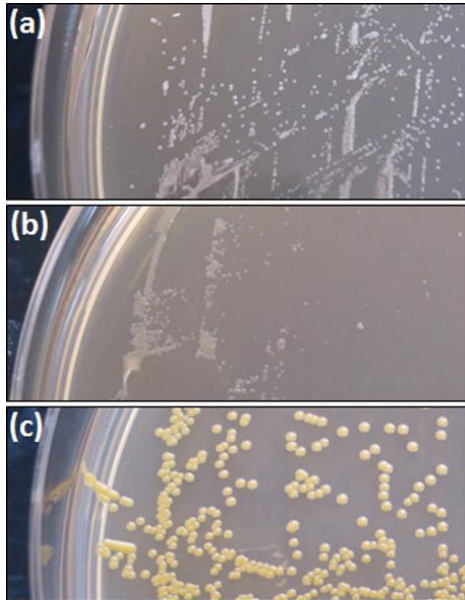
SCV1 was observed to be a catalase positive, coagulase positive and haemolysis positive strain with haemin/menadione auxotrophy. SCV2 was determined as a catalase negative, coagulase positive and haemolysis negative strain with thymidine auxotrophy. P1 was identified to be catalase positive, coagulase negative and haemolysis positive with haemin/ menadione auxotrophy.

Figure 39. S. aureus small colony variant SCV1 (a), SCV2 (b) and parent strain P1 (c).

As depicted in Figure 40, during 24 hours SCV1 and SCV2 showed a slower growth rate and a lower OD 600 value than the parent strain P1. While SCV1 reached stationary and decline phase after 24 hours, SCV2 continued to grow reaching an OD600 of 0.95 after 40 hours. Loops taken after 24 hours revealed a small colony morphology for both SCV1 and SCV2, while after 40 hours SCV2 presented as a mix of small colonies and very few normal sized colonies. SCV1 showed a small morphology after 40 hours.
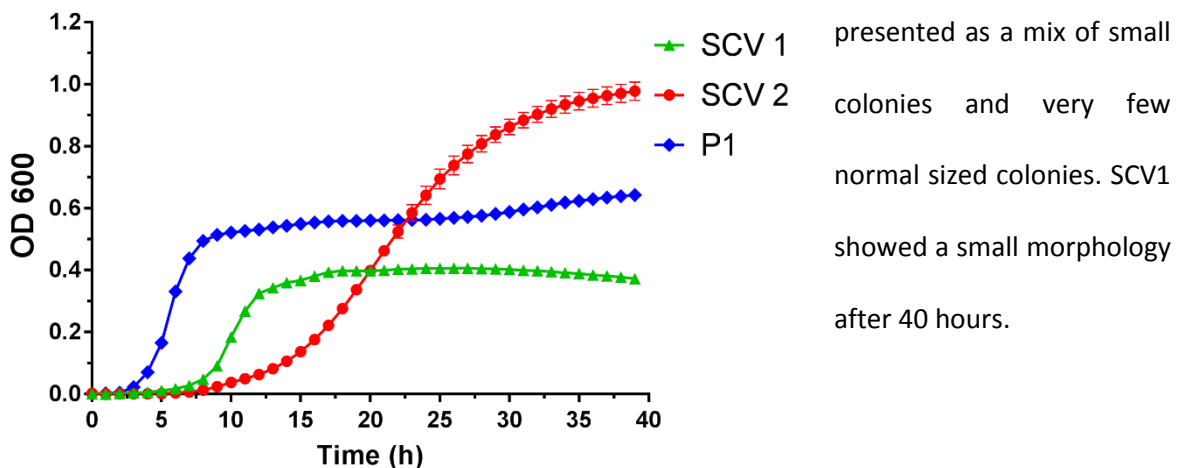
Figure 40. Growth curves of small colony variant SCV1 (green triangles), SCV2 (red dots) and parent strain P1 (blue diamonds).

SCV1 and SCV2 were 4- and 16-fold more susceptible to Def and 4- and 8-fold less susceptible to Cip and Gent compared to the parent strain P1. SCV1 and P1 had low MICs for GaPP (6.25 µg/ml and 12.5 µg/ml, respectively), while SCV2 was observed to have a MIC above 50 µg/ml for GaPP. The MICs for the Def-GaPP combination were typically lower than the MICs for individual compounds, however, the extent of this difference was strain-dependent.

As displayed in Table 5, SCV1 was susceptible to Mup and Van, and showed increasing MIC values for Ceph, Doxy, and Chlor, and was not susceptible to Amoxi and Strep. SCV2 was susceptible to Doxy, less susceptible to Chlor and not susceptible to Mup, Ceph, Van, Amoxi, and Strep. P1 showed low MIC values for Doxy and Mup, and increasing MICs for Van, Ceph, Amoxi, and Chlor, and was not susceptible to Strep.

Table 5. Characteristics of small colony variant SCV1, SCV2 and parent strain P1, including catalase, coagulase and haemolytic activity, auxotrophy type, as well as MICs (in µg/ml) of deferiprone (Def), gallium-protoporphyrin (GaPP), the combination of both compounds, ciprofloxacin (Cip), gentamicin (Gent), mupirocin (Mup), doxycycline (Doxy), chloramphenicol (Chlor), cephalexin (Ceph), vancomycin (Van), amoxicillin (Amoxi) and streptomycin (Strep).

	SCV 1	SCV 2	P1
Catalase	positive	negative	positive
Coagulase	positive	positive	negative
Haemolysis	positive	negative	positive
Auxotrophy	haemin/menadione	thymidine	haemin/menadione
Def	1392	348	5568
GaPP	6.25	>50	12.5
Def-GaPP	348/3.125	174/1.56	1392/12.5
Cip	2	4	0.5
Gent	16	16	2
Mup	0.25	>32	1
Doxy	4	0.5	0.25
Chlor	8	4	8
Ceph	2	>32	4
Van	1	>32	2
Amoxi	32	>32	4
Strep	>32	>32	32

3c.7.2 Colony biofilm model

The blank gel and the Def gel showed no antibiofilm activity (data not shown), while the GaPP gel demonstrated a concentration- and strain-dependent effect. Gel loaded with a low concentration of GaPP (100 µg/ml) showed a $\log_{10}$ reduction of 1.7 and 1.8 against biofilms of SCV1 and its parent strain P1, respectively, but no antibiofilm activity against SCV2 (data not shown), while at 500 µg/ml GaPP showed a $\log_{10}$ reduction of 4.3, 1.4 and 2.0 in SCV1, SCV2 and P1 biofilms (Figure 41). Cip and Gent loaded gels were observed to have only a minor effect against SCV1, SCV2 and P1 ($\log_{10}$ reduction of 0.4, 0.1 and 1.1, respectively for Cip and 0.2, 0.8 and 1.1, respectively for Gent). A small colony morphology of both SCV1 and SCV2 has been observed when analysing CFUs after treatment exposure.

The haemin auxotroph SCV1 (Figure 41a) showed increased susceptibility to gel loaded with Def-GaPP100-Cip, Def-GaPP100-Gent, GaPP 500 and Def-GaPP500 ($\log_{10}$ reduction of 3.4, 5.4, 4.3 and 4.4, respectively), and decreased susceptibility to monotherapy with Cip or Gent compared to its parent strain P1. Interestingly, Def-GaPP100 combined with Gent showed a high degree of synergy (Figure 42) and significant activity against the highly Gent-tolerant SCV1 compared to Gent alone and Def-GaPP100 ($\log_{10}$ reduction of 5.4 for Def-GaPP100-Gent *versus* 0.2 for Gent and 1.3 for Def-GaPP100, p<0.0001).

Figure 41. $\log_{10}$ reduction of small colony variant SCV1 (a), SCV2 (b) and parent strain P1 (c) colony biofilms after exposure to drug loaded hydrogels compared to untreated control. 1: Gentamicin (Gent) 100 µg/ml (light grey), 2: Ciprofloxacin (Cip) 5 µg/ml (purple), 3: Deferiprone (Def, 20 mM)-Gallium-protoporphyrin (GaPP) 100 µg/ml (blue), 4: Def-GaPP100-Cip (black), 5: Def-GaPP500 (red), 6: GaPP 500 (orange), 7: Def-GaPP100-Gent (dark grey). Data represent the mean ± SD of 3 biological replicates. O p<0.001 # p<0.0001 ns-not statistically significant

The thymidine auxotroph SCV2 (Figure 41b) showed elevated susceptibility to gel incorporating Def-GaPP100-Cip, Def-GaPP100-Gent, GaPP 500 and Def-GaPP500 (log_{10} reduction of 3.1, 1.7, 1.4 and 2.5, respectively), and no susceptibility to Cip or Def-GaPP100. Notably, Def-GaPP100 combined with Cip showed high synergy (Figure 42) and significant activity against SCV2, manifestly exceeding the effect of the individual compounds and Def-GaPP100 (log_{10} reduction of 3.1 for Def-GaPP100-Cip *versus* 0.1 for Cip and 0.1 for Def-GaPP100, $p < 0.0001$).

Against the haemin auxotroph P1 (Figure 41c) the combination of Def-GaPP100 gel with Cip or Gent showed significantly higher activity than the antibiotics alone (log_{10} reduction of 1.8 for Def-GaPP100-Cip *versus* 1.1 for Cip, $p < 0.001$; and 1.9 for Def-GaPP100-Gent *versus* 1.1 for Gent, $p < 0.0001$). However, no synergistic effect was observed compared to the Def-GaPP100 gel (1.8 log_{10} reduction). The highest activity against P1 was achieved with Def-GaPP500 gel (2.4 log_{10} reduction).
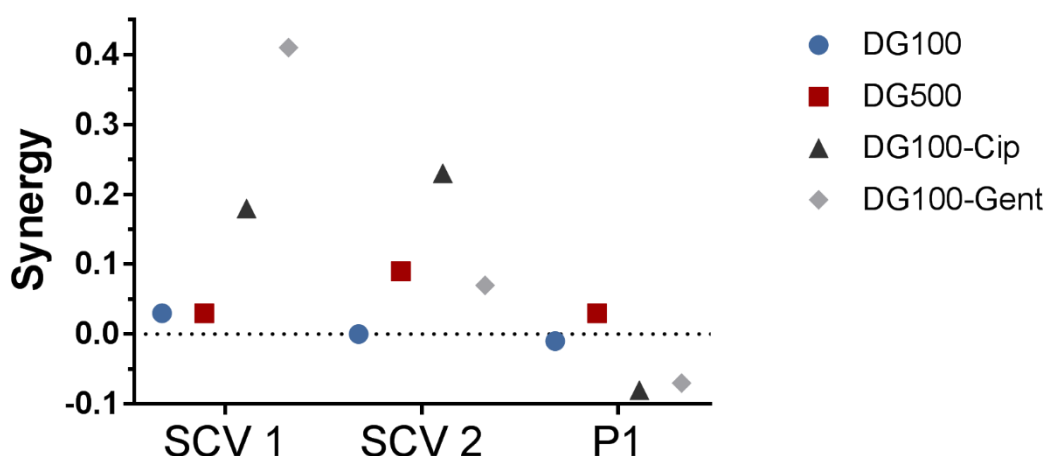
Figure 42. Synergy of treatment combinations against small colony variant SCV1, SCV2 and parent strain P1 colony biofilms. Deferiprone 20 mM (Def)-Gallium-protoporphyrin (GaPP) 100 μg/ml (blue circles), Def-GaPP500 μg/ml (red squares), Def-GaPP100-ciprofloxacin 5 μg/ml (black triangles), Def-GaPP100-gentamicin 100 μg/ml (grey diamonds). The higher the value the higher the degree of synergy.

3c.7.3 Macroscopic biofilm analysis

The antibiofilm activity of loaded hydrogels was macroscopically analysed over 5 days of treatment exposure. All biofilms grew extensively when exposed to blank gel and gel incorporating Def (not shown), Cip and Gent (Figure 43). A concentration- and strain-dependent antibiofilm effect was apparent after exposure to gels incorporating GaPP (not shown), Def-GaPP, Def-GaPP-Cip and Def-GaPP-Gent. While Def-GaPP100 gel moderately inhibited bacterial growth, the combination of Def-GaPP100 with either Cip or Gent resulted in a substantial antibiofilm effect (Figure 43).
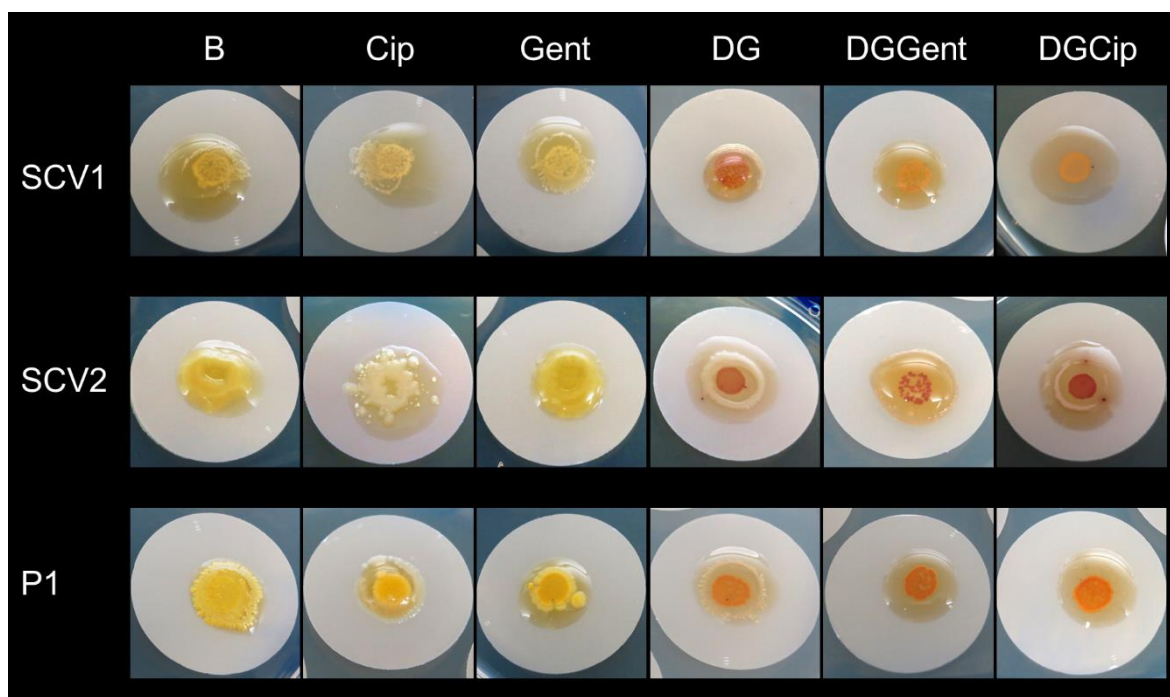
Figure 43. Inhibitory effect of drug loaded hydrogels on biofilms after 5 days exposure. Elevated biofilm inhibition was observed for gels containing deferiprone, gallium-protoporphyrin and ciprofloxacin or gentamicin (DGCip, DGGent). Strains used: Small colony variant SCV1, SCV2 and parent strain P1. Hydrogels- B, Blank control gel; Cip, Ciprofloxacin 5µg/ml; Gent, Gentamicin 100µg/ml; DG, Deferiprone 20 mM-Gallium-protoporphyrin 100µg/ml; DGGent, Def-GaPP100-Gent; DGCip, Def-GaPP100-Cip.

3c.7.4 Artificial wound model

Biofilms grown on an artificial dermis were exposed to drug loaded hydrogels to determine the antibiofilm activity in an *in vitro* wound model (Figure 44). The visual analysis of CFUs after treatment exposure confirmed a small colony morphology for the majority of both SCV1 and SCV2. The untreated control and the blank gel showed similar growth of all biofilms, indicating no

antibiofilm effect of the blank gel. The Def gel demonstrated substantial antibiofilm activity against SCV1 (log_{10} reduction of 0.9), but failed to be effective against P1 and SCV2. The GaPP 500 gel showed minor antibiofilm activity with a 0.2-0.4 log_{10} reduction. In contrast, Def-GaPP500 gel showed significant antibiofilm effects against SCV1, SCV2 and P1 with a 1.4, 1.0 and 0.9 log_{10} reduction, respectively, thereby demonstrating significantly higher activity than the individual compounds ($p < 0.05$-0.0001) and slightly higher activity than Cip gel (Figure 44).
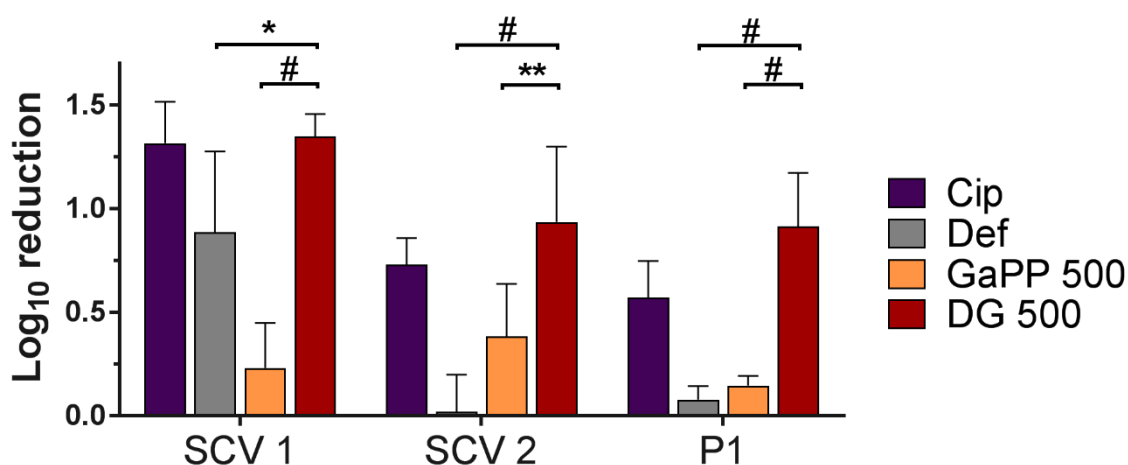
*Figure 44. Effects of hydrogels in an artificial wound model compared to untreated control. Log_{10} reduction of small colony variant SCV1, SCV2 and parent strain P1 after exposure to hydrogels loaded with ciprofloxacin 5 µg/ml (purple), deferiprone 20 mM (Def, grey), gallium-protoporphyrin 500 µg/ml (GaPP, orange) and Def-GaPP500 (red). Data represent the mean ± SD of 3 biological replicates.   * $p < 0.05$ ** $p < 0.01$ # $p < 0.0001$*

3c.7.5   *In vivo* infection model in *C. elegans*

Nematodes were infected with bacteria and their survival rate was determined with and without Def, GaPP 500 or Def-GaPP500 treatment (Figure 45). The worm killing was strain-dependent with 75%, 25% and 57% survival in SCV1, SCV2 and P1 infected worms, respectively, while uninfected controls showed 88% survival over 3 days. When worms were exposed to Def, 45%, 73% and 71% of SCV1, SCV2 and P1-infected nematodes survived, while 73%, 48% and 81% infected worms survived when treated with GaPP 500. The combination of Def-GaPP500 showed a similar survival rate in SCV1 and P1 infected worms as GaPP 500 alone (71% and 87% survival, respectively). In contrast, the survival rate of SCV2 infected worms was substantially higher (86% survival) when
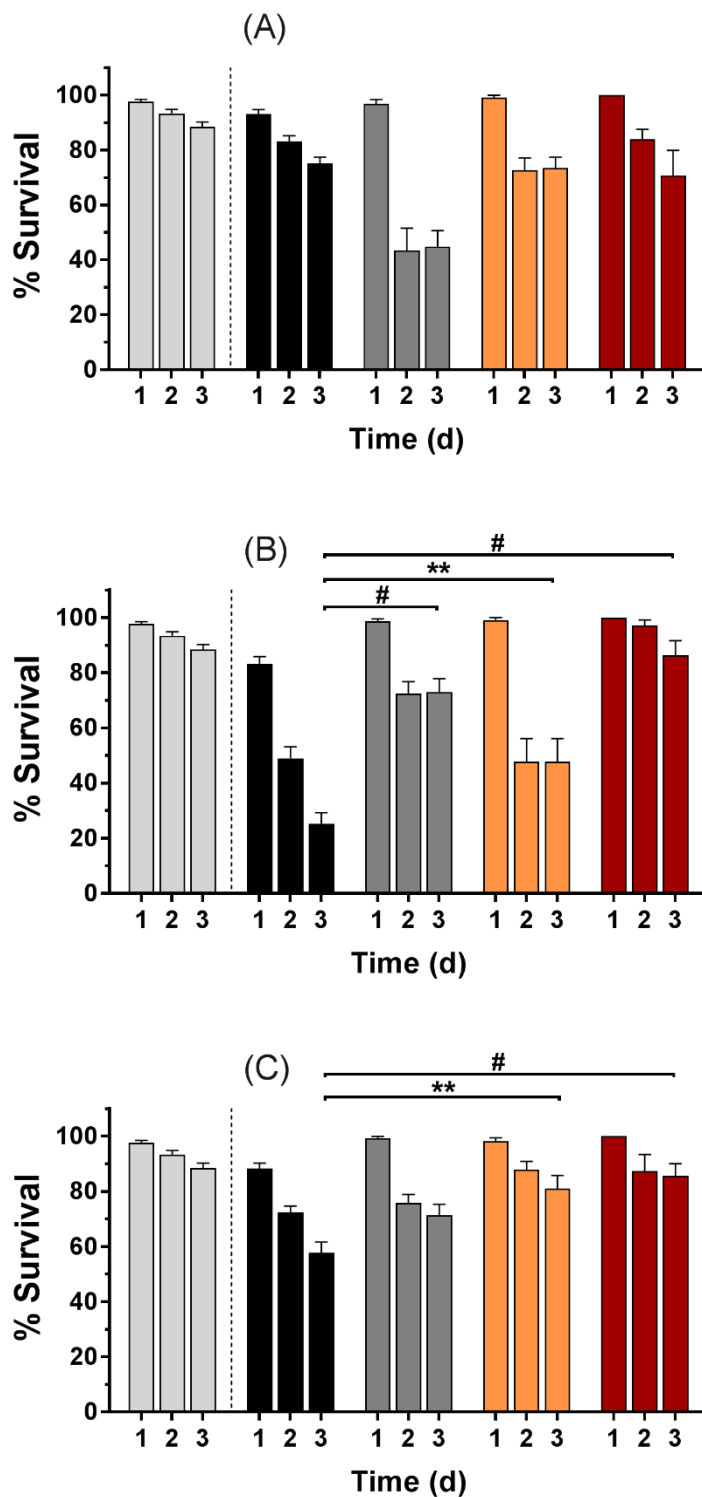
treated with Def-GaPP500 compared to the individual treatments. Compared to the uninfected control the Def-GaPP500 treatment achieved similar survival rates in SCV2 and P1 infected worms. Furthermore, the survival of SCV1 and P1 infected worms after GaPP 500 treatment was not different to uninfected controls (Figure 45).

*Figure 45. C. elegans survival (%) over 3 days in uninfected controls (light grey) and after infection (black bars) with small colony variant SCV1 (a), SCV2 (b) or parent strain P1 (c) and treatment with loaded hydrogels: deferiprone 20 mM (Def, dark grey), gallium-protoporphyrin 500 µg/ml (GaPP, orange) and Def-GaPP500 (red). Data represent the mean ± SEM of at least 6 biological replicates. ** p<0.01 # p<0.0001*

(A)

(B)

(C)
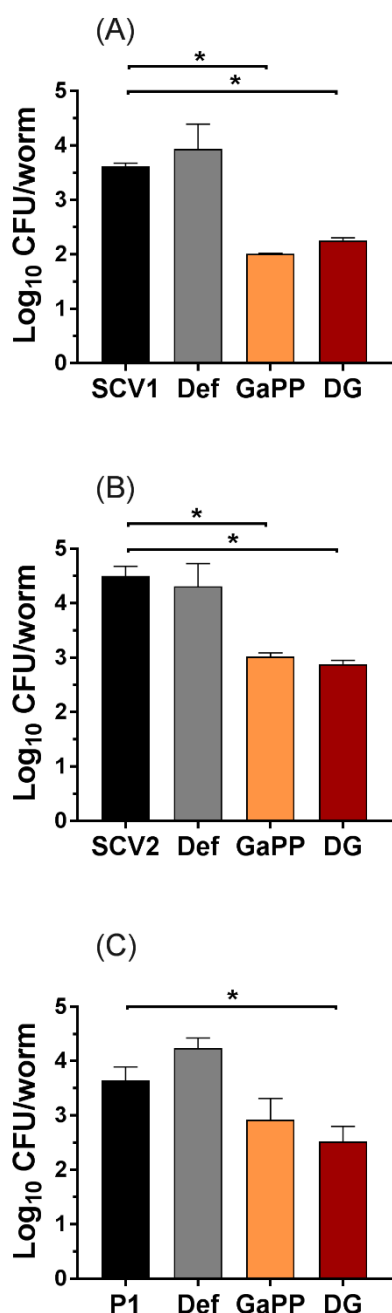
Following 3 days of infection the bacterial load per worm was quantified (Figure 46) by enumeration of CFU. Small colony phenotypes of SCV1 and SCV2 were observed. Consistent with results in the colony biofilm model and wound model, the treatment with Def alone showed no significant effect and failed to reduce the CFU per worm. In contrast, both GaPP 500 and Def-GaPP500 showed a significant ($p<0.05$) reduction of the bacterial load, resulting in a $\log_{10}$ of 2.0 for GaPP and 2.3 for Def-GaPP in SCV1 infected worms (SCV1 infection control: $\log_{10}$ of 3.6 CFU/worm), 2.9 and 2.5 in P1 infected worms (P1 infection control: $\log_{10}$ of 3.6 CFU/worm), and 3.0 and 2.8 in SCV2 infected worms (SCV2 infection control: $\log_{10}$ of 4.5 CFU/worm).

*Figure 46. $\log_{10}$ of CFU per C. elegans worm after 3 days infection (black bars) with small colony variant SCV1 (a), SCV2 (b) or parent strain P1 (c) and treatment with drug loaded hydrogels- Def: deferiprone 20 mM (grey), GaPP: gallium-protoporphyrin 500 µg/ml (orange) and DG: Def-GaPP500 (red). Data represent the mean ± SD of at least 6 biological replicates. * p<0.05*

3c.8 Discussion

S. aureus SCVs in planktonic and biofilm form have a global significance in the clinical environment being associated with treatment failure and recurrence of disease[37,112]. Treatments are mostly based on antibiotics, however, the low growth rate, reduced antibiotic susceptibility and emerging resistance of SCVs pose a challenge for efficient medical therapies. In the present study, an alternative treatment that relies on the disruption of bacterial iron metabolism was evaluated. As previously described, the iron chelator Def and the haem analogue GaPP show synergistic effects

144

against *S. aureus* biofilms *in vitro*[303]. Herein, the activity of Def-GaPP against planktonic and biofilm-associated SCVs and a parent strain with elevated antibiotic tolerance/resistance was evaluated *in vitro* and *in vivo*.

The Def-GaPP treatment exhibits antimicrobial activity based on the initial iron-chelation by Def, followed by iron depletion-induced upregulation of the bacteria's iron acquisition systems[46]. The latter are exploited by the haem analogue GaPP mimicking haem as the preferred iron source of *S. aureus*[319,321,337]. Inside bacteria, GaPP disrupts the iron metabolism vital for bacterial growth, survival and virulence[319,388]. Unlike haem, GaPP lacks the ability to transfer electrons, hence, GaPP cannot be part of redox reactions required for respiration, ATP production and DNA synthesis. Furthermore, GaPP cannot be cleaved by bacterial enzymes and cannot be utilised as nutrient source leading to starvation. In addition, GaPP is able to block efflux pumps essential for haem homoeostasis resulting in an intracellular accumulation of redox-active molecules[45,354]. The subsequent antibacterial effects are based on starvation, limited respiration and elevated production of reactive oxygen species contributing to DNA and protein damage and ultimately cell death.

As shown in the present study, the combination of Def and GaPP exhibited significant antibacterial and antibiofilm activity against clinical isolates of *S. aureus* SCVs and a parent strain. In the colony biofilm model, where compounds were delivered in a surgical wound gel, a dose-dependent antibiofilm effect of GaPP and Def-GaPP was observed (Figure 41 and 43). A colony biofilm comprises of a heterogeneous consortium of sessile cells with a mixture of metabolic active bacteria on the biofilm surface and metabolic retarded/inactive bacteria on the inside. The metabolic activity arises due to different oxygen levels (aerobic conditions on the biofilm surface, micro-aerobic/anaerobic inside the biofilm) and different nutrient availability according to the location bacteria occupy. While sessile bacteria on the biofilm surface can relatively easily be targeted, bacteria deeper in the biofilm are less exposed to compounds and show reduced susceptibility[385]. The blank gel and Def gel are thought to interfere only with the biofilm surface by attaching to

bacterial cell wall proteins and depriving nutrients, respectively, resulting in minor antibiofilm activity. Likewise, gels incorporating low concentrations (100 µg/ml) of GaPP showed limited activity, indicating an insufficient GaPP penetration into the colony biofilm and/or a suboptimal GaPP concentration. A higher GaPP concentration in gels (GaPP 500, Def-GaPP500) resulted in higher antibiofilm activity. It was observed that the presence of Def was crucial to facilitate a significant activity of GaPP against SCV2 biofilms, which is likely based on a Def-induced upregulation of haem acquisition systems elevating GaPP uptake into bacteria for an improved antibiofilm effect. Another reason for differences in susceptibility of the strains used in this study can rely on auxotrophy[112]. SCVs can be classified in two types of auxotrophs, namely haemin/menadione auxotrophs and thymidine auxotrophs. This in return affects the susceptibility towards antibiotics and other antibacterial compounds[112]. SCV2 was observed to be auxotrophic for thymidine, hence, less dependent on haemin/iron which can explain the poor GaPP susceptibility. SCV1 was shown to be haemin/menadione auxotrophic, thus, more susceptible to the iron depriving and haem mimicking Def-GaPP treatment.

SCVs are electron-transport-defective strains with a reduced transmembrane potential, which impedes the penetration and activity of membrane active compounds and some antibiotic classes, such as aminoglycosides and antifolate agents[133,398,399]. Therefore, Gent was expected to show low activity against both planktonic SCVs as confirmed in Table 5, and sessile SCVs as seen in Figure 41 and 43. Surprisingly, when Gent was combined with Def-GaPP, the triple combination showed significant antibiofilm activity (Figure 41 and 43), indicating a potentiation of Gent by Def-GaPP even against the Gent-tolerant SCV1 strain. This result implies that by disrupting bacterial iron metabolism Def-GaPP made the bacteria vulnerable and increased their susceptibility to Gent for a synergistic antibiofilm effect as confirmed in Figure 42. Similar results were observed for the treatment with Cip. In line with the literature, planktonic SCVs showed higher MIC values for Cip than the parent strain[400] and biofilm-associated SCVs were also poorly susceptible to Cip. In contrast, the combination of Def-GaPP-Cip showed synergistic effects (Figure 42) resulting in significantly higher antibiofilm activity, even when treatment with the individual compounds was

146

unsuccessful (Figure 41, SCV2). This confirms that the combination of drugs with different modes of action can be effective in treating bacteria with poor antibiotic susceptibility. The higher efficacy of the triple combinations Def-GaPP-Cip and Def-GaPP-Gent might rely on the synergy between reactive oxygen species (resulting from the Def-GaPP treatment) and antibiotics, as described elsewhere[112,401].

The antibiofilm activity of Def-GaPP was furthermore determined in an *in vitro* wound model where biofilms were grown on an artificial dermis and exposed to loaded gels. In line with results obtained in the colony biofilm model, Def-GaPP gel showed substantial antibiofilm effects, though to a lower extent. This may be the result of a nutrient-rich environment with blood/haem as favourable iron source. Bacteria recognise the tetrapyrrole ring of haem and GaPP, but can distinguish between both compounds[337,355]. Therefore, GaPP gel showed only minor antibiofilm activity, in contrast to the Def-GaPP combination. The Def-induced chelation may have deprived bacteria of nutrients and increased the uptake of GaPP as haem-mimicking agent for a substantial antibiofilm effect of this "Trojan Horse" compound.

In an *in vivo* infection model in the nematode *C. elegans* the antibacterial effect of Def-GaPP was assessed. It was observed that the survival rate of infected nematodes were strain-dependent (Figure 45). Literature described that haemin/menadione auxotrophic strains were less virulent than parent strains and thymidine auxotrophic strains[402]. In line with this the order of virulence in the present study was observed to be SCV1 (haemin/menadione auxotroph) being the least and SCV2 (thymidine auxotroph) being the most virulent strain (Figure 45). The auxotrophy type can influence the strain's susceptibility to antibacterial compounds. In the colony biofilm model SCV1 was more susceptible to GaPP and Def-GaPP than SCV2, which required the combination of Def-GaPP for a substantial antibiofilm effect. Consistent with these observations similar results were observed *in vivo* in the *C. elegans* model. While infected nematodes died over time due to bacterial colonisation, biofilm formation and toxin production[305], the exposure to Def-GaPP prolonged the lifespan of all infected nematodes (Figure 45) and reduced the bacterial burden per worm (Figure

46). Treatment with GaPP alone only increased the survival rate of SCV1 and P1 infected worms, but failed in SCV2 infected worms. Therefore, combining GaPP with Def appeared to be crucial for an elevated antibacterial activity against *S. aureus* SCVs of any auxotrophy type.

3c.9 Conclusion

The present study confirmed strong antibacterial and antibiofilm properties of Def-GaPP against *S. aureus* SCVs *in vitro* and *in vivo*. When applied in a surgical hydrogel, Def-GaPP has potential to complement the gel's wound healing properties with antibacterial and antibiofilm effects and could serve as an alternative treatment for biofilm and SCV-related infections. Due to the risk of emerging antibiotic resistance, which is in particular associated with prolonged antibiotic treatment, the combination of drugs with different modes of action is advantageous. The combination of Def-GaPP with antibiotics may facilitate a multi-pronged approach to increase the treatment efficacy against otherwise antibiotic tolerant/resistant *S. aureus* SCVs. While the results of the current pilot study are encouraging, the broader applicability of Def-GaPP for the treatment of SCVs derived from other species is on the way to be validated.

3c.10 Acknowledgements

3c.11 Funding information

had no role in study design, data collection, analysis and interpretation of data, or the decision to submit the work for publication.

3c.12   Disclosures

Competing financial interests: PJW holds a patent on the chitosan-dextran hydrogel. PJW and SV hold a patent application on the treatment combination of deferiprone and gallium-protoporphyrin. All other authors: none to declare.

Chapter 4.

Conclusion, future perspectives and translational prospects

The emergence and spread of antibiotic resistant bacteria over the past decades has large societal, economic and medical implications, posing a global threat to humans and putting a tremendous strain on health care systems worldwide. While novel antibiotics are urgently needed, the antibiotic pipeline in pharmaceutical industry contains few molecules with a novel mode of action. There is an unmet need for innovative therapeutic strategies not based on traditional antibiotics to combat and control antibiotic resistant bacteria. Several approaches have been proposed to date, however, there are complex scientific, technical, cultural, regulatory, diagnostic, and intellectual property hurdles to overcome before novel treatments can be commercialised. Generally only 20% of potential therapeutics that enter phase I clinical trials are ultimately approved for use in patients[403]. Treatment success is depending on multiple factors, such as the type and severity of disease, susceptibility and escape mechanisms of pathogens, drug delivery and the application route, applicability in clinical settings and economic factors.

The growing awareness about the role biofilms play in infectious diseases has increased the interest in the development of antibiofilm approaches to treat infections. These approaches aim to address the challenge that biofilms require up to 1000-fold higher drug concentrations for eradication than planktonic bacteria. Drug delivery via the oral route frequently cannot achieve the drug concentrations needed at the site of infection without causing severe side-effects and an imbalance of the gut microbiome. Depending on the type of biofilm infection, a topical treatment as alternative route can deliver higher drug concentrations to facilitate improved antibiofilm activity directly at the infection site. At the same time side-effects can be reduced, a healthy gut flora maintained, interactions with other drugs or food diminished and the first pass metabolism in the liver and potential drug-degradation in the gastro intestinal tract circumvented. However, topical treatments have their limitations and can only be used for a small range of biofilm infections, including chronic rhinosinusitis. Internal biofilm infections or device-related biofilm infections require different smart medicine approaches.

Novel antibiofilm strategies include new antimicrobials with higher antibiofilm activity as traditional antibiotics, discovery of novel antibiotic classes with a different mechanism of action to current antibiotics, agents that inhibit the bacterial attachment and biofilm formation, quorum sensing inhibitors, dispersing agents, compounds repurposed as antibiofilm agents or strategies that combine multiple mechanisms of action[93]. Although many studies have confirmed the *in vitro* and *in vivo* antibiofilm activity of innovative approaches, one of the biggest challenges is to develop a translational strategy suitable for an application in clinical practice and everyday life. Moreover, much needs to be learned about the implications of medical therapies on the human microbiome.

In this thesis, two alternative approaches for the topical treatment of biofilm-related infections have been described, namely colloidal silver nanoparticles and the treatment combination of deferiprone and gallium-protoporphyrin.

Colloidal silver nanoparticles

In chapter 2, the synthesis, physico-chemical properties and antibiofilm activity of colloidal silver nanoparticles of different morphology (i.e. spheres, cubes and stars) were described. After successful *in vitro/in vivo* evaluation, silver nanoparticle spheres were found to show significant antibiofilm activity against *S. aureus*, MRSA and *P. aeruginosa*, while being not toxic in two cell lines. Furthermore, extended studies over 6 months confirmed that silver nanoparticle spheres were physically stable in a "ready-to-use" suspension with no observed loss in antibiofilm activity over time.

This research has been instrumental for a phase I human clinical trial currently being carried out at The Queen Elizabeth Hospital, Woodville, SA, Australia. Following Good Laboratory Practice procedures an upscaled in-house production of silver nanoparticle spheres at the Basil Hetzel Institute for Translational Health Research/University of Adelaide has been implemented. The silver nanoparticle production is coordinated with clinicians to accommodate the demand required for the ongoing clinical trial that commenced in October 2016 (Trial number: AU/1/657826, title: The

effect of colloidal silver sinonasal rinses in recalcitrant chronic rhinosinusitis). The silver nanoparticles are applied as a nasal rinse after sinus surgery to defeat residual pathogens and prevent biofilm-associated recurrence of disease. Preliminary data showed a substantial reduction of the bacterial burden on the sinonasal mucosa following silver nanoparticle flushes. This is expected to considerably enhance the quality of life of chronically infected patients and delay/prevent revision surgery, thereby improving health of patients and reducing both direct and indirect healthcare costs. Clinical trial results will be analysed and published in the near future as part of another PhD project carried out at The Queen Elizabeth Hospital. Moreover, the impact of silver nanoparticle flushes on the sinonasal microbiome is under investigation.

This approach may be beneficial for the lives of approximately 3 million Australians suffering from recalcitrant chronic rhinosinusitis. Ultimately, the silver nanoparticles can be utilised for other topical infectious diseases, for example as a lavage for chronic wound infections and burn wound infections, thereby potentially improving the conditions of more suffering patients.

Def-GaPP

A fundamental step towards improvements in antimicrobial health care also includes the development and validation of treatment strategies with a non-conventional mode of action. Unaffected by traditional antibiotics, bacterial iron metabolism presents a unique alternative target that is vital for virtually all human pathogens. While bacteria have established resistance to antibiotics, iron is always required for bacterial survival and virulence.

In chapter 3, significant activity of the iron chelator deferiprone and the haem analogue gallium-protoporphyrin against *S. aureus* and other clinically relevant pathogens was described. Synergistic effects of Def and GaPP were shown against biofilms, small colony variants and multidrug resistant strains, which largely contribute to therapeutic failures, infection relapse and exacerbation. It was demonstrated that this novel treatment shows greater antimicrobial effects than routinely used antibiotics, while being not toxic in human cell culture. This research has been instrumental for the

approval of a patent, which is currently in PCT phase (international application number: PCT/AU2016/050811).

Furthermore, a formulation to apply Def and GaPP in a clinical setting was investigated. To date, sinus surgery is an inevitable intervention for the treatment of recalcitrant chronic rhinosinusitis. In clinical practice of surgeons in our Department a chitosan-dextran gel is instilled in the sinuses post-surgery to aid wound healing, while corticosteroids and oral antibiotics still remain the standard follow up treatments. However, these therapeutic options are associated with severe side effects and treatment failure due to emerging antibiotic resistant bacteria.

By incorporating Def and GaPP into the wound healing gel, the properties were complemented with strong antimicrobial activity without the utilisation of antibiotics and corticosteroids. The Def-GaPP gel is nontoxic, biocompatible, biodegradable and facilitates post-operative wound healing by promoting homoeostasis. In an artificial wound model, the Def-GaPP gel showed significant antibiofilm activity against clinically relevant biofilms and SCVs. Moreover, strong activity of Def-GaPP against both intracellular and extracellular SCVs in a human bronchial epithelial cell infection assay with gentamicin-induced *S. aureus* SCVs was observed in preliminary studies. Importantly, Def-GaPP combined with gentamicin resulted in complete eradication of the highly gentamicin-tolerant SCVs *in vitro* (Figure 47, unpublished). This finding highlights the potential of Def-GaPP as novel treatment against intracellular SCVs.
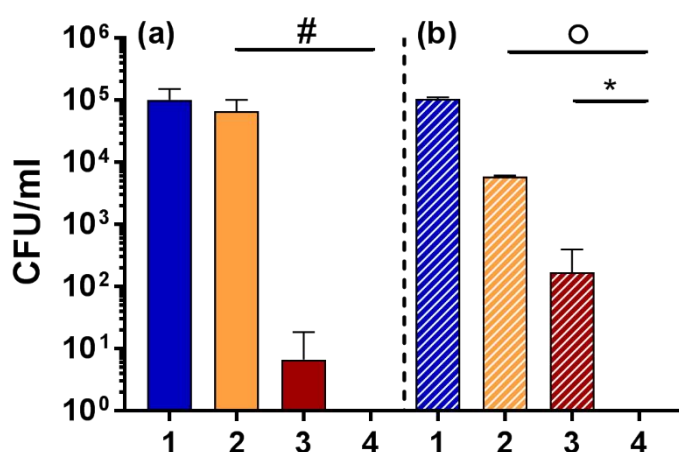
Figure 47. Number of (a) intracellular and (b) extracellular SCVs in a human bronchial epithelial cell infection assay (1: untreated control) after treatment with 2: gentamicin 100 µg/ml, 3: deferiprone 20 mM + gallium-protoporphyrin 100 µg/ml, 4: combination of 2 and 3. Data represent the mean ± SD of 3 biological replicates.   * p<0.05   O p<0.001   # p<0.0001

An animal study has been carried out in a sheep model of sinusitis that confirmed *in vivo* safety and efficacy of Def-GaPP gel. The results of this study will be published in the near future as part of another PhD project carried out at The Queen Elizabeth Hospital (manuscript currently in preparation). The gel delivers Def and GaPP directly to the site of infection, thereby combating residual bacteria without causing side effects. Furthermore, the topical application prevents both drug degradation in the gastro intestinal tract and interactions with medical treatments and food, which could diminish the antibacterial activity of Def and GaPP. The translation of Def-GaPP gel into a pilot study in humans is envisaged in the near future, initially focussing on the treatment of chronic rhinosinusitis and the effect on the sinonasal microbiome. The gel can be endoscopically instilled in the sinuses and has potential to prevent the progression of disease and advance clinical outcomes after sinus surgery. This can improve the quality of life of 3 million chronic rhinosinusitis patients in Australia and reduce healthcare costs associated with surgery and follow up treatments.

Ultimately, this topical strategy can be refined and tailored for a wide range of biofilm and SCV-related diseases, such as implant associated infections and as a wound dressing for chronic and burn wound infections. Furthermore, Def-GaPP could be incorporated in other drug delivery systems and pharmaceutical formulations to engineer the drug release, improve drug solubility and stability, and increase bioavailability for an enhanced antimicrobial activity.

Developing proof-of-concept by elucidating the activity of Def-GaPP against more pathogens and SCVs than the ones described in this thesis is warranted. In addition, the correlation between the efficacy of Def-GaPP and iron levels, as well as the effect of Def-GaPP on host cell iron metabolism and cell functionality needs to be explored. Furthermore, the underlying mechanism of action of Def-GaPP on a molecular level remains to be investigated. The molecular understanding of bacterial and host cell responses to Def-GaPP and the implication of their interactions is essential in order to translate this innovative treatment for biofilm and SCV-associated infections towards phase I clinical trials.

Based on the activity against multidrug resistant strains and intracellular SCVs, this novel approach has potential to contribute tackling the looming threat of antibiotic resistance. Collaborative practise and inter-professional care therefore embrace the development of the innovative antimicrobial treatment resulting from this thesis. A rapid translation of novel insights from research to practice has potential to supply health care providers with an innovative therapy enhancing treatment options for recalcitrant, debilitating infections arising from biofilms and SCVs.

Multi-pronged strategies

Finally, an exciting option is combining several novel approaches outlined in this thesis. For example, the combination of Def, GaPP and HAM was observed to exceed the antibiofilm activity of the individual compounds and the antibiotic control against *S. aureus* biofilms (Richter and Coenye, Figure 48, unpublished), indicating that such combinations may lead to increased antibiofilm activity.
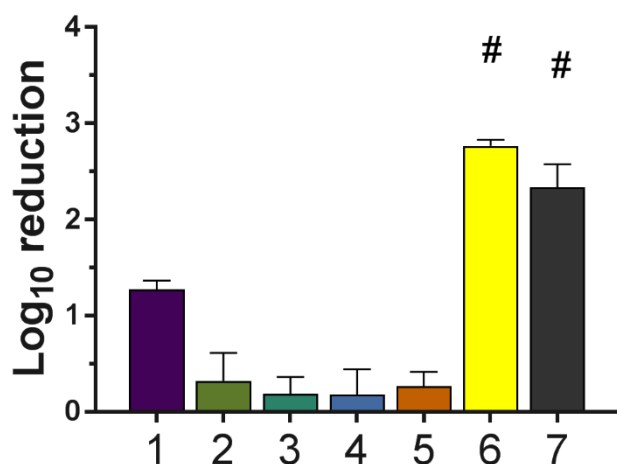
Figure 48. Log_{10} reduction of S. aureus colony biofilms after exposure to loaded hydrogels compared to untreated controls. 1: ciprofloxacin 5 µg/ml (Cip), 2: deferiprone 20 mM (Def), 3: gallium-protoporphyrin 100 µg/ml (GaPP), 4: Def-GaPP 100, 5: hamamelitannin 250 µg/ml (HAM), 6: Def-GaPP-HAM, 7: Def-GaPP-Cip. Data represent the mean ± SD of 3 biological replicates. Statistical comparison to Cip-loaded gel.   # p<0.0001

Moreover, the ability to potentiate the effect of antibiotics with Def-GaPP is another interesting path. As described in chapter 3, Def-GaPP increased the activity of ciprofloxacin and gentamicin even against strains with elevated antibiotic tolerance/resistance. The possibilities to combine different compounds are manifold and combination therapies with Def-GaPP may open the road towards further pharmaceutical developments to expand the medical armamentarium against biofilms and SCVs.

Combination therapies offer high potential to improve antimicrobial activity while reducing the risk for resistance. By selecting compounds with different mode of action, combination therapies are able to target pathogens from various sides, combining the advantages of single compounds for a potentially higher efficacy. Such approaches could target active and dormant cells, breakdown the biofilm matrix and effectively kill vulnerable bacteria, inhibit intercellular communication and destroy uncoordinated microbes, disperse bacteria and control the residual as well as the released cells. Moreover, by using combination therapies conventional antibiotics could regain their efficacy. A multi-pronged approach of different technologies may bring the urgently needed help to fight the emerging threat of multidrug resistant bacteria encountered in clinical practice. A plethora of novel antibiofilm strategies are available, the remaining challenge is to translate these ideas into pharmaceuticals for utilisation in every day practice.

Appendix

Publication: "Innovative approaches to treat *Staphylococcus aureus*

biofilm-related infections"[1]

Statement of authorship

Title of Paper	Innovative approaches to treat *Staphylococcus aureus* biofilm-related infections
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Richter K, Van den Driessche F, Coenye T. Innovative approaches to treat Staphylococcus aureus biofilm-related infections. Essays in Biochemistry. 2017;61(1):61-70.

Principal Author

Name of Principal Author (Candidate)	Katharina Richter		
Contribution to the Paper	Conception and design of the project, drafting the article		
Overall percentage (%)	60		
Certification:	This paper presents an invited review article I wrote during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	7/4/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

    i.   the candidate's stated contribution to the publication is accurate (as detailed above);

    ii.   permission is granted for the candidate in include the publication in the thesis; and

    iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Freija Van den Driessche		
Contribution to the Paper	Drafting the article		
Signature		Date	10/4/2017

Name of Co-Author	Tom Coenye		
Contribution to the Paper	Conception and design of the project, drafting the article, critically revising the article		
Signature		Date	10/4/2017

Publication title page

PORTLAND PRESS

Review Article

Innovative approaches to treat *Staphylococcus aureus* biofilm-related infections

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Many bacterial infections in humans and animals are caused by bacteria residing in biofilms, complex communities of attached organisms embedded in an extracellular matrix. One of the key properties of microorganisms residing in a biofilm is decreased susceptibility towards antimicrobial agents. This decreased susceptibility, together with conventional mechanisms leading to antimicrobial resistance, makes biofilm-related infections increasingly difficult to treat and alternative antibiofilm strategies are urgently required. In this review, we present three such strategies to combat biofilm-related infections with the important human pathogen *Staphylococcus aureus*: (i) targeting the bacterial communication system with quorum sensing (QS) inhibitors, (ii) a 'Trojan Horse' strategy to disturb iron metabolism by using gallium-based therapeutics and (iii) the use of 'non-antibiotics' with antibiofilm activity identified through screening of repurposing libraries.

Richter, K., van den Driessche, F. and Coenye, T. (2017). Innovative approaches to treat Staphylococcus aureus biofilm-related infections. *Essays in Biochemistry,* 61(1), 61-70.

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