THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Amphiphilic hydrogels functionalized with antimicrobial peptides for wound care

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Cover: An illustration of a hydrogel sheet, and a zoomed in section of the surface of the hydrogel functionalized with antimicrobial peptides, which kills a bacterial cell when it comes in contact with the surface.

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Abstract

Bacteria have the potential to cause significant harm to us when found in places where we do not want them, such as in wounds. Through the last century, the gold standard of eradicating bacteria in these cases has been with antibiotics. However, this treatment is at the risk of being severely impeded by antibiotic resistance, requiring alternative ways of dealing with bacteria. Antimicrobial peptides (AMPs) have shown promise as such an alternative with its broad-spectrum and rapid antibacterial activity, with a lower risk of inducing resistance.

The aim of this thesis was to investigate the covalent attachment of AMPs to the surface of Pluronic F127 based amphiphilic hydrogels and validate the same *in vitro*, *in vivo*, and clinically for wound care applications. A few variants of the material were created, with hydrogel discs being the most common as a convenient base for evaluation. The material was also prepared as a wound dressing where it was evaluated in a human intact skin study for a clinically relevant investigation. Furthermore, the material was also made into particles as a platform for treating deeper wounds as well as in liquid formulation or as a coating.

Overall, the AMP-functionalized hydrogels showed a potent antibacterial activity against both gram-negative and gram-positive bacteria, and some antibiotic resistant strains among them. At the same time, the materials did not show any signs of cytotoxicity against fibroblasts or erythrocytes. Furthermore, the AMP-functionalized hydrogels showed a potential to reduce the endotoxin levels released by *Pseudomonas aeruginosa*, a property that might assist further with combating the adverse effects of a wound infection and improve healing outcomes. The material also showed a significant antibacterial effect against the bacteria naturally present on our skin when evaluated clinically on healthy volunteers.

A main limitation behind the clinical use of AMPs is that they have a low biostability and are rapidly degraded by proteolytic enzymes. By covalently attaching the AMPs to a solid substrate they should gain steric protection against degradation. That was also the case observed for the AMPs when covalently attached to the hydrogels, as they retained their antibacterial activity for several days, both in serum, and implanted in an infected rat model. The covalent attachment of the AMPs also resulted in a contact killing mechanism, suitable for a local antibacterial effect.

Keywords: Antimicrobial peptides, antibacterial surfaces, hydrogels, amphiphilic polymers, lyotropic liquid crystals, wound care, infections, surface functionalization

LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text, * indicate shared first authorship:

I **Antimicrobial peptide-functionalized mesoporous hydrogels**
Saba Atefyekta*, Edvin Blomstrand*, Anand K. Rajasekharan, Sara Svensson, Margarita Trobos, Jaan Hong, Thomas J. Webster, Peter Thomson, Martin Andersson
ACS Biomaterials Science & Engineering 7.4 (2021): 1693-1702.

II **Cross-linked lyotropic liquid crystal particles functionalized with antimicrobial peptides**
Edvin Blomstrand, Anand K. Rajasekharan, Saba Atefyekta, Martin Andersson
International Journal of Pharmaceutics 627 (2022): 122215.

III **Clinical investigation of use of an antimicrobial peptide hydrogel wound dressing on intact skin**
Edvin Blomstrand, Saba Atefyekta, Anand K. Rajasekharan, Martin Andersson
Journal of Wound Care 32.6 (2023): 368-375.

IV **Antibacterial and hemolytic activity of antimicrobial peptides in solution and attached to a hydrogel surface**
Edvin Blomstrand, Elin Posch, Annija Stepulane, Anand K. Rajasekharan, Martin Andersson
Submitted manuscript.

V **In vivo biostability and endotoxin binding properties of a hydrogel functionalized with antimicrobial peptides**
Edvin Blomstrand, Saba Atefyekta, Anand Rajasekharan, Sara Svensson, Margarita Trobos, Peter Thomson, Martin Andersson
Manuscript in preparation.

CONTRIBUTION REPORT

I Made all materials and sample preparation, performed the LIVE/DEAD antibacterial evaluation, zone of inhibition, serum stability assessment, and MTT assay, also performed the data evaluation of the same. Assisted during the *in vivo* pilot study and assisted the writing.

II Made all materials and sample preparation and performed all antibacterial evaluation, serum stability, SAXS as well as the hemolysis and cytotoxicity evaluation. Assisted during the following experiments: DLS, Raman, FTIR, and Cryo-EM. Performed data analysis for all tests, wrote the first draft and was responsible for the manuscript.

III Made all materials and sample preparation, was involved in the planning, and had the role of collecting the data in the intact skin study. Performed the data analysis of all tests, wrote the first draft and was responsible for the manuscript.

IV Made all materials and sample preparation and performed all tests except for the MIC study (which I instead supervised), and the 2D surface Zeta potential measurements. Performed all data analysis, wrote the first draft and was responsible for the manuscript.

V Made all materials and sample preparation, performed the endotoxin tests, and was involved in planning of *in vivo* study and had an assisting role during the surgeries and data collection. Performed all data analysis, wrote the first draft and was responsible for the manuscript.

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1 Introduction

Microbes are the oldest form of life found on Earth. While being the least complex form of life, the sheer quantity of them, together with random mutations, have allowed them to adapt to the most extreme environments and situations. Humans have only existed during a fraction of the timeframe of microbes, and our relationship is complicated to say the least. Bacteria are most certainly the class of microorganism with which we have the most interactions. While some companies have devoted all their resources to kill bacteria as efficiently as possible, others sell dairy products with added bacteria in order to improve your well-being. We also like to use them to refine things such as sour dough bread and wine. So are bacteria good, bad, or just ugly? Well, it depends on the situation. The most well-known case of a mutualistic relation is that of more than a kilogram of bacteria we have living in our guts [1]. We provide them with a safe secluded environment with a steady flow of nutrients, and they digest food that we have difficulties dealing with ourselves into components we need in order to stay healthy.

The problems arise when the wrong bacteria end up in places where we do not want them. If opportunistic pathogenic bacteria find their way into a wound, for instance, they will try and colonize the new habitat [2]. This will of course not go unchallenged, as our bodies have elaborate immune systems whose main job is to take care of instances like this. Our defense systems are equipped with both an innate immune response that rapidly deals with intruders with a broad-spectrum, utilizing non-specific targeting, and an adaptive immune system that is dealing with repeat offenders in a more specific manner. However, when the immune system

is compromised, or when the intruding bioburden is too large, an infection will occur, and we might need help to not fall severely ill.

Humans do not have the numbers or turnover to deal with external factors in the fantastic way that bacteria can mutate and thrive. Instead, we must take to knowledge and technology. By learning how different microbes behave and infect us, we have changed our ways and reduced the occurrence of numerous diseases. Where infections still happen, scientific discoveries such as antibiotics have been instrumental to assist our bodies in killing off the intruders. However, the ability of microbes to push through a challenge by numbers and random mutations have ever since the introduction of penicillin over 100 years ago gained resistance against it, as well as many antibiotics that have come after. That is why alternatives to antibiotics are in high demand and constitute such a large research area as it is today.

There are thankfully a range of innovative approaches, and most come with benefits but also limitations. The substance utilized in this work is called antimicrobial peptides (AMPs for short). These are originally a part of our, and most other multicellular organism's, innate immune response [3]. That means that they need to have a broad-spectrum activity and a non-specific target, as they are meant to be able to deal with unknown intruders of all sorts and shapes. This target is frequently the actual membrane of the microbes [4]. However, there are a lot of different AMPs and a lot of different suggested mechanisms of action, but what most of them have in common is just that they are membrane active. Broad-spectrum and rapid antimicrobial activity are of course very nice benefits for an antibacterial compound to have, but as mentioned before, most approaches also come with limitations. AMPs are no exception. One limitation is that AMPs are very sensitive to compounds found in our plasma and do not have a very long lifespan in our bodies [5]. A narrow therapeutic window before toxicity can also be an issue that needs to be considered. The research being performed on AMPs is therefore usually addressing these issues, which is also the case in this thesis. The approach utilized in this work is to covalently attach the AMPs to the surface of a material in order to protect them.

More specifically, the AMPs in this work are attached to soft hydrogels made from cross-linked amphiphilic polymers. This material mainly has uses within biomedical applications, which is also the intended area for its implementation. For instance, hydrogels have been found to have several benefits within wound care, as the soft material with a high-water content have shown to provide a moist environment, known to promote wound healing, and also provide pain relief [6, 7]. Furthermore, while attaching AMPs to a surface will severely limit their degree of freedom, by attaching the AMPs to a soft material there is potential to retain more mobility compared to being attached to a stiff material like titanium.

This thesis will first go through some background of the areas mentioned above, followed by brief explanations of the methodology used in the papers the thesis is based on. At the end, key results, and discussion of the same is presented.

2 Aims and objectives

The work summarized in this thesis had the overall aim of investigating the covalent attachment of antimicrobial peptides to the surface of amphiphilic hydrogels with wound care as the intended field of application. Furthermore, some additional desired properties for the developed materials are also discussed. These properties primarily focused on three aspects: ensuring no release of AMPs for a local contact-killing antibacterial effect; maintaining no toxicity towards our own cells; and providing AMP functionalization that ensured higher stability in the presence of plasma components compared to free AMPs. The thesis will therefore also discuss the importance of these features as well as the influence of different properties of the AMP and hydrogel on the final material. The different papers that this thesis is based on investigates different important aspects in the development of antibacterial materials. The individual aims of each paper were as follow:

For **Paper I** the main aim was to investigate whether the covalent attachment of a specific end-tagged AMP to an amphiphilic hydrogel would result in an effective contact killing antibacterial material. Attempts to gain information on the mechanism of action was also a goal as well as to investigate proteolytic stability of the functionalization.

The same concept was utilized in **Paper II**, where the main objective was to investigate the potential of the hydrogel and AMP combination to be used as a particle system. This was evaluated by material characterization, as well as investigation of mode of action, antibacterial potency, serum stability, and toxicity.

For **Paper III,** the concept from **Paper I** was developed into a material that had the intended use as a wound dressing. The aim of this paper was to evaluate whether the contact killing mechanism of the functionalized hydrogels could decrease the bioburden found naturally on healthy intact human skin. Another objective of the study was to evaluate the biocompatibility of the developed material.

The goal of **Paper IV** was to investigate the role of different AMPs with varying molecular structures, and how they affected the AMPs antibacterial potency and hemolysis, when free in solution compared to when attached to the amphiphilic hydrogel discs.

Finally, **Paper V** had two main objectives. The first objective was to evaluate the biostability of the AMP functionalized hydrogels described in **Paper I**, in an infected rat model. The second objective was to investigate the interaction between endotoxins released by pathogenic gram-negative bacteria and the hydrogels functionalized with AMP, as endotoxins can cause considerable harm for a host if released in high loads.

3 Background

3.1 Bacteria – the coreless wonder

Only a few microns in size, bacteria might be the first and simplest form of life on Earth. This does not stop them in the slightest from inhabiting nearly all habitats on earth. They can both thrive as individual units or as communities, even between species. Without a nucleus, their genetic material is typically based on a single circular chromosome as well as plasmids. The fact that bacteria proliferate at a rapid pace and that some bacteria can share their plasmids through horizontal gene transfer have led to a vastly diverse and ever-changing form of life [8]. This does pose a certain challenge for our human fondness of categorizing. For instance, two bacterial cells within the species we have called *Escherichia coli* can have around 30% variation in their genome across their own species [9]. The varied genetic material does of course result in bacteria with properties just as varied. Nonetheless, there is one major characteristic that we tend to divide the bacteria in which is based on a physical trait. Their outer shell tends to take one out of two forms. We call these two forms gram-negative or gram-positive based on a technique that differently stains these, first developed in the 1800s [10]. A very simplified graphic of the major components found in the cell envelope of gram-negative and gram-positive bacteria is presented in Figure 1, along with a simplified graphic of the membrane of our own cells, to highlight the differences.

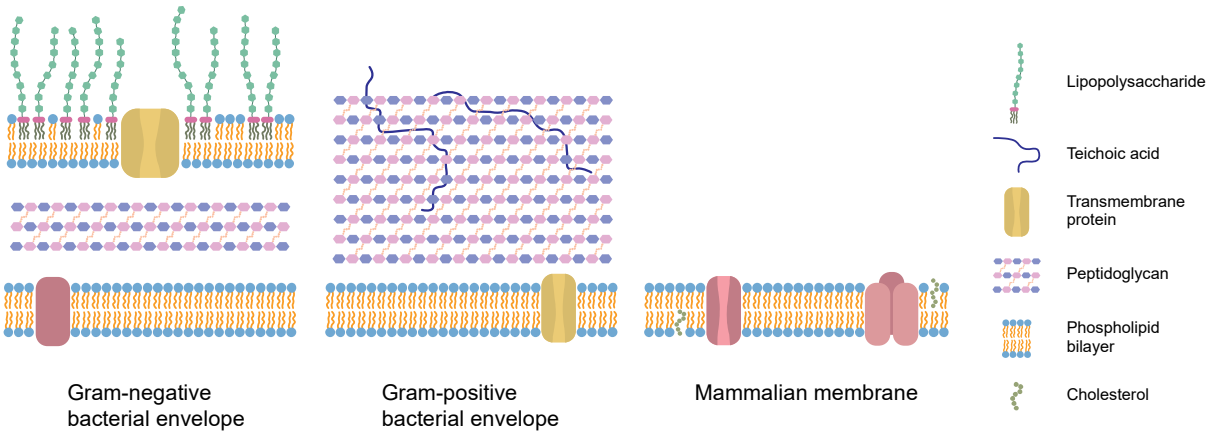

Figure 1. A simplified graphic highlighting the major differences of the cell envelope of gram-negative bacteria (left), gram-positive bacteria (middle), and the membrane of mammalian cells (right).

Gram-negative bacteria have two membranes with a thin peptidoglycan layer in between. The outer membrane has quite a different composition compared to the inner membrane, with more porins for transport as well as being packed tight with lipopolysaccharides on the outer surface [11]. Gram-positive bacteria, on the other hand, completely lack a second outer membrane but instead possess a much thicker peptidoglycan layer for rigidity. The peptidoglycan layer is further supported by the presence of teichoic acid, a copolymer that assist with regulation of the cell wall shape and cell division [12]. The added layer above the inner membrane is vital for bacteria as they rely on it for structural integrity, and a transmembrane potential for osmotic regulation, among other functions [11]. The destruction of the outer membrane or peptidoglycan layer is therefore a death sentence for the bacteria. The membrane of our own cells looks quite bleak in comparison, but we rely on having friendly neighbors (our other cells) compared to bacteria that need to be able to exist in inhospitable environments. A key feature of high importance for this thesis, is that the lipopolysaccharides of the gram-negative bacteria and the teichoic acid of the gram-positive bacteria provides the bacteria with a net negative charge. This is not found for mammalian cells as the outer surface of the membranes consists of zwitterionic phospholipids giving in an overall neutral characteristic.

3.2 Bacteria and humans

As long as humans have walked the earth, bacteria have been floating around, and within us. There is even compelling evidence that the mitochondria, famously the powerhouse of the cell, originally was a bacterial cell that got engulfed by a bigger cell. But instead of being broken down as substance, a symbiotic relationship was formed [13]. In the coexistence between animals and bacteria, there are still symbiotic relationships found between organisms. For instance, in our guts and on our skin, resides quantities of bacteria similar to the number of cells that make up our whole bodies. The gut microbiota, helps us breaking down

macromolecules into components vital for our metabolism and immune system [14]. In return, we provide the bacteria with a cozy and safe environment with a steady inflow of nutrients. On our skin, bacteria coexists together with some species of yeast and viruses which make up the skin microbiota [15]. If the skin microbiota is disturbed this can give rise to a number of diseases [16]. This is mainly due to the fact that more pathogenic microorganisms will inhabit the skin if the commensal microbiota is removed.

The skin microbiota in healthy individuals is dominated by gram-positive bacteria. While the composition will vary depending on body site, as moist, dry or oily skin will be preferential for different species, there are three genera most likely to top the charts: *Propionibacterium*, *Staphylococcus*, and *Corynebacterium* [15, 17]. The most prominent species of *Propionibacterium* is *P. acne* (also known as *Cutibacterium acnes*), which mainly resides in the sebaceous glands of hair follicles. As the name suggests, it has been linked to acne vulgaris, but is generally considered as a commensal bacteria [18]. From the *Staphylococcus* genus, *S. epidermidis* is without a doubt the most common species to inhabit our skin. In healthy humans they can be mutualistic as it has been shown that *S. epidermidis* actively fight off more pathogenic bacteria, and even killed the more pathogenic *Staphylococcus aureus* when they were reintroduced to patients with atopic dermatitis [19]. While *S. aureus* is part of the microbiota, they are more or less contained to the nasal microflora of healthy individuals and not the whole population carries it [18]. Species from *Corynebacterium* mainly reside in moist environments like the armpits, behind the knee, and the crease of the elbow [17]. *Corynebacterium* appear to be less involved with adverse conditions in humans than other parts of the skin microbiota, but some evidence points towards some role of commensalism by keeping more pathogenic bacteria away [20]. In general, a healthy microbiota leads to a healthy human, but bear in mind, a lot of seemingly harmless bacteria are opportunistic pathogens.

As most people are well aware of, the coexistence between humans and bacteria is not always sunshine and rainbows. A strong driving force for bacteria is to spread and colonize new habitats. They can spoil our food and infect our bodies unless counteractions are taken. Even *S. epidermidis*, that appear to be friendly in their protection of the skin, is one of the leading causes behind biomaterial-associated infections [21]. The skin is our first line of defense, as it creates a barrier between our tissues and the outside environment. As we cannot exclude ourselves completely from the world, there are other defensive mechanisms to attempt to remove pathogens. You get tears if you get something in your eye, your mucus in your throat trap microbes and irritants and you cough to get it out, while saliva and vaginal secretions also use chemical barriers to actively kill microbes [22, 23]. If these defenses are broken, we have an elaborate immune system that deals with foreign objects and pathogenic microorganisms. The immune system is divided into two parts: the innate and the adaptive. The innate immune system is the defense that we were born with, and has the role of broad, non-specific activity. As pathogens are introduced into the body, there are certain pathogen associated molecular patterns present, which are highly conserved within the microbes and is a telltale sign that intruders are near since they are not expressed by our own cells [24]. These molecules are recognized by pattern recognition receptors present on both immune cells like macrophages and dendritic cells, but also on epithelial cells, endothelial cells, and fibroblasts [25]. Once the

receptors have been activated, signaling pathways call upon more immune cells to deal with the pathogens. Phagocytes, like macrophages, engulf the pathogens and internally lyse and digest the invaders [26]. Granulocytes like neutrophils, able to perform phagocytosis, are also loaded with toxic compounds that can kill bacteria once engulfed, but also extracellularly by release of the bactericidal compounds from the granules as well as the formation of neutrophil extracellular traps (NET) [27]. The adaptive immune system, on the other hand, has the role of dealing with recurring pathogens. As a threat is detected, the innate immune system recruit cells of the adaptive immune system by presenting antigens specific to a certain pathogen through antigen presenting cells [28]. When activated, the B and T cells are effectively targeting the specific pathogen and the antigens are after an infection stored in memory B and T cells. Once the pathogen infects our bodies again, the ligands are recognized and antibodies specific to those antigens are released, which gives the other cells of the immune system a good target for a faster and more specific response.

3.3 Skin wounds and bacteria

The skin is our largest organ and is a vital barrier that encloses our cells in their own domain, away from our surroundings. It provides protection against dirt, microbes, water, UV-light and much more, but it can still release sweat through pores when necessary to keep us at a functional temperature. However, since it covers almost all of our body externally, it is also subjected to frequent minor and major trauma of all sorts. If the skin gets damaged to the point that a wound is formed, a portal is formed for the outside elements to gain access to our tissue. Luckily, there is a system in place to deal with this. We call it wound healing, and it is typically divided into four phases: hemostasis, inflammation, proliferation, and maturation, illustrated in Figure 2. If the skin gets damaged to the point of rupture, so are the blood vessels in the underlying tissue. Hemostasis is the process in which the bleeding is stopped by coagulation by the formation of blood clots. These are created through the clotting cascade by the activation of platelets as well as the activation of thrombin, by exposure to extravascular tissue, which converts plasminogen to fibrin [29]. Activated platelets and fibrin then coagulate together and stop the bleeding. Growth factors and cytokines released by activated platelets as well as by damaged cells, attract immune cells that in the inflammatory phase remove foreign objects, damaged cells and intruding pathogens by mechanisms such as phagocytosis and NET formation [30]. When the bleeding has been stopped and the area is cleared from outside elements, the proliferation phase commences. Here, fibroblasts create components of the extracellular matrix, mainly collagen, but also components like elastin and proteoglycans [31]. At the same time, vascularization takes place, and endothelial stem cells from the wound edges proliferate and the daughter cells migrate into the newly formed matrix and start to re-epithelialize the wound [32]. This builds up the different layers of keratinocytes forming the outer barrier. This newly formed tissue is called granulation tissue and while being quite sensitive, is a clear sign of healthy wound healing taking place. Lastly comes the maturation or remodeling of the wound. The now redundant immune cells are removed by apoptosis and the rapidly formed extracellular matrix is remodeled to try and mimic the non-wounded tissue. For smaller wounds this is usually very

successful but for larger wounds, a scar will remain on the skin that might fade with time but can also last for life.

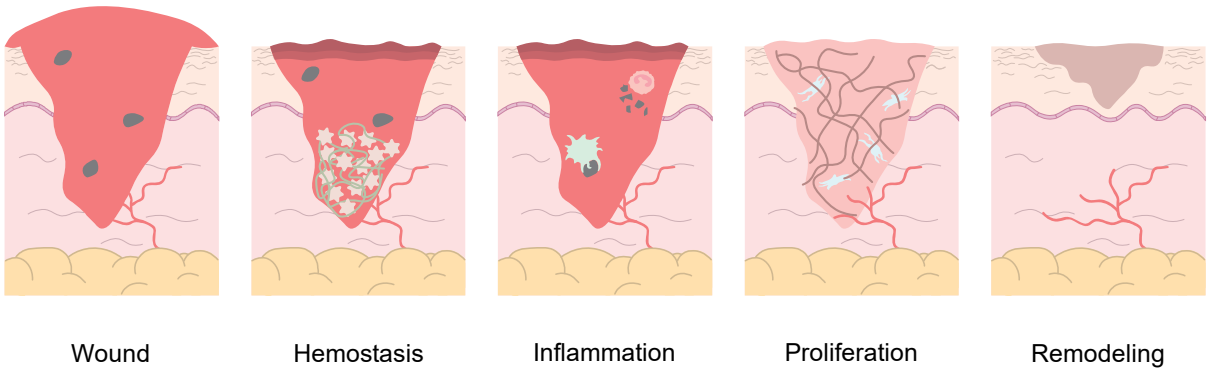

Figure 2. An illustration of the wound healing process. A *wound* is formed by a skin rupture and debris contaminates it. The *hemostasis* stops the bleeding by forming a blood clot. During *inflammation* the debris is removed by immune cells. A new extracellular matrix is formed by fibroblasts in the *proliferation* phase. The newly formed matrix is remodeled to more resemble undamaged skin in the *remodeling* phase.

Most of the time this process works well. However, there are a few factors that can cause the wound healing to not make it through one phase of the wound healing to the next. The focus of this thesis is when the invading bioburden becomes too great for the immune system to deal with, and a wound infection takes form. Depending on how and where the wound was formed there are several paths in which bacteria may get introduced into the wounds. As mentioned before the skin is the natural habitat for a lot of bacteria and is not an unusual source of infection both as an endogenous source, but also from the skin of others (mainly observed as nosocomial, hospital acquired) [33, 34]. However, it does not appear to be quite so simple that the most abundant bacteria on the skin are the most commonly found in wound infections. While *S. epidermidis* (among other coagulase negative staphylococcus) and *P. acne* are the most commonly occurring bacteria on the skin, their prevalence in wound infections is quite low. On the other side of the spectrum, another gram-positive bacteria, *S. aureus,* is by far the most common bacterial species found in infections of all type of wounds; acute, chronic, and surgical [35-37]. While *S. aureus* is indeed a part of the natural skin flora of a portion of the population, endogenous sources for infections are not fully understood but has been observed to be quite common in several studies [33, 38, 39]. The second most commonly found bacteria in infected wounds is the gram-negative bacteria *Pseudomonas aeruginosa*. Naturally occurring in soil and water, especially drains, *P. aeruginosa* have also been linked to frequent nosocomial infections and was found in higher abundance in hospital areas compared to non-hospital areas [40, 41].

When bacteria are infecting a wound, the wound healing will get stuck in the inflammatory phase as they never signal that things are well and done. This is usually recognized by redness, swelling, heat and pain in the affected area, which does not get better within days. Most of the symptoms experienced by us are not caused by the bacteria per se, but rather the immune

system's response to the infecting bacteria. This is very evident in the case of endotoxins. Endotoxins are another name for the lipopolysaccharides found in gram-negative bacteria. They have been associated with severely over activating the immune system, with the risk of causing septic shock if found extensively in the blood system [42, 43]. In wound infections, elevated endotoxin concentrations have been associated with reduced healing as it reduces cellular migration [44, 45]. In general, the infecting bacteria will try to consume nutrients, proliferate, and avoid the host defense. To ensure a steady supply of nutrients, bacteria like *S. aureus* will secrete exotoxins that will damage and kill host cells, turning them into precious food [46]. The same molecules can also cause damage to cells of the immune system, reducing their ability to fight the invaders. Furthermore, in chronic wounds, the wound healing is not progressing as usual and infection in these cases is guaranteed, with an even worse prognosis in terms of healing and fighting infections. If the bacteria are left unchecked, a lot of them have the tendency to form communities inside a self-produced slime consisting of extracellular matrixes, found in the vast majority of chronic wounds [47]. These formations are known as biofilms, and if they are formed in a wound, any bactericidal compounds have a very difficult time penetrating the slime and killing the bacteria which here have further protection due to a varied metabolism [48]. This is evident from the current recommended treatment of an established biofilm in wounds, which is debridement by cutting and scraping away the infected tissue [49].

3.4 Antibiotics and resistance

The gold standard for treating bacterial infections for the last 100 years has undoubtedly been with antibiotics. While there are several classes of antibiotics which work in different ways, most of them inhibit a specific function in the bacterial cell by interfering with the components necessary for the critical biochemical reactions to take place. For example, tetracyclines binds to different sites of the bacterial ribosomes and inhibits the function of translating mRNA to proteins [50]. The most frequently used antibiotic class, β-lactams, function by inhibiting the transpeptidase enzymes, which are involved in the final step of the peptidoglycan synthesis, effectively killing the bacteria during their cell division [51]. These kinds of mechanisms have been proven to be extremely effective at killing bacteria very selectively. Sadly, the specificity has also proven to be quite susceptible to mutations and other defense mechanisms employed by bacteria, causing resistance against the antibiotic. This can happen in various ways: efflux pumps that recognize the antibiotics can actively eject the antibiotics from the bacterial cells; enzymes designed to cleave the antibiotics can be secreted; the active sites of the enzymes which the antibiotic binds to and inhibit can be altered, rending the antibiotics without a target [52].

Bacteria have proven to be very effective at adapting and developing resistance to almost all antibiotics we have implemented in the clinic today, and together with their ability to perform horizontal gene transfer, even between species, the antibiotic resistance genes can spread fast and wide [53]. An increasingly common concern, especially for wound infections, is methicillin resistant *S. aureus* (MRSA). While MRSA does exist in several variants, they

exhibit some form of resistance towards β-lactams. Depending on where you are in the world, MRSA might even be the most common *S. aureus* isolate found in infections [54]. The way that we have used antibiotics historically and to this day has further been speculated to significantly influence and increase the rate by which bacteria have developed resistance. By using antibiotics as a growth stimulant in the animal agriculture industry where antibiotics are pumped into animals acts as a reservoir for antibiotic resistance to form [55]. There is also an ignorance or knowledge deficit in the use and prescription of antibiotics within healthcare around the globe and antibiotics are routinely used in cases where it is unnecessary, further driving resistance [56].

Regardless of how the resistance is caused, the effects have been all to noticeable. A recent study showed that in 2019, almost 5 million deaths were associated with antimicrobial resistant bacteria, with 1.3 million deaths directly attributed to antimicrobial resistant bacteria [57]. That is almost 10% of the total 55 million deaths reported that year. The World Health Organization (WHO) have recognized antibiotic resistance as a major global health concern and that implements are required to limit spread and to create new ways of treatment. Within all areas where infection is a factor, there is an evident need for alternatives or complements to conventional antibiotic usage.

The area of wound care has seen a lot of new interventions, most likely due to the ease of access to wounds and by the frequency they occur. Cuts, scrapes, and burns are parts of our normal lives without being too much of a daredevil, and every time a surgery is performed an incision is created, which should not get infected. These areas are of course much easier to reach with topical innovations than, for instance, to reach internal organs like the lungs to treat pneumonia. This does mean there is likely to be a higher demand for devices within this particular field. Especially important in the case of preventing infection since the active parts of the wound care product should not interfere with the wound healing. One antibacterial component used in wound care is honey. While this is not exactly a new innovation, it has been incorporated into modern wound care dressings to prevent bacterial colonization by high sugar content, acidity, and active components like hydrogen peroxide and proteins [58]. Honey can also promote the wound healing by stimulating the release of tumor necrosis factor alpha (TNF-α) and by anti-inflammatory properties [59]. However, one of the limitations with honey is that the antibacterial effect comes from a dosage factor, which can be diluted due to exudate. The dressings must therefore be continuously exchanged. Another well-known antibacterial substance frequently used within antibacterial wound care is silver. The silver ions and silver nanoparticles directly kills bacteria by destabilizing the cell membrane after an accumulation of silver, as well as the direct interactions with other internal components like nucleic acids, and the production of reactive oxygen species [60]. The antibacterial effect of silver is without a doubt quite effective. However, a limitation with silver is that the ions have to be released, which in combination with a debated cytotoxic potential might limit their usage [61, 62]. While it might still prove effective in established infections, prevention might not be the best area of use as our cells in wounds are already under considerable stress. There are also technologies that has shown to reduce infection not by killing the bacteria but binding them to the wound dressing in an attempt to remove them from the wound [63, 64]. This technology is based on

dialkylcarbamoyl chloride coatings, to which the bacteria irreversibly bind. The claimed benefits of this way of dealing with the bacteria is that if they are not killed, they will not release toxic compounds like endotoxins into the wound, limiting the harm caused. In contrast, the antibacterial substance used in the work presented in this thesis is antimicrobial peptides, which may attract bacteria and kill them.

3.5 Antimicrobial peptides

Antimicrobial peptides (abbreviated AMPs) are short sequences of amino acids found in most multicellular organisms as an antimicrobial defense component. In some frogs, AMPs are found in high concentrations on the skin used to fight off intruding microbes, and some bacterial cells even utilize AMPs to kill competing microbes [65, 66]. For humans, AMPs are a part of our innate immune system where it is mainly released by neutrophils to fight an ongoing infection and by cells present on our skin and salivary glands for a more preventative strategy [67]. AMPs are well suited for the innate immune system due to the broad-spectrum activity they usually exhibit. Apart from the direct bactericidal effect, AMPs of the innate immune system have also been found to perform several other features illustrated in Figure 3 [68]. Cathelicidins like LL-37 can activate other immune cells by binding to G-protein coupled receptors and work as a chemoattractant, they can also stimulate mast cells to release histidine [69]. β-defensins can regulate inflammatory response as they have been shown to both induce and suppress a proinflammation response, they have also been shown to bind to endotoxins and lower the risks associated with these [70].

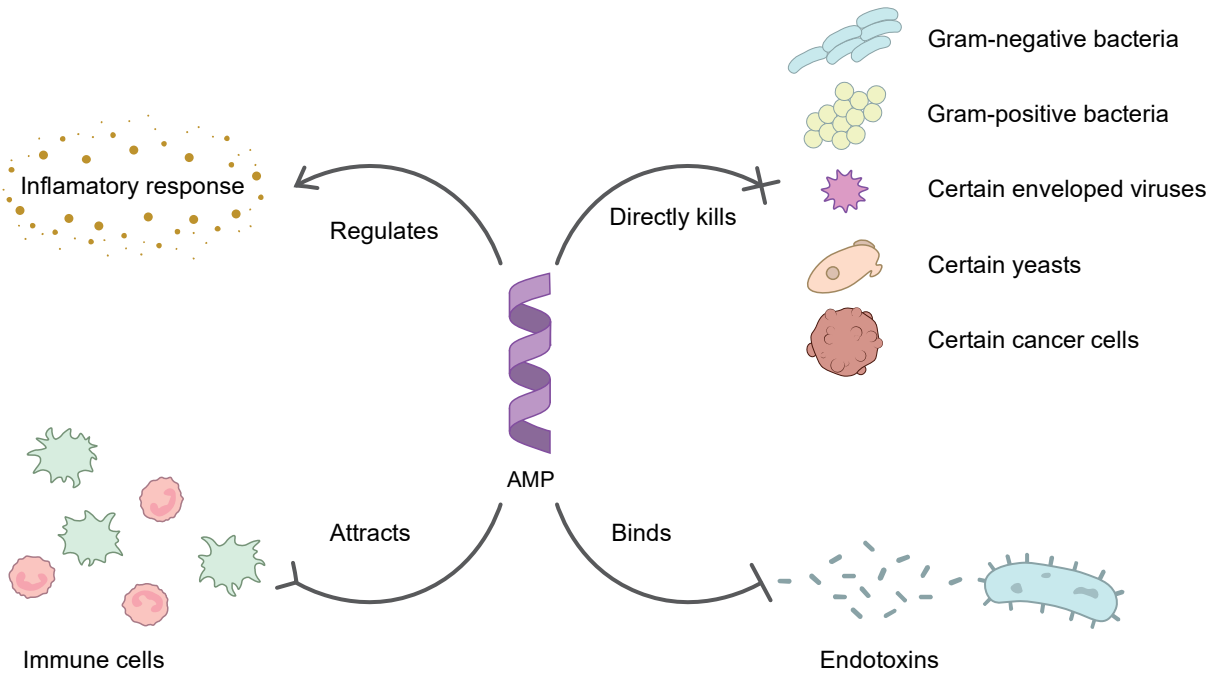

Figure 3. Some of the roles and functions that the AMPs of the innate immune system provide the host with.

There are thousands of AMPs discovered that exist naturally, and even more AMPs produced synthetically. While all these AMPs do not share the very same antimicrobial mechanism, there are some common features found in most AMPs which reveal their mode of action. A cationic charge is usually present due to a considerable portion of lysin, arginine and histidine, which provides the AMPs with a selectivity towards the negatively charged bacteria [71]. As mentioned in 3.1, both gram-negative and gram-positive bacteria carry a net negative charge on their surface, not found on our own cells. A selective killing effect has also been observed against some enveloped viruses, yeast, and cancer cells, believed to be due to the very same reason of a net negatively charged surface [72-74]. Furthermore, AMPs possess a high content of hydrophobic amino acids, which in combination with the cationic amino acids (and other polar residues) can provide the AMPs with an overall amphiphilic structure. This makes the AMPs membrane active, which is also how most of them are hypothesized to exert their antimicrobial effect. While it is quite challenging to study in detail, it has been observed that AMPs can destabilize the membrane by membrane thinning, forming pores by barrel stave or toroidal pore models, or by dissolving the membrane in a detergent like manner [75-77]. In some cases, the AMPs adopt a secondary structure, usually an α-helix, upon contact with the membrane. This means that the hydrophobic domains orientate along the secondary structure first at contact with the membrane, something which should also allow for better transport in the extracellular matrix [78]. In the cases where internal components like genetic material, ribosomes or chaperons are targeted, the AMPs are still membrane active in the sense that they translocate through the membrane to gain access to those components [76, 79]. Furthermore, it is not a specific binding or inhibition of those components as with conventional antibiotics, but a non-specific damaging mode of action.

This non-specific mode of action, for all AMPs, means that there are no mutations that can occur to change an active site. While efflux pumps may not affect the AMPs targeting the membrane or cell wall to a large extent, AMPs targeting internal components are subjected to their activity [75]. One strategy that bacteria can employ in order to reduce the effectiveness of AMPs is to modify their cell envelope structure. A d-alanylation of teichoic acid has for instance shown to reduce the initial attraction of some AMPs, and for gram-negative bacteria the inclusion of amine-containing molecules in lipid A of the LPS has also been associated with lower AMP effectiveness [80-82]. However, the negative charge found on both gram-negative and gram-positive bacteria is a highly preserved feature as they for example use it to attract cations vital for their existence and help stabilize the cells [12, 83]. Incorporation of resistance genes has also been linked to a lower viability and fitness [84]. The other main method by which bacteria can increase resistance towards AMPs is by creating enzymes that cleave the AMPs in pieces [85]. This is barely something that the bacteria need assistance with as AMPs are by nature quite sensitive to proteolysis by enzymes present in our plasma and can also be inactivated by the ionic strength present [5]. These stability challenges are one of the main reasons why AMPs have seen limited success in the translation into medicine, and the major research area involving AMPs is to improve the stability. The other main issue is that at higher concentrations, the charge-based selectivity of AMPs is reduced, and they show

cytotoxicity towards human cells. This means that increasing the concentration to deal with the stability concern is not a viable option, and we are therefore left with a narrow therapeutic window.

In order to combat this inherent stability issue, there are several techniques employed by researchers. One method is to include versions of amino acids that do not exist in abundance in nature, or at all. This would be D-amino acids or other non-natural amino acids, which makes it more difficult for proteolytic enzymes to find attachment points [86, 87]. Cyclization has also shown to be effective at increasing the stability against proteolysis due to limited attachment sited of the proteolytic enzymes [88]. It is not by chance that the two medically approved AMP derivatives colistin and daptomycin adapt a circular structure. Another method, which is also used for the AMPs in this work, is end-capping where the C- and N-terminals are modified, again to lower the ease of enzymatic attachment [88]. Furthermore, the main method used throughout this work is to covalently attach the AMPs to a surface to provide steric protection against proteolysis.

The different AMPs used in this thesis are presented in Table 1. Their sequence, net charge at pH 7, and hydrophobicity calculated based on the Wimly White experimentally determined scale is presented [89]. In **Paper I**, **II**, **III**, and **V** only one AMP was used, R9W4 (shown in Figure 4). This AMP is synthetically made and consists of a tryptophan end tagging to increase the hydrophobicity and antimicrobial properties as well as a section of proline arginine-rich end leucine-rich repeat protein (PRELP) that has the function of heparin binding [90, 91]. Heparin binding peptides has earlier been linked to also possess antibacterial potential, most likely correlating to the negative charge of heparin, and these peptides might also work multifunctionally [91, 92]. In **Paper IV** the aim was to investigate the six different AMPs shown in Table 1 in terms of their antibacterial and hemolytic activities free in solution and when attached to a hydrogel. Three additional versions of the standard AMP were included with just small amino acid alterations. The alterations revolved around changing hydrophobicity by including more tryptophan groups and the cationic group, changing from arginine to lysine to include more primary amine groups, which are used in the attachment chemistry [93]. One AMP called Omiganan was included as it has shown a potent antibacterial effect in solution and is suggested to have a membrane destabilization after saturation mode of action [94]. Piscidin 1 was included as it should have a very potent antibacterial effect in solution, might form pores as its mode of action, and is known to adapt an α-helical structure upon contact with membranes, whose effect would be of interest to study when attached to the hydrogel surface [95].

Table 1. Structural information about the AMPs used in this work. All peptides are amidated on their C-terminal. The hydrophobicity is calculated based on the Wimly White scale.

Name	Amino acid sequence	Net charge at pH 7	Hydrophobicity [kcal mol^{-1}]
R9W4	RRPRPRPRPWWWW	+6	1.55
K9W4	KKPKPKPKPWWWW	+6	0.65
KR9W4	KKPRPRPRPWWWW	+6	1.19
R9W5	RRPRPRPRPWWWWW	+6	3.40
Omiganan	ILRWPWWPWRRK	+5	3.95
Piscidin 1	FFHHIFRGIVHVGKTIHRLVTG	+4 (+8 at pH<6)	1.07

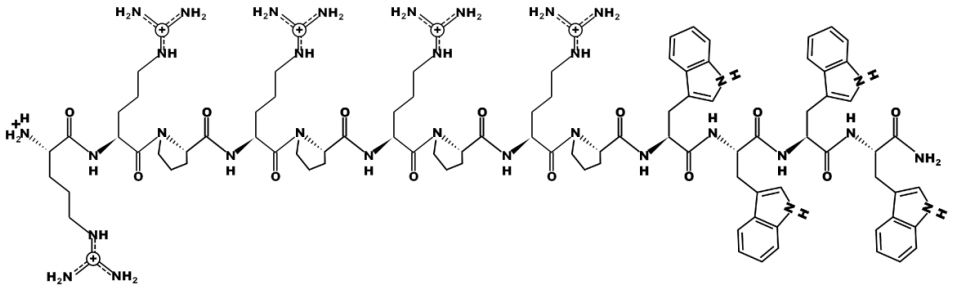

Figure 4. Molecular structure of the peptide R9W4 at pH 7.

3.6 Antibacterial surface functionalization

The functionalization of medical devices with an antibacterial contact killing agent has several key benefits. First, the antibacterial effect will be contained locally, close to the material, and not affect the microorganisms that we have a beneficial coexistence with to a large extent. This is beneficial since the usage of systemic treatments have been shown to be able to greatly affect our microbiota, which have led to long-lasting detrimental health impacts [96, 97]. A second benefit is that a material which is difficult to reach, such as an implant, can gain protection against bacterial colonization continuously while in use. When an implanted medical device is infected, most likely by a biofilm, it is usually too late to start a treatment and device removal tends to be the only option [98]. Infection prevention is in this case vital, which surface functionalization can provide, both by anti-fouling but also by incorporating antibacterial components. Thirdly, especially in the case of AMPs, the attachment to a surface can also be beneficial to the antibacterial component as the steric hindrance should make it difficult for proteolytic enzymes to attach and cleave. Finally, by relying on an antibacterial approach that does not leach out the active substance to the environment, development of antimicrobial resistance is hypothesized to be further reduced [99].

A range of approaches has successfully been used to functionalize the surfaces of medical devices. One commonly used group of antibacterial substances is quaternary ammonium compounds (QACs), which has been shown to retain a good antibacterial effect when covalently attached to a wide range of materials [100]. While the mechanism of action is not completely understood it is believed to rely on strong interactions between the positively charged ammonium and the negatively charged bacterial cell walls subsequently causing significant membrane damage, similar to the mechanism of AMPs [101]. N-chloramine is another substance used to create bactericidal surface modifications which is believed to exert the antibacterial effect by chlorine transfer to the outer proteins of the bacteria [102]. Another approach is a nanostructured surface that limit the attachment of bacteria by killing them upon contact. One example is nano-patterned arrays on titanium devices inspired from the wings of dragonflies and cicada which cause membrane deformation [103].

The use of AMPs to functionalize surfaces of medical devices has steadily been increasing through the last two decades. Given the benefits discussed in the previous section combined with the limited half-time and potential cytotoxicity at high concentration, surface functionalization is a great option. AMPs have successfully been attached to titanium, contact lenses, cellulose, glass and more [104]. However, there are some key features that need to be taken into consideration for this application, showcased in Figure 5. As mentioned before, there are plenty of different AMPs, and while membrane interactions are deemed to be the leading mode of action, there is a larger variety at play. An AMP that does not exert its effect on the membrane when in free form, but instead targets internal components, is not likely to retain its mode of action when attached to a surface. Furthermore, AMPs that do target the membrane might still lose their antibacterial effect depending on how that mechanism is displayed. AMPs that create pores by aligning in a barrel-stave fashion might find it much more difficult to do so when tethered to a surface. That being said, there is also a significant chance that an antibacterial effect is observed for a tethered AMP, but with a completely different mode of action compared to when in its free state.

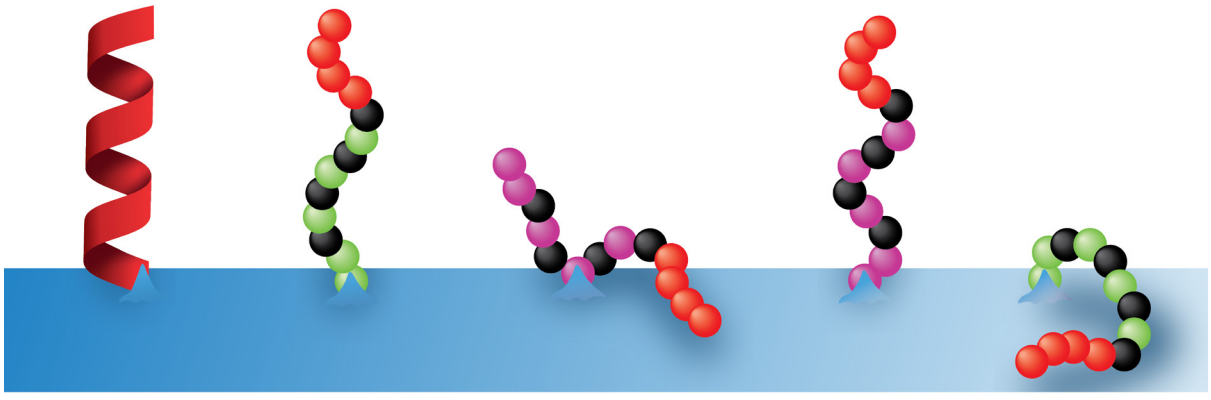

Figure 5. An illustration of some of the different behaviors AMPs might adapt when covalently attached to the hydrogel and need to be taken into consideration. The attached AMPs might adapt a secondary structure, there might be several attachment points, and the AMPs may stand up or lie down. All can play a role in the antibacterial activity of the attached AMPs.

The orientation of the AMP on the surface might also play a significant role in the antibacterial effect. This can be related to the attachment site on the AMP but also depending on interactions with the material itself, for instance if it is laying down or standing up [105, 106]. It is also rather common that AMPs adapt a secondary structure of α-helices and β-sheets, and the retention of this ability have been linked with a preserved antibacterial effect [105]. Furthermore, which base material the AMP is attached to will also play a large role in the success of the functionalization. For a stiff material, like titanium, the mobility of the AMPs will be much lower than a more flexible material, like elastin. If the AMPs need to aggregate or orientate in a specific way corresponding to the cell wall, it will have a greater chance of doing so when attached to a softer and flexible material. Usually, the material is set and cannot be changed except for limited surface chemistries; a titanium screw as a whole cannot be altered because the AMPs does not work. A method devised to assist with this is the use of spacers between the base material and the AMPs. A few kDa long PEG spacers is a common practice to include as it has been improving the antibacterial effect observed by AMPs attached to resin beads and chitosan [107, 108]. In this present work, the base material has been that of a hydrogel based on a polymer that might in itself act as a spacer, discussed further in the following chapter.

3.7 Amphiphilic polymer hydrogels

To start this chapter off, a clarification of the term hydrogel relevant to this work is in order. Hydrogels contain mainly water, which is held in place by a three dimensionally cross-linked polymeric network. When hydrogel is mentioned in this work, think of it not as a fragile substance, but rather as a cohesive material that can be interacted with, without it falling to pieces. The hydrogels used in this work is shown in Figure 11 and Figure 12, found in the result and discussion section.

Hydrogels have shown great promise within biomedical applications, mainly due to their high-water content and tunable softness, which can be made to match well with our own soft tissues [7]. Combined with the possibility for further modifications in terms of bioactivity, biodegradability, and water absorption, this allows for more advanced utilization. Hydrogels have for instance found success within wound care, as the hydrogels can provide a moist environment which promotes wound healing while at the same time providing pain management, especially important when treating burn wounds [6, 109]. While some absorption of exudate is possible, it is relatively low compared to some super absorptive wound dressings. This means hydrogels should not be used by themselves on highly exuding wounds as this might instead cause maceration [110, 111]. Apart from the beneficial interactions with our soft tissues, hydrogels are utilized within wound care as they are susceptible for drug loading and other modifications. Components such as growth factors and antibiotics have been physically loaded onto hydrogel wound dressings, which can then be released into the wound for infection treatment or wound healing stimulation [112].

The hydrogels in this work are all made with the same amphiphilic polymer, Pluronic F-127, also known as Poloxamer 407. This is a block copolymer constituted by a block of polypropylene oxide flanked by two blocks of polyethylene oxide. To enable cross-linking, the very ends of the polymers have been modified to incorporate acrylate groups, which by radical polymerization can bind to each other. This results in a polymer with the structure shown in Figure 6. This class of polymers have some extra interesting features as they can self-assemble into structures with a long-range order depending on concentration, solvent, and temperature. This is because the polypropylene oxide is more hydrophobic and the polyethylene oxide more hydrophilic making the overall structure amphiphilic. When in an aqueous solution, there will be a driving force to limit the interactions between water molecules and hydrophobic entities. This goes for all non-polar molecules solved in water and is due to the hydrophobic effect. To accommodate a non-polar component in water, the water molecules have to form a cavity and rearrange around the molecule which significantly lower the amount of hydrogen bonding they can do [113]. It is therefore beneficial for the whole system to limit this interaction by aggregating the hydrophobic sections. The temperature dependance is believed to mainly be due to the presence of ethylene oxide in the structure. In non-ionic surfactants the temperature dependence has been linked to conformational changes of the ethylene oxide, adapting a conformation with a higher dipole moment at lower temperature and a conformation with a lower dipole moment at higher temperatures [114]. Effectively this means that the same structure is more hydrophilic and solves easier in water at low temperatures and vice versa at higher.

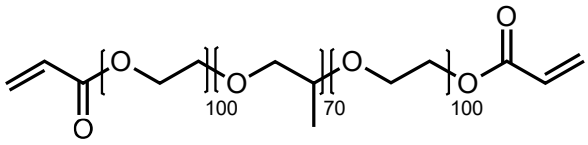

Figure 6. The molecular structure of Pluronic F-127 modified with acrylate groups at both ends.

The interesting case is when the hydrophobic sections are covalently connected to more hydrophilic sections as is the case with our polymer. An illustration is shown in Figure 7 (a) for the cases where only water is used as the solvent. At lower concentrations the polymer self-assembles to form a solution of micelles (L_1), with an increase of polymer concentrations the polymers self-assembles into lyotropic liquid crystals (LLCs), first as micelle cubic phase (I_1) and with a further concentration increase hexagonal phase (H_1) [115]. By changing the solvent to include the non-polar butanol, Pluronic F-127 can also form LLCs with a lamellar phase (L_α) or reverse micelle solutions (L_2). The phase diagram of these systems is displayed in Figure 7 (b). However, this is only at a specific temperature with changes of the concentrations and solvents, but as mentioned before the self-assembly also responds to temperature changes. This is actually one of the most commonly used features of Pluronic F-127. It can be used as a bioink scaffold that you can print with in room temperature (or higher) and then wash away by simply adding cold water, or by injecting a liquid at room temperature that sets to a viscous gel at physiological temperature for applications within our bodies [116,

117]. In this work this effect is mainly used to make pristine hydrogels with a long-range order that has the potential to be loaded with hydrophobic components down the road. However, possible interactions between the hydrophobic domains of the polymer and the amphiphilic AMPs are not excluded.

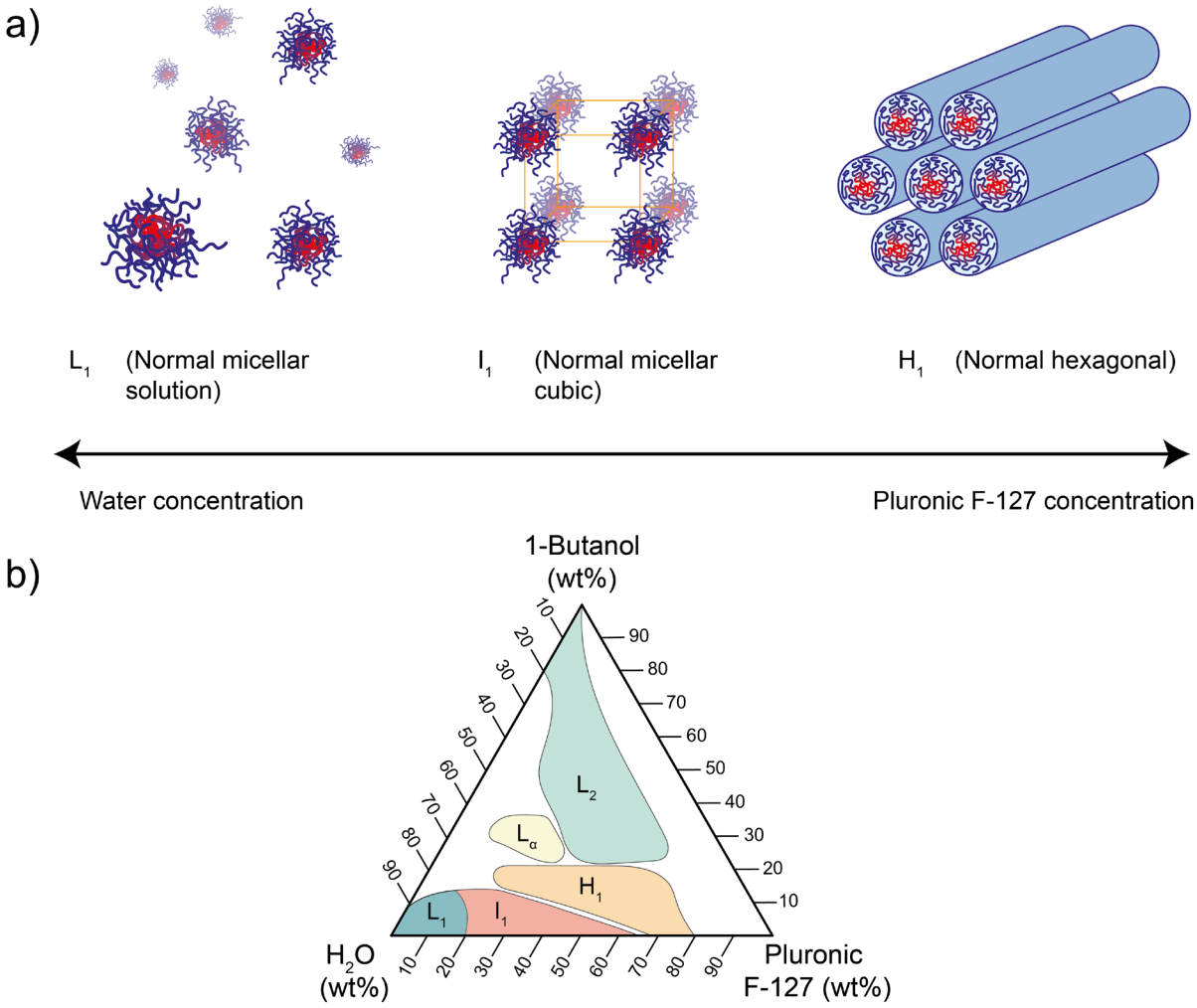

Figure 7. Concentration dependent self-assembled structure formed by Pluronic F-127. (a) show the different structures possible with just water and Pluronic and (b) show a phase diagram of Pluronic F-127, water, and 1-butanol.

4 Methodology

The following sections will very briefly go through the basics of techniques used in this work. The motivation for why they were used is presented as well as an overview of how they were performed. For more detailed information, please read the papers the thesis is based on.

4.1 Material synthesis

In all five papers the same polymer was used to make the hydrogels and in all papers except **Paper IV**, the same peptide was attached to the hydrogels. However, as time passes, experience and knowledge are gained, and because of this, the material synthesis was slightly changed between the works discussed. In **Paper I** and half of **Paper V**, for instance, the end-acrylation modification of the polymers was done in house, ending up with an unknown and lower purity than used in the other papers. This was performed by dissolving the Pluronic F-127 in chloroform with added trimethylamine acting as a buffer (neutralizing HCl formed during the synthesis). Acryloyl chloride was added dropwise to the system which was then left to react under nitrogen atmosphere for 24 h. The mixture was washed thrice with Na_2CO_3 and then dried over anhydrous $MgSO_4$. Finally, the organic phase was separated, and the polymer obtained by rotary evaporation. The AMPs were purchased from companies that synthesized them with solid phase synthesis i.e. amino acids are in solution added one at a time to the peptide which is anchored to a solid support.

The polymer concentration also changed over the course of time, although only from 40 wt% to 30 wt%, the rest being water. At these concentrations the polymer should according to literature adapt LLCs with a micellar cubic phase [115]. A photoinitiator was added to the mixtures which were left to rest in the dark for at least 2 days in order for the polymer to adapt into their LLCs. For **Paper I** and half of **V**, this was done at RT after centrifugation while in the rest of the papers the gels were first kept in a fridge (4 °C, dark). The mixtures were then cross-linked into hydrogel sheets by exposure to UV-light. This causes a radical polymerization of the vinyl groups, but also starts to form carboxylic acid groups by breaking down some of the ester groups, also done by hydrolysis. These carboxylic acid groups were subsequently utilized for the attachment of the AMPs by EDC and NHS coupling. This works by activating the carboxylic acid group which forms a peptide bond to a primary amine group, found on the AMPs. The C-terminal of the peptides was amidated in order to not create polypeptides, leaving only one primary amine group at the N-terminal for the standard AMP used in all papers except for **Paper IV**. In **Paper IV** one of the goals was to investigate the presence of primary amine groups at other locations as the side group of the amino acid lysine also contains a primary amine. Practically this was done by placing the hydrogels in MES solution (pH 5.5-6) with EDC and NHS for 30 min, followed by washing with water. Then adding AMPs solved in phosphate buffered saline (PBS, pH 7.4) to the hydrogels for 2 h, followed by washing.

The sheets (before AMP functionalization) were in **Paper II** used to create particles by a stepwise reduction of size in a top-down approach. The sheets were first manually torn into small pieces, blended using a hand-held kitchen blender, reduced further in size by as ultra-turrax (a kind of homogenizer) and finally ultrasonicated. The AMP functionalization was performed in a similar way as for the discs but using higher concentrations.

4.2 Water absorption and AMP attachment analysis

As evident from the name, an important feature of a hydrogel is the water content of the material. This is especially important with the intended area of use within wound care, because while a moist environment provide the optimal healing conditions, a wet environment is bad. Too much liquid around a wound can cause maceration which is why a highly exuding wound needs to be covered with a highly absorptive dressing. The water content of the hydrogels was therefore determined by weighing the materials at fully swollen state as well as fully dried state after drying in 37 °C incubator for hydrogel discs and by freeze-drying for the particles.

In order to analyze the extent of AMP functionalization of the different materials, analysis by UV-vis spectroscopy was implemented. This was done since the indole group of the tryptophan amino acid has a very strong absorption peak at around 280 nm. Together with standard curves the concentrations in the solutions before and after functionalization could be determined and the amount of AMP that attached to the hydrogels could be calculated. While this was also attempted for Piscidin 1 in **Paper IV**, it was not possible since the signal of the phenylalanine group was not distinguishable from the background

4.3 Molecular and structural information by Raman, FTIR, and SAXS

In order to investigate the steps in the material synthesis, Raman spectroscopy and Fourier-transform infrared (FTIR) spectroscopy were implemented. These techniques provide information about the functional groups and bonds that are present in the samples. This was performed on the particles in **Paper II**, and because of the high-water content of the hydrogels, which will engulf most of the signal in the FTIR, the particles were freeze-dried before measurements. FTIR is based on the principle that bonds within molecules vibrate, and these vibrations can be excited by IR light. By exposing a sample to IR light and then measuring the wavelength of the light exciting the sample, a fingerprint of the present functional groups is obtained.

Raman spectroscopy is also based on the vibrations of molecules, and that these can get excited into virtual energy states. Raman spectroscopy is however utilizing the fact that the excitement is somewhat inelastic and that sometime the scattering light does not always have the same energy as the incoming light. This is known as Raman scattering. This is also specific for the different vibrations and therefore the different functional groups present in a molecule and can therefore be used to gain structural information. In general, Raman spectroscopy is good at detecting symmetrical groups while FTIR is good at detecting asymmetrical groups, meaning that they make excellent complementary techniques. Following sample preparation, the actual measurements for both techniques are not too complicated as the samples are placed in line of a laser which goes to a detector and a spectrum of absorbance and transmission is obtained. The trickier part is to analyze the content of these spectra by identifying the origin of the different peaks.

Also performed in **Paper II** was the investigation of the long-range order of the samples. The LLCs formed by the polymers as they self-assemble should have a repeating structure throughout the material if this is maintained in the synthesis of the material. In order to investigate this, small angle X-ray scattering (SAXS) was implemented. This is a powerful technique that utilize that X-rays gets scattered by materials and based on scattering intensity at different angles, information regarding the structure can be obtained. For instance, all LLCs have distances in between the structures, and scatterings that are caused by these distances will be repeated more frequently, showing up as a peak in the diffractograms. Practically this was performed by placing a control hydrogel, control particles, freeze-dried AMP-functionalized particles, and swollen AMP-functionalized particles in between tape and adhered to a sample holder. The sample holder was placed in line of the X-ray, in vacuum as the samples were exposed to the radiation, and the scattering detected.

4.4 Qualitative antibacterial analysis by fluorescence microscopy and cryo-EM

A commonly used method to get a visual display of the antibacterial effect of a surface is the use of fluorescence microscopy in combination with staining bacteria depending on their viability. This is done by using a LIVE/DEAD staining which consists of two dyes which function is illustrated in Figure 8. One is SYTO-9, which is membrane permeable and therefore enters all cells and can be excited to emit a green light. The other dye is called propidium iodide, which can only enter damaged membranes, i.e., dead cells, and can be excited to emit a red light. By staining bacteria with these dyes and then using a fluorescence microscope, the proportions of alive and dead cells can therefore be observed as they show up as green or red cells.

LIVE/DEAD staining was utilized in **Paper I** to evaluate the AMP functionalization of the hydrogels as they were cultured for 24 h in well plates together with *S. aureus*, *S. epidermidis*, *P. aeruginosa*, MRSA, and MDR-*E. coli* (multidrug resistant *E. coli*). After a 24 h incubation in tryptic soy broth (TSB), the hydrogels were gently washed and then stained with the LIVE/DEAD staining and observed with a fluorescence microscope. When compared to a control, any antibacterial activity present on the surfaces could easily be observed as the proportion of cells stained red and green changes. An attempt to calculate the fraction of the surfaces that was stained red compared to green was also performed using a macro in the software imageJ. Although the LIVE/DEAD staining of the bacteria might be more suitable to investigate whether any antibacterial activity is present than to investigate the strength of the antibacterial effect as there are several factors not considered.

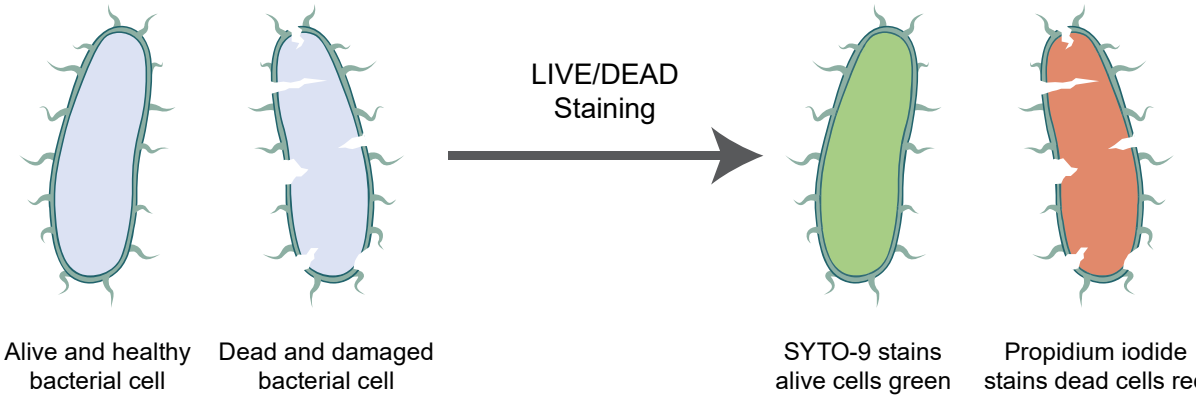

Figure 8. An illustration of how LIVE/DEAD staining works. Alive and healthy bacteria stains get stained green by SYTO-9, while dead bacteria get stained red by propidium iodide.

Another technique that was used in **Paper II**, in order to get more information on how the antibacterial activity was exerted was cryo-EM. This was performed by incubating AMP-functionalized particles (a subsection of smaller size, separated by filtration) with *S. aureus* for 1 h followed by a plunge freeze in liquid ethane. This freezes all interactions in place in amorphous ice and allows for an observation of the same without any coating, meaning that

interactions with the cell envelope of the bacteria should be possible. The samples were observed in a transmission electron microscope (TEM) while constantly being kept at cryogenic conditions with circulating liquid nitrogen.

4.5 Quantitative antibacterial analysis by counting colonies

In order to get a quantitative measure of the antibacterial activity, a very useful method is to count colonies. This is done by diluting solutions of bacteria, placing some of that solution on to agar plates which are incubated overnight. The individual bacterial cells that are spread on the agar plate will grow into a colony of bacteria that are visually observable if that bacterial cell is healthy and proliferating. This is called a colony-forming unit (CFU). By counting the number of colonies on the agar plate and back-calculating to the original solution, a concentration in CFU/ml is obtained. In **Paper II** this was used to assess the antibacterial potency of AMP-functionalized particles compared to control particles in an agar plate study where *S. aureus* was spread onto agar plates and the control and AMP-functionalized particles were spread on top. After incubation, samples were punched out together with the agar using biopsy punches, and the CFU of the whole biopsy punches were evaluated.

In **Paper IV** the technique was used to evaluate six different AMPs after they were covalently attached to hydrogel discs. In brief, this was performed by placing a 10 µl drop of highly concentrated bacterial solution (10^9 CFU/ml) on top of the different hydrogels in well plates, covering the hydrogels with cover slips to ensure a good contact. After a short 30 min incubation period the hydrogels were transferred to glass vials, and PBS was added to the vials for extraction of the bacteria to the PBS by vortexing and sonication. The CFU of the PBS solutions were then evaluated by CFU measurements and the antibacterial activity of the surface bound AMPs could be evaluated by comparing the CFU to that of control hydrogels to get a measure of bacterial reduction. This was performed against both *S. aureus* and *P. aeruginosa*.

4.6 Measuring potency by inhibiting bacterial growth

A powerful method to try out the potency of antibacterial substances in solution is minimum inhibitory concentration (MIC). It was used in **Paper II** to assess the antibacterial efficacy of the AMP-functionalized particles compared to free AMP, and in **Paper IV** in order to evaluate and internally compare six different AMPs in their antibacterial potency free in solution. In short, MIC tests are performed by serial diluting the substance of interest in Muller Hinton broth and then adding the bacteria of interest (cultured and diluted to required concentration, here 10^6 CFU/ml) in equal amounts to each dilution. The bacteria are allowed to grow overnight in an incubator. If the antibacterial substance is potent enough to inhibit the bacteria to grow, the solution will stay transparent after the cultivation, and if the antibacterial substance is not potent enough to inhibit bacterial growth, the solution will be cloudy after incubation. By doing

this at several dilutions, the lowest concentration of a substance that inhibits bacterial growth can be found, which is called the minimum inhibitory concentration. The lower the MIC the more potent the antibacterial substance is. However, this technique is only possible to use for substances in solution so it cannot be used to assess larger solid materials.

4.7 Proof of concept by agar plate studies

In order to study the release of antibacterial substances, a disc diffusion assay is a commonly implemented tool. In **Paper I**, a version of disc diffusion was implemented in a zone of inhibition study. This was done in order to investigate whether the AMPs released from the hydrogels and to demonstrate if the covalent attachment of the AMPs were in fact true and not simply absorbed into the hydrogels. AMP-functionalized hydrogels were prepared as described above as well as a set of samples to which the AMPs had not been covalently attached but simply physically loaded into (no EDC/NHS activation step). These two versions along with control hydrogels were placed on agar plates previously streaked with *S. aureus* and were subsequently incubated overnight. If a significant release of AMPs occurs, this will diffuse out to the surrounding agar, killing the bacteria present there, causing a zone of inhibition to form around the sample. This is visually observable as the non-affected bacteria will grow into a bacterial lawn, seen as a slimy layer on top of the agar, while the zone of inhibition will stay as clear agar. In a disc diffusion assay, the strength of the antibacterial substance as well as the release of the same can be evaluated by measuring the size of the zone of inhibition as the concentration will decrease further away from the disc. Meaning that a more potent antibacterial substance should result in a larger zone of inhibition. Since the hydrogels are not any kind of model substrate it is more unclear whether the strength of the substance can be obtained in this way. However, where a clear zone of inhibition is present it is at least clear that the AMP present is leaking out from that sample at effective concentrations.

An adaptation of this was also investigated in **Paper II** in an agar plate study. The goal of that study was mainly to measure the bacterial presence under the AMP functionalized particles by CFU analysis to investigate the antibacterial potency. However, to observe whether any leaching occurred was a sub goal of the study.

4.8 Evaluating the stability of the functionalization

As mentioned in the Background, the main limiting factor for AMPs in the translation into medicine is the low stability observed in biological environments. This is also one of the key features of why the AMPs have been covalently attached to the hydrogels as the supporting theory is that this should increase the stability of the AMPs, mainly by steric hindrance, depicted in Figure 9. In order to evaluate if this holds true, several serum stability tests were performed *in vitro* in **Paper I**, **Paper II**, and also *in vivo* in **Paper V** (this is presented more thoroughly in section 4.11). For the *in vitro* tests, a simulated environment was evaluated by

placing the hydrogel discs and particles in a solution with 20% human serum. After a certain amount of time, 2 h up to 2 days, the antibacterial effect of the AMP-functionalized hydrogels were evaluated. For the hydrogel discs of **Paper I**, this was performed by removing the discs from the serum, washing them, and then cultivating them with *S. aureus* for 24 h followed by antibacterial evaluation by LIVE/DEAD staining and fluorescence microscopy. The fraction of green and red of the images were then evaluated by a macro, and presented as such, for an easier comprehension. For the particles of **Paper II**, bacteria were added to the solution with particles and serum present. The solutions were then incubated for 1 h before the alive bacteria present in the solution was quantified by CFU measurements. This procedure was also performed for free AMPs in order to better analyze the effect of the serum inhibition as well as the protection provided of the AMP attachment to the particles.

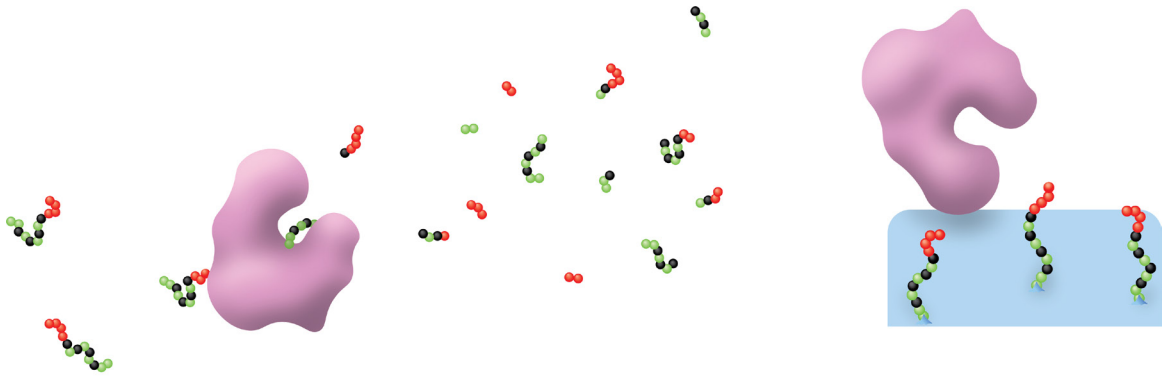

Figure 9. Free AMPs in solution are rapidly degraded by proteolytic enzymes present in serum (left). When covalently attached to a solid substrate, the AMPs should gain protection against the proteolytic enzymes due to steric hindrance (right).

4.9 Toxicity investigations by determining hemolysis and MTT-assay

For a material that has an intended use within applications that come in contact with our bodies, it is extremely important that they do not cause harm to our own cells. Especially since the main purpose of the material in question is to kill bacterial cells. Usually, the tricky part is not to kill the bacteria per se, but to do so without causing severe damage to the mammalian cells close to the bacteria.

The cell types that a wound care product will encounter the most are the cells that are present in blood, foremost erythrocytes (red blood cells). In both **Paper II** and **IV**, a test that measures the amount of lysis caused in erythrocytes (hemolysis) was therefore implemented. Hemolysis is studied by measuring the hemoglobin levels in a sample and by comparing it to a negative control (erythrocytes in PBS), which is set to 0% hemolysis, and to a positive control (erythrocytes in 0.1% Triton X-100, rest PBS) which is set to 100% hemolysis. The hemoglobin is released from inside the erythrocytes when the membrane is compromised, something that is extremely important to make sure not to happen when antimicrobial peptides are used, since

they are membrane active. The hemoglobin levels are obtained by measuring the absorbance at 540 nm by UV-vis spectroscopy in samples where that have been centrifuged so that the cells do not absorb the light. Practically this was done by incubating the AMPs and AMP-functionalized particles and hydrogels with 1 vol% erythrocytes obtained from defibrinated horse blood. Incubation took place for 1 h with frequent rotation to limit sedimentation, followed by centrifugation to obtain the released hemoglobin.

Cytotoxicity towards other cell types can be more difficult to study in cases where a material is of interest. Especially in this case where an integration of the material with the surrounding tissue is not of interest, because proliferation on top of the material would be undesirable. Instead, the standard is to check the cytotoxicity of substances potentially leached from the material after a period of extraction. This is also what was performed in this work, on the hydrogel sheets in **Paper I** and **Paper III** and on the particles in **Paper II**. The materials were placed in full media and incubated at 37 °C for 3 days for potential leachable to be extracted. This media was then used to cultivate fibroblast cells (primary human dermal fibroblasts in **Paper I** and cell line L929 in **Paper II**) with in well plates, as this is a very important cell of wound healing. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is then added to all the wells and viable and healthy cells will reduce the MTT to formazan which concentration can be detected using UV-vis spectroscopy. By comparing the formazan concentration of cells cultivated with the media used in extraction to when the cells were cultivated with normal media, a direct cell viability percentage can be obtained.

4.10 Investigating interactions with endotoxins

A subject frequently brought up by clinicians when discussing bactericidal compounds designed to be used in patients is the potential burst release of endotoxins. Endotoxin is a component of the outer membrane of gram-negative bacteria. When these are released in large quantities inside the body, they can over activate the immune system and cause severe conditions like endotoxemia and disseminated intravascular coagulation. However, endotoxin is a synonym for lipopolysaccharide, the very same substance that provides gram-negative bacteria with the negative charge of their outer membrane and a target for AMPs.

In **Paper V** a study was performed to evaluate what happened to the endotoxin levels after the pathogenic bacteria *P. aeruginosa* was cultivated on the AMP-functionalized hydrogels. This was done by adding 50 µl drop of *P. aeruginosa* (10^7 CFU/ml) on top of the AMP-functionalized hydrogels as well as control hydrogels, a standard absorbent pad (viscose/polyester nonwoven) as well as just in a glass vial. After 3 h and 24 h, the endotoxins were extracted by vortexing for 10 sec followed by filtration. The quantity of endotoxins in the different samples was subsequently quantified by a chromogenic LAL endotoxin-detection assay.

4.11 Infected *in vivo* rat model

In vitro simulations of a biological environment with culture media and added serum is a great first step to investigate the behavior with those elements. However, it is important to know that there are a lot of components missing from those environments that exist *in vivo*. In order to get more information on how the AMP functionalization survives biological environments, an infected *in vivo* rat study was implemented, an overview is illustrated in Figure 10. A pilot study was presented in **Paper I** which was performed to get a first assessment and to set the parameters for the full study presented in **Paper V**. The tests were performed by shaving the backs of female albino rats, making incisions, and creating pockets by blunt dissection in the soft tissue. The test materials were then placed in the pockets with and without *S. aureus* (50 µl 10^6 CFU/ml), suturing the pockets up, and then leaving them be for 24 or 72 h. After the designated time periods, the animals were sacrificed and the pockets were opened, materials removed and collected, and exudate in the pockets collected. The bioburden in the exudate and around the implanted materials were then determined to analyze whether any antibacterial effect was observed for the AMP-functionalized hydrogels. This was achieved by vortexing the solutions, sonicating the implant containing solutions, serial dilution and finally plating on CHROMagar, incubation and CFU determination. 32 rats were included in the larger study of **Paper V**, and for ease of operation, the study was divided up into two sets with 16 rats at the time.

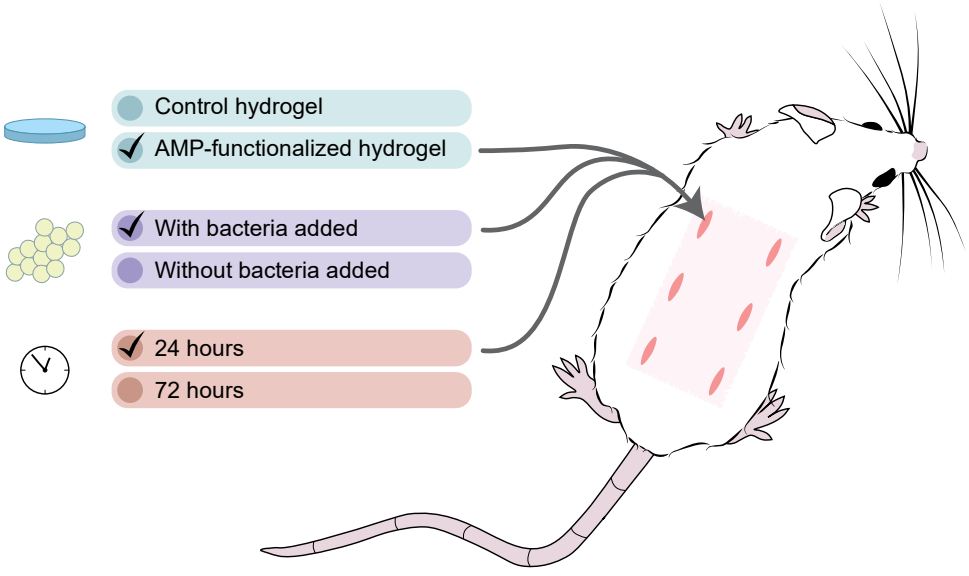

Figure 10. An overview of the infected rat model and the different parameters evaluated. Hydrogels were placed in the separate pockets made on the backs of the rats. For half of the rats, bacteria was added to the pockets, for the other half of the rats, bacteria was not added. Half of the rats were evaluated after 24 h while the other half was evaluated after 72 h.

4.12 Intact skin clinical study

The objective of **Paper III** was to investigate the antibacterial activity of the AMP-functionalized hydrogels formulated as wound dressings on the intact skin of healthy volunteers. This is a good model on the way to see how the AMP-functionalized hydrogels behave in a more clinically relevant setting. While the covalent attachment does come with a lot of benefits, there are also some significant limitations with it, mainly as the antibacterial effect is only maintained on the surface of the hydrogels. It was therefore of interest to evaluate whether this contact killing antibacterial effect was enough to reach and eradicate the bacteria naturally present on the skin. Since the bacteria will be present in a wide variety of strains (there should be other microbes present) and reside in pores, creases, and hair follicles, this could have a significant limiting effect on the activity.

In the study, the forearms of 40 healthy volunteers got exposed to control hydrogel dressings as well as AMP-functionalized dressings for 3 h. An area under the dressings as well as an untreated area got scrubbed with a retrieving solution (0.1% Triton X-100 in PBS) and the solutions were collected in separate Eppendorf tubes. The procedure is presented in Figure 11. The bioburden was analyzed by plating the solutions on agar plates. After incubation, a blinded analysis was performed by counting the colonies and calculating the CFU/cm^2 present on the skin of the volunteers. After unblinding, the bioburden under the AMP-functionalized hydrogel, control-hydrogel and on the untreated skin could be compared and the antimicrobial effect of the AMP-functionalized hydrogel dressing could be evaluated.

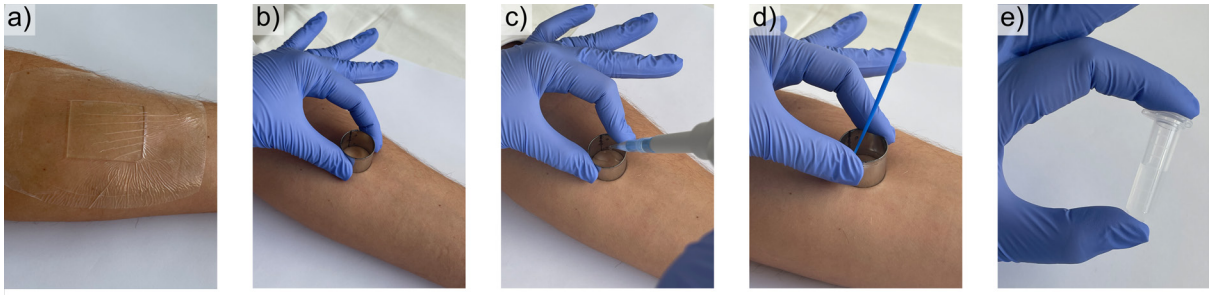

Figure 11. Images taken of the different steps of the intact skin test procedure. The hydrogel dressing was placed on the forearm and covered with a transparent adhesive dressing (a). A steel ring was placed on the forearm and held in place tightly where the hydrogel had been in contact with the skin (b). 1ml of retrieving solution was added into the steel ring (c). The area inside of the steel ring was scrubbed using an inoculation loop (d). The 1ml solution was collected for plating (e)

5 Results and discussion

5.1 Material characterization

In **paper I**, **IV**, and **V** hydrogel discs were used, which appearance can be observed in Figure 12. The disc in the middle is the appearance just after it is punched out from the hydrogel sheets (70% water), on the left is the appearance if it is left to dry, and on the right is the appearance after it being positioned in water for 3 days, forming a swollen state containing 85-90% water. For the AMP functionalization and all experiments evaluating activity in those three papers, it is the swollen version that is used. In Figure 13, the preparation of the hydrogel sheets and particles used in **Paper II** is presented. The appearance of the 30 wt% Pluronic F-127 in water just after mixing is a very foamy, white paste (a), which turns into a transparent liquid at 4 °C (b) and a very viscous gel at room temperature (c). This counterintuitive effect of gelling at higher temperatures is due to the thermo responsive self-assembly of Pluronics discussed in chapter 3.7. After the gel was placed between glass slides and cross-linked, hydrogel sheets (d) were obtained which were used to make both hydrogel discs as seen in Figure 12, and particles. After processing, the fully swollen particles (e) are the ones used for antibacterial and cytotoxicity experiments while the freeze-dried particles (f) are used for material characterization. Most of the particle mass was found in particles with a size between 100 – 500 µm, but in sheer numbers, the majority of the particles had a size of 2 – 30 µm. Furthermore, DLS measurements showed that a size distribution between 20 – 350 nm was also present if the solution was passed through a filter.

Figure 12. Appearance of the hydrogel discs used throughout the work. The dry hydrogel (left) is after 5 days of drying at 40 °C, where only the polymer remains. The just prepared hydrogel (middle) is the appearance just after it has been punched out from a sheet. The swollen hydrogel (right) is the appearance after being stored in excess water for 3 days, which is the most used version.

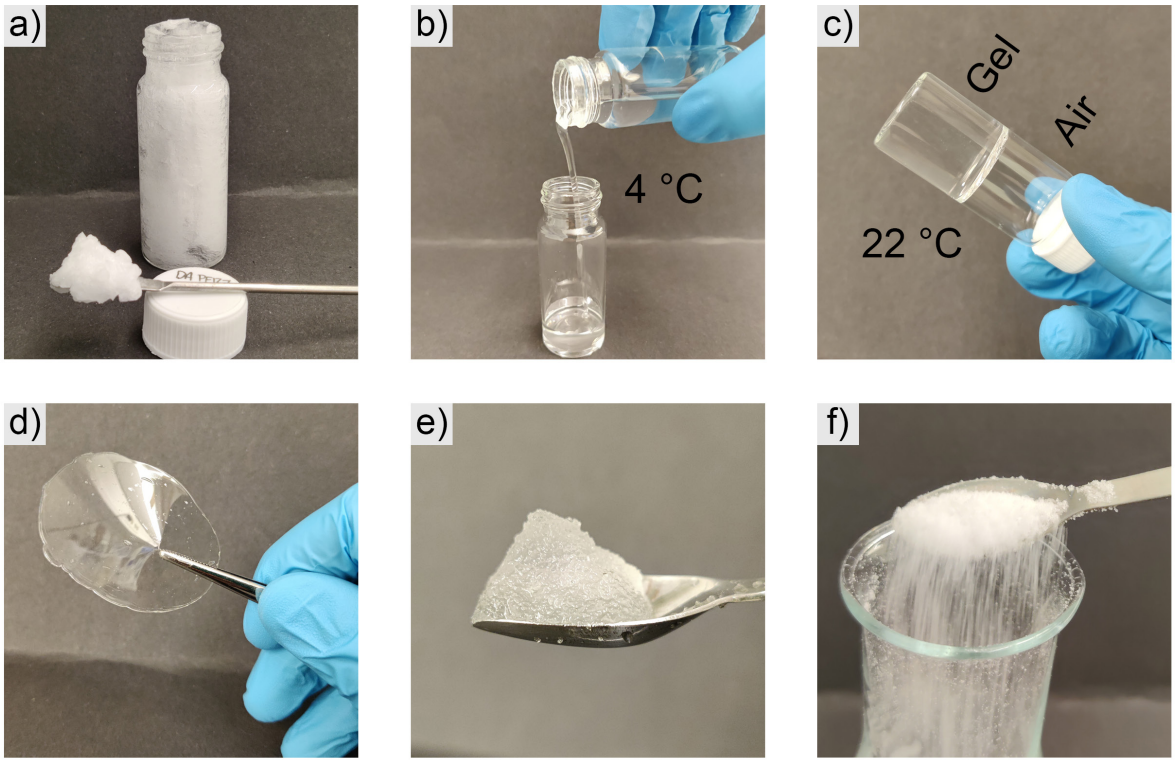

Figure 13. Different steps of the hydrogel synthesis. (a) show the gel after mixing 30 wt% Pluronic with 70 wt% water. (b) show the gel after 2 days at 4 °C where it is liquid, (c) show the same gel as it is left to reach room temperature. (d) show the cross-linked sheet of hydrogel. (e) show particles made from the sheets, when fully swollen, and (f) show the particles after they are freeze-dried.

The attachment of six different AMPs onto hydrogel discs was studied by UV-vis spectroscopy in **Paper IV** and is presented in Table 2. The amount of attached AMP did not differ vastly between the AMPs as all fell within the range of 119 – 166 nmol per disc. Furthermore, the results strongly indicated that the hydrophobicity of the AMPs seemed to be more of a driving force for higher attachment than the prevalence of available attachment site. As the AMPs containing lysine have more primary amine groups than their arginine counterpart, this should promote attachment in the case that the availability of the primary amine group of the N-terminal is limited. Since the AMP with most lysine instead had the lowest attachment, this does not appear to be the case, instead the hydrophobicity of the AMPs appears to be a driving force. This could either be because more hydrophobic compounds tend to yield to a stronger surface activity, promoting migration away from the solution, towards surfaces. Another explanation could be due to the hydrophobic domains in the center of the micelles formed by the polymers could have a significant interaction with the AMPs, especially the more hydrophobic versions.

Adding to this, in **Paper II** it was found that hydrogel discs was saturated with 1 mg of the AMP R9W4 per 100 mg of hydrogel (dry weight) while the particles were saturated with 3.3 mg of the AMP R9W4 per 100 mg of particles (dry weight). This showed that the much larger specific surface area of the particles increased the amount of AMP that was used up in the functionalization, indicating that the available surface was another driving force for AMP attachment. This does make sense since the surface is the most likely place on the hydrogels to find carboxylic acid groups, as this should be the first site where the ester groups of the acrylates should be broken down by both hydrolysis and exposure to UV-light.

Table 2. Attachment of five different AMPs onto 14 mm Ø hydrogel discs. The presented numbers are the average of 18 samples ± the standard deviation.

AMP name	**Attached AMP [nmol]/ hydrogel disc**
K9W4	119 ± 14
KR9W4	136 ± 19
R9W4	130 ± 23
R9W5	166 ± 28
Omiganan	147 ± 26

To further investigate the synthesis steps, Raman spectroscopy and FTIR was implemented in **Paper II**. This was performed on the polymer itself, freeze-dried hydrogel particles (no AMP), freeze-dried AMP-functionalized particles, and on the pure AMP. The results from this are presented in Figure 14, with Raman spectroscopy in (a) and the FTIR region corresponding to the carbonyl stretching in (b). From the Raman spectroscopy it was evident that the cross-linking made the vinyl groups of the acrylate react, as the two small peaks around 1700 cm^{-1} disappeared moving from the polymer to the control particles. Between the control particles

and the AMP-functionalized particles, three new small peaks appeared, which aligned very well with three peaks observed in the pure AMP sample, most likely due to the indole group of tryptophan. The AMP presence in the functionalized particles was also evident from the FTIR results. The strong peak(s) at 1650 cm^{-1} of the pure AMP corresponds to amide groups which there are plenty of in peptides, it is also clear that this is present in the AMP-functionalized particles sample but not in any other. While this shows that the AMP indeed is present, it does not show exactly how it is present. There is a clear peak shift going from the polymer sample to the cross-linked hydrogel particle which most likely is due to the carbonyl moving from a conjugated ester at the carbonyl side to a non-conjugated ester which should correspond to a peak shift to higher wavenumber. The presence of carboxylic acids and esters within this peak is difficult to evaluate, as well as the prevalence of carboxylic acids from this peak forming amide bonds with the AMP and jumping to lower wavenumbers.

By assuming that all the AMPs that is used up in the functionalization is covalently attached, and that all carboxylic acids present is used in the reaction, the theoretical carboxylic acids present can be calculated. For the hydrogel discs with 1 mg AMP per 100 mg hydrogel, 3.3% of the carbonyls should be carboxylic acids and the rest esters. For the particles that the measurements were performed on, with 3.3 mg AMP per 100 mg particles, that number should be 11.1%. With a higher surface area for the particles these numbers do feel reasonable in that the hydrogels are still maintained as hydrogels with that amount of ester bonds broken. However, if the assumption of the calculation holds, 11.1% does not appear to be significantly large to show a clear separate peak. Arguments might be made for that the appearance of the peak at 1735 cm^{-1} looks different for the AMP-functionalized particles compared to the control particles, but it is not clear enough to draw any certain conclusions.

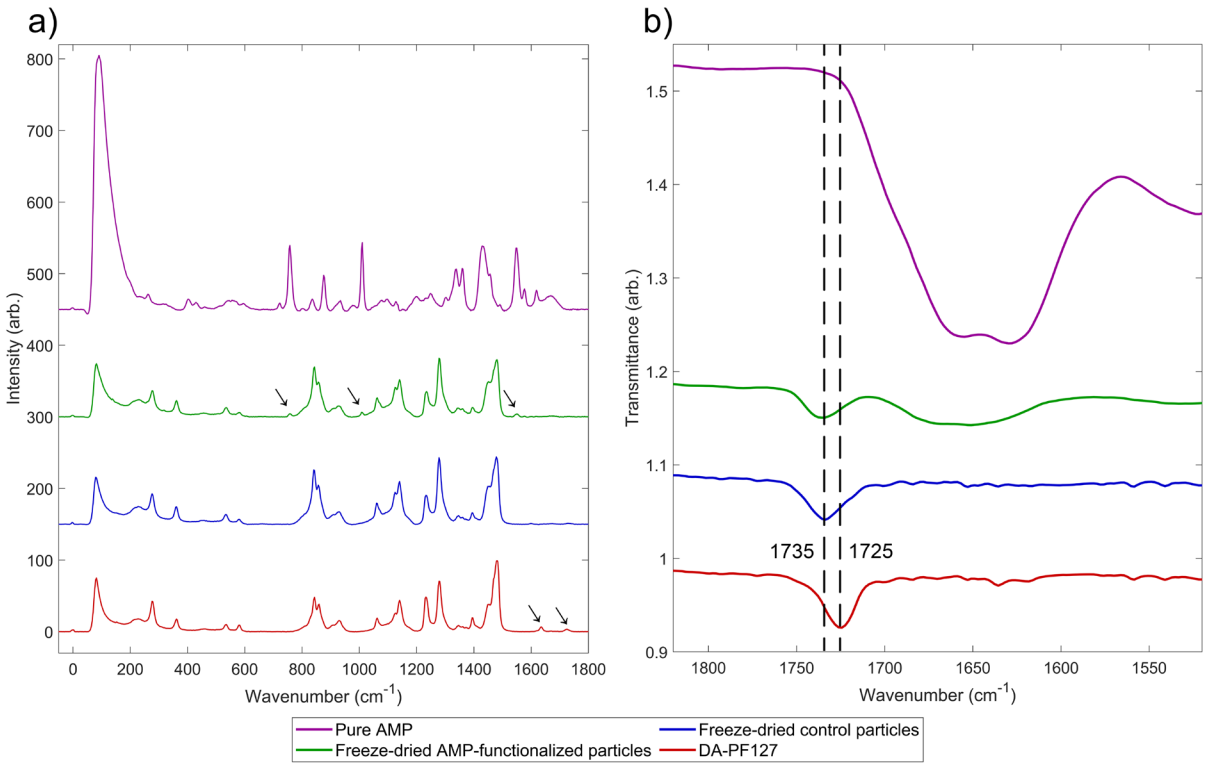

Figure 14. (a) Results from the Raman spectroscopy of pure AMP (purple, top), freeze-dried AMP-functionalized particles (green, second from the top), freeze-dried control particles (blue, second from the bottom), and the polymer Pluronic F-127 diacrylate (red, bottom). (b) A section of the FTIR measurements corresponding to carbonyl stretching with the same samples and order as in (a).

The diffractograms obtained by SAXS measurements of the samples are shown in Figure 15. A clear peak was observed in all the samples evaluated, which strongly indicates that a structure with long-range order was present. Since no noticeable difference was observed between the control hydrogel disc, control particles, or AMP-functionalized particles it is assumed that neither the preparation of particles nor the AMP-functionalization of the same affect the ordered structure present. A small peak shift to a higher q-value was observed for the swollen AMP-functionalized particles which indicated slightly reduced distances in the sample. This is most likely due to the micelles swelling as they absorb water, but as they are cross-linked together, they cannot move as much in relation to each other, resulting in the distance effectively becoming smaller. One of the benefits of analyzing LLCs with SAXS is that the phase of the structure can be determined by the presence of secondary peaks and the relative patterns they make. However, this was not possible in these samples as there were no secondary peaks present. This could be due to lower sensitivity of the instrument or in-situ drying causing a higher probability of random scattering. Regardless, the concentration used and the location of the observed peak points towards that the ordered structure is of micellar cubic nature.

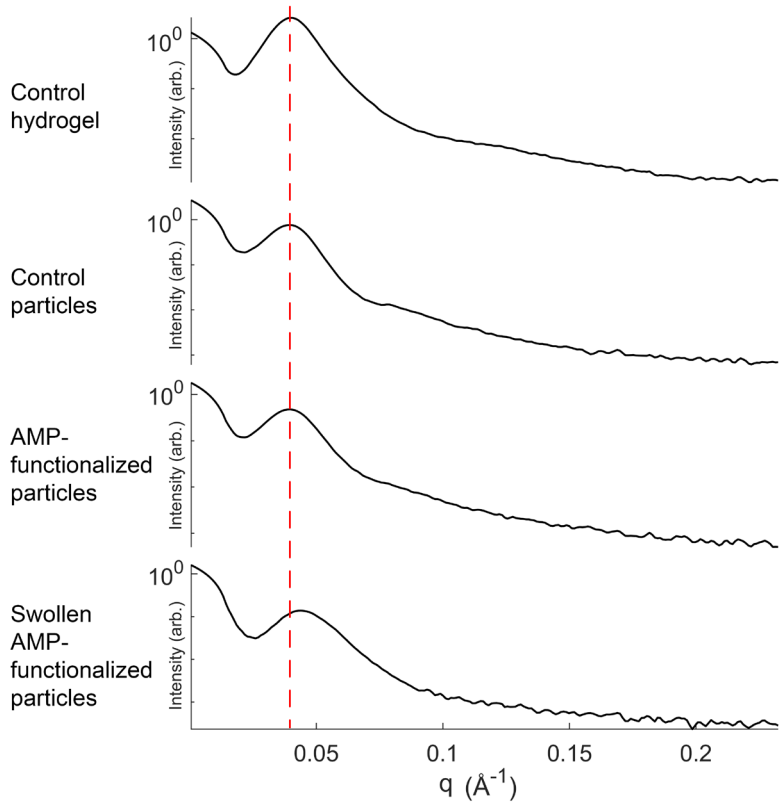

Figure 15. SAXS diffractograms of cross-linked hydrogels (not swollen), freeze-dried control particles, freeze-dried antimicrobial particles, and fully swollen control particles. All samples were measured in between tape, which has been removed as a background from the other samples.

5.2 Antibacterial evaluation *in vitro*

The first indications on whether an antibacterial effect was obtained for the AMP-functionalized hydrogels based on R9W4 were presented in **Paper I.** Here several different bacterial strains were cultivated in well plates together with control and AMP-functionalized hydrogels for 24 hours. Afterwards the bacteria present on the surfaces were stained green if they were alive and stained red if they were dead. Representative example images of the different bacteria on the two surface types can be observed in Figure 16. While some bacteria, mainly the gram-negative, did not prefer to attach and grow on the controls to a large extent, a clear antibacterial effect was still evident on the AMP-functionalized hydrogels against all five strains tested. The reason why some bacteria did not prefer to attach to the control hydrogels might be found in the polymer structure as the hydrophilic section, polyethylene oxide also known as polyethylene glycol, is commonly used as an antifouling surface modification. The bacteria that do attach to the controls are for the most part thriving, and in most of the *Staphylococci* cultures, the bacteria appeared to start forming biofilms. For the AMP-functionalized hydrogel the situation was quite the opposite since most of the bacteria present on the surfaces were dead. This was the case for common pathogenic bacteria *S. aureus, S.*

epidermidis, and *P. aeruginosa* but also against the antibiotic resistant strains MRSA and MDR *E. coli*. MRSA obtains its resistance by producing other proteins that take the role of transpeptidase, and MDR *E.coli* obtains its resistance by the presence of a gene that code for NDM-1, an enzyme that hydrolyses most β-lactam based antibiotics, rendering them ineffective [54, 118]. Since these mechanisms should not have any effect on AMPs it should therefore not be a surprise that the antibacterial effect is maintained. But it does show the potential of the technology.

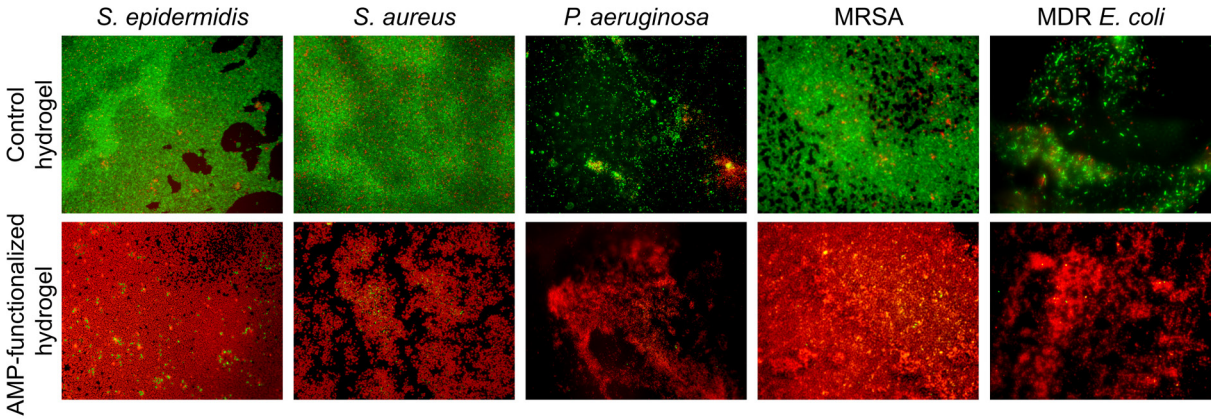

Figure 16. Bacteria stained green if alive and red if dead after 24 hours cultivation on control hydrogels (top row) and AMP-functionalized hydrogels (bottom row).

To study the impact of the choice of AMP for surface functionalization, six different AMPs were evaluated both free in solution and attached to the hydrogels, as detailed in **Paper IV**. The minimum inhibitory concentration (MIC) of the peptides against *S. aureus* and *P. aeruginosa* are presented in Table 3. Here it was evident that substituting arginine to lysin had a negative effect on the antibacterial effect in solution, as K9W4 and KR9W4 showed quite high MIC values compared to R9W4. Adding an extra tryptophan to the sequence introduced stability/solubility issues as the AMPs precipitated out from the media and resulted in higher MIC values for R9W5 compared to R9W4. Both Omiganan and Piscidin 1 showed quite low MIC values against both strains, especially Piscidin 1 which showed a very low MIC against *S. aureus*.

Table 3. Measured MIC values for six different peptides against *S. aureus* and *P. aeruginosa*. Three independent sets of the measurements were performed, and if different MIC values were reported for the different runs, a span is presented in the table.

AMP	**MIC, *S. aureus* [μM]**	**MIC, *P. aeruginosa* [μM]**
K9W4	32 – 64	64 – 128
KR9W4	8 – 16	16 – 64
R9W4	8	16
R9W5	16	32 – 64
Omiganan	4 – 8	32
Piscidin 1	1 – 2	8 – 16

The antibacterial effect observed by the AMPs when attached to the hydrogel surfaces was evaluated by incubation with bacteria for 30 min where the bacteria was just present in a thin liquid layer on top of the hydrogels. The viability of the bacteria was then evaluated by CFU measurements which are presented in Figure 17. Against *S. aureus*, the activity followed the same MIC trend found when in solution apart from R9W5 and Omiganan. R9W5 had a very high antibacterial effect, most likely due to the stability/solubility issue not being as much of an issue when attached to the surface. Omiganan showed a relatively low antibacterial activity against both strains, which might be due to its suggested mode of action where saturation of the membrane is required before destabilization. A feat which might be considerably more difficult to achieve when attached to a surface.

Against *P. aeruginosa* the antibacterial activity of the AMPs did not follow the same trend as the MIC in solution. The two AMPs that had the highest MICs, K9W4 and KR9W4, had the lowest number of viable bacteria present closely followed by R9W4. There is no straight forward explanation to why this is the case. Then again, the mode of action of these peptides are not established and the fact that *P. aeruginosa* is a gram-negative bacteria could indicate that certain AMPs have a more difficult time to kill these bacteria when attached to a surface. Furthermore, from the LIVE/DEAD staining presented in Figure 16 it was evident that *P. aeruginosa* in general interacted less favorably with the hydrogel itself compared to *S. aureus* and it might be that this presents a further challenge for some AMPs. What is evident from this is that it is very difficult to guess the antibacterial activity of an AMP when attached to a surface based on its activity when free in solution. Furthermore, these antibacterial results might very well be specific to when attached to the amphiphilic hydrogel and might differ substantially if attached to another material.

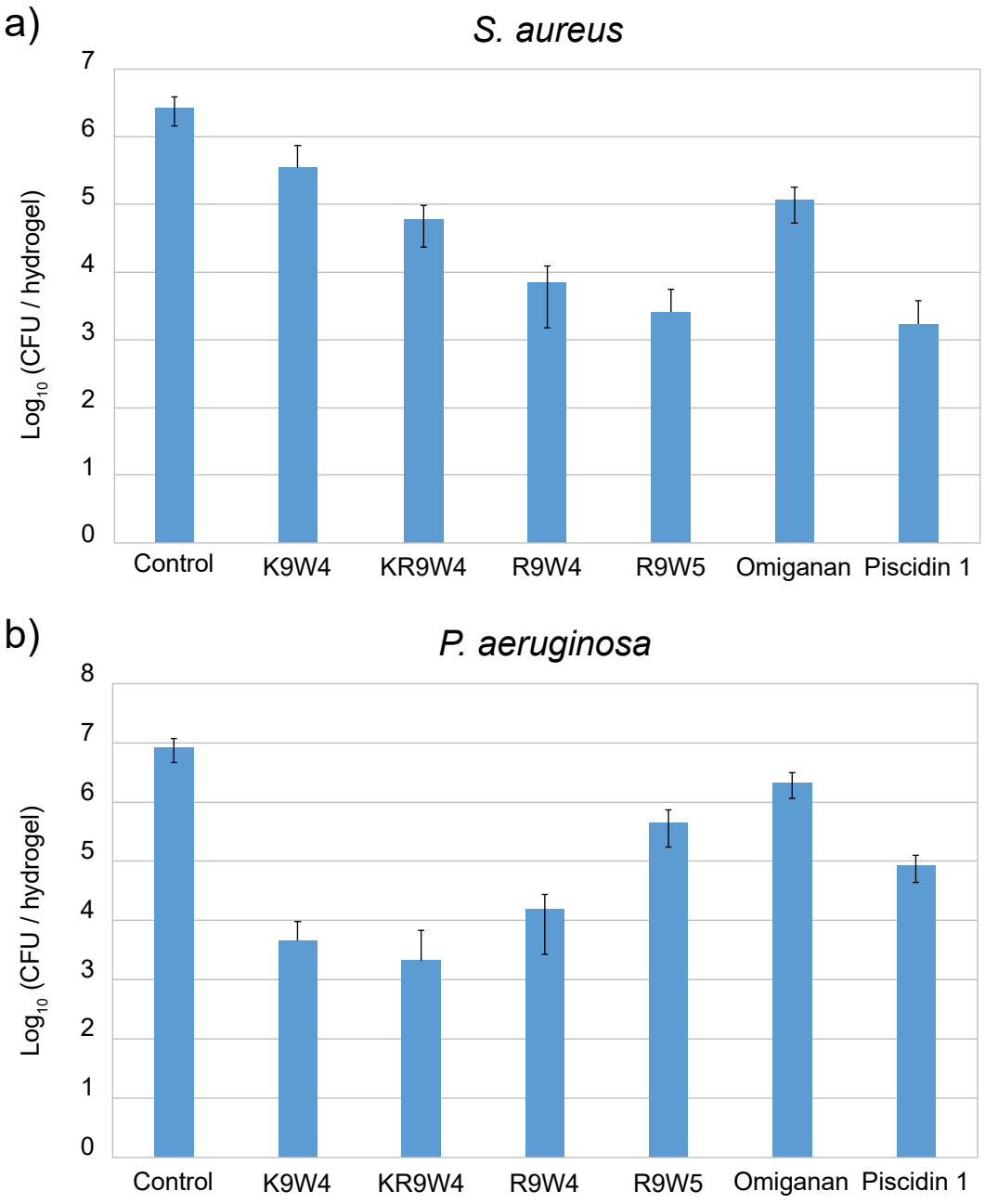

Figure 17. CFU observed on the hydrogel surfaces functionalized with six different AMPs as well as a control hydrogel surface against *S. aureus* (a) and *P. aeruginosa* (b). The data presented is the average of three runs performed in triplicates (n = 9) and is shown in log10. The error bars are the standard deviation.

The antibacterial activity of the AMP-functionalized particles was evaluated both in a MIC test where the activity was compared to the AMP when in free state, and in an agar plate study. The observed MIC values against six bacterial strains, including two MRSA strains, are presented in Table 4. From here it was clear that the AMP-functionalized particles had an evident antibacterial activity, which mostly followed the same trends as the free AMPs. The amount of AMP in µg/ml was also calculated for the free AMPs as well as the AMP-functionalized

particles. From here it was also evident that in this setting, it requires more AMP for the particles to inhibit bacterial growth, since the amount of AMP at MIC was 10-30 times higher for the AMP-functionalized particles. This is not too surprising seeing that the AMPs lose a lot of their mobility when attached to the surfaces. Free AMPs will in a much higher extent travel to the bacteria and exert their antibacterial effect, for the particles the situation is most likely the opposite since the particles present in the MIC solutions were much larger than the bacteria. Furthermore, a substantial portion of the weight of the particles in this test was constituted of quite large particles that rapidly sedimented and contributed more to the higher MIC. Particles with a size distribution consisted of smaller particles should result in lower MIC values, at least in terms of weight.

Table 4. MIC values against six bacterial strains. The tests were performed in three independent experiments and if different concentrations were observed at different separate runs, an interval of concentrations is presented.

	Free AMP		**AMP-functionalized particles**	
	[μM]	[μg/ml] of AMP	[mg/ml]	[μg/ml] of AMP
S. aureus	1.6	3	0.8 – 3.1	26 – 103
S. aureus MRSA, 41586	1.6 – 3.1	3 – 6	0.4 – 0.8	13 – 26
S. aureus MRSA, 74135	1.6 – 6.2	3 – 12	0.4 – 1.6	13 – 52
S. epidermidis	3.1	6	0.8 – 1.6	26 – 52
E. coli	1.6 – 3.1	3 – 6	1.6 – 3.1	52 – 103
P. aeruginosa	6.3 – 12.5	12 – 24	12.5 – 25	413 – 825

Apart from in a solution, the antibacterial effect of the particles was also evaluated more as a paste that might be applied topically. This was done in an agar plate study against *S. aureus* from which the observed results are presented in Figure 18. In (a) the control particles (left) and AMP-functionalized particles (right) can be seen after overnight incubation. A clear difference in appearance was observed as the AMP-functionalized particles retained more of their transparency, while the control particles became more cloudy, likely due to the presence of bacteria. This was supported by the CFU determination of biopsy punches taken through the middle of the spreads, including the agar beneath. The AMP-functionalized particles showed a reduction of bioburden present after incubation by a factor of 10^4 compared to the control particles.

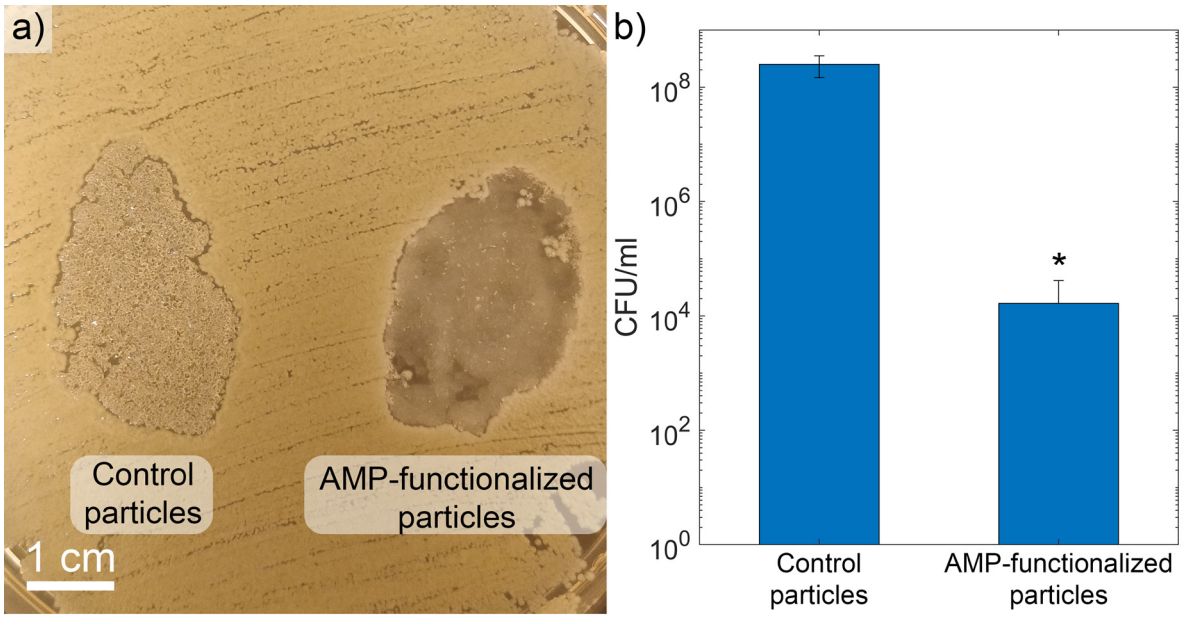

Figure 18. (a) An image of the top of an agar plate cultivated over night with *S. aureus*. (b) The measured CFU/ml of biopsy punches taken from the middle of the spreads shown in (a). n = 9 and the error bars show the standard deviation. The '*' indicates a statistically significant difference compared to the control with a confidence interval of 95%.

5.3 Indications of mechanism

From the agar plate study presented in Figure 18 it was clear that a zone of inhibition was not present around the AMP-functionalized hydrogels. This strongly indicated on an actual contact-killing mode of action and not by any release of AMP. This was also studied in a zone of inhibition test for the hydrogel discs in **Paper I**. Here hydrogel discs were activated by covalent attachment through the usual EDC/NHS activation, but a version of physically absorbed AMPs was also included where the hydrogels were just soaked in the AMP solution without being exposed to EDC and NHS prior. The AMP in this version was therefore only physically loaded onto the hydrogel. Control hydrogels were also included to make sure that any effect observed was from the inclusion of AMPs and nothing else.

Representative images from this study are shown in Figure 19 which depicts the hydrogels after overnight incubation on top of agar plates previously streaked with *S. aureus*. The results clearly showed that the covalently attached AMPs were in fact covalently attached and did not cause an evident zone of inhibition around the hydrogel. On the other hand, a zone of inhibition was clearly present around the hydrogel with physically loaded AMPs. Indicating that AMPs that are loaded onto the hydrogel construct have the potential to leach out at concentrations capable of antibacterial effect. While the FTIR and Raman spectroscopy results were inconclusive in the presence of a covalent attachment, the zone of inhibition study clearly show that when the EDC/NHS activation step is not performed, the AMPs are leaching out which is not the case when the EDC/NHS step is performed. This strongly indicates that the AMPs are

in fact covalently attached to the hydrogels and that bacteria found dead on the hydrogel has most likely been eliminated by a contact killing mechanism.

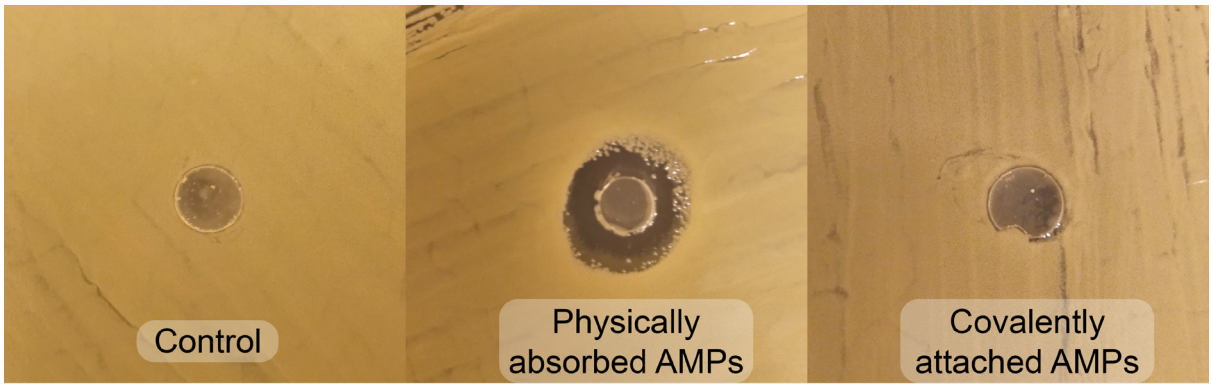

Figure 19. Images from the zone of inhibition study against *S. aureus* with control hydrogel (left) hydrogel with physically loaded AMPs (middle) and hydrogel with covalently attached AMPs (right). The images are taken after overnight incubation.

In order to further search for indications regarding how the contact killing mechanism takes form, cryo-EM experiments were performed on the AMP-functionalized particles in **Paper II**. In this experiment, a subsection of the very smallest particles was obtained by filtration and subsequently functionalized with the AMPs. After a short incubation with *S. aureus* the solution was plunge frozen and observed in a TEM under cryogenic conditions. The samples were then scanned for interactions between particles and bacteria and one example can be observed in Figure 20. The set up contained several factors that made the interactions difficult to study. Mainly the size of the bacteria which with its roughly 1 µm diameter built up a thicker layer of ice around it, making everything dark close to the cell, but also the relatively low particle concentration, limiting the number of interaction available for study. Regardless, in the image it does appear to be a strong interaction between the particle and the bacteria, as the particle is deformed along the bacterial surface. Furthermore, there might even be some deformation of the cell envelope close to the particle, but again, the size of the cell made it difficult to observe clearly. There are some mutated bacteria, like mini-*E. coli*, that might be better suited to study the interaction, but those bacteria usually have a lower viability and have a lower fitness, and the idea here was to study the interaction with bacteria of more relevance. However, this resulted in images with a much lower clarity. While the bactericidal effect appears to have to do with direct interactions with the cell envelope, whether the effect is reached by creating ruptures in the membrane/peptidoglycan layer or by other means is still up for debate.

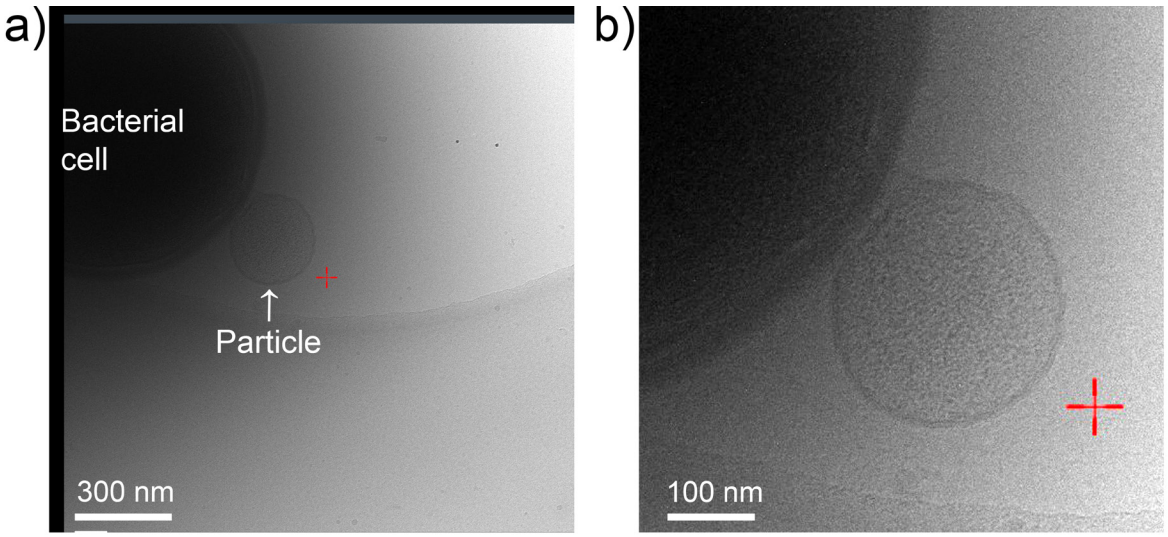

Figure 20. An interaction between a particle and an *S. aureus* bacterial cell. The right image is a zoomed in section of the left image. The red crosshair is a result of the image capture process and is to be ignored.

5.4 Stability

As the main motivation behind the attachment of AMPs to a surface is to increase their stability against components of our plasma, this is also an important feature to study. In **Paper I** this was evaluated by placing the AMP-functionalized hydrogels and control hydrogels in 20% human serum and then evaluating antibacterial effect by cultivating the hydrogel with *S. aureus* for 24 h followed by LIVE/DEAD staining. For easier evaluation, a macro was used to determine how much of the images were red and how much of the images were green. The results from this study can be observed in Figure 21, where the fraction of dead cells in the images are presented against the amount of time spend in serum prior to cultivation with the bacteria. Here it was clear that a potent antibacterial effect was maintained for the first 10 h of serum exposure. After 1 and 2 days of serum exposure the potency of the antibacterial effect was somewhat reduced, but a significant effect was still observed which did not change between 1 and 2 days.

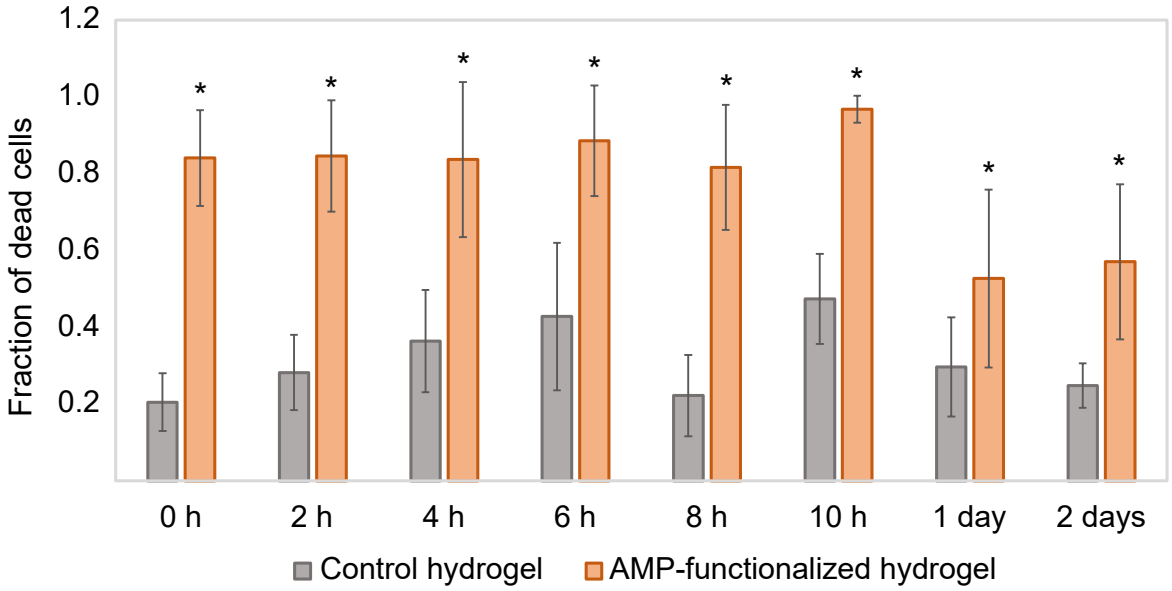

Figure 21. Fraction of dead *S. aureus* on control hydrogels and AMP-functionalized hydrogels after 24 h cultivation. Prior to cultivation, the hydrogels had been exposed to 20% human serum for up to 2 days. Each bar represents the average of two experimental set ups with duplicates, and 6 images per surface. '*' indicates a statistically significant difference with a confidence interval of 95%.

While the experiment presented above showed that an antibacterial effect was maintained on the hydrogels after exposure to serum, there were some questions that remained unanswered. The first was how well the attachment to the surfaces provided the AMPs with protection in comparison to their free state. Another question was the effect of ionic strength and protein adsorption onto the surface. Since the hydrogel discs were washed before exposure to bacteria, there is a chance that barely any serum components remained at that stage, and that the main loss of antibacterial effect was due to proteolysis. In order to take these parameters into consideration, a test was devised in **Paper II** where the antibacterial activity of the free AMPs was compared to the AMP-functionalized particles against *S. epidermidis*.

The antibacterial activity was determined for solutions containing free AMP and AMP-functionalized particles which were compared to a PBS solution. The antibacterial activity was evaluated by CFU determination after a 10 min cultivation with bacteria with the following conditions: without serum, just after serum was added, after the solutions had been standing for 1 day with serum, and after the solutions had been standing for 2 days with serum. The results from this study are presented in Figure 22. (a) show the rapid antibacterial activity of the free AMPs and AMP-functionalized particles without the presence of serum. From (b), where serum is added to the solutions at a final concentration of 20%, this has a major impact on the antibacterial activity. While the AMP-functionalized particles became less effective, the free AMPs showed a 2 log higher bacterial count compared to the case without serum present. Furthermore, after 1 day in serum (c), the free AMPs showed no antibacterial activity whatsoever, while the AMP-functionalized particles retained all antibacterial activity. The same case was observed after 2 days in serum (d).

These results strongly indicate that by attaching the AMPs to the amphiphilic hydrogels, the antibacterial activity can be preserved for relevant time frames. Proteolysis of the AMPs when attached to the hydrogel surfaces appear to be significantly reduced while the deposition of serum components onto the surface and the presence of physiological ionic strength does not significantly hinder the bactericidal effect of the AMPs.

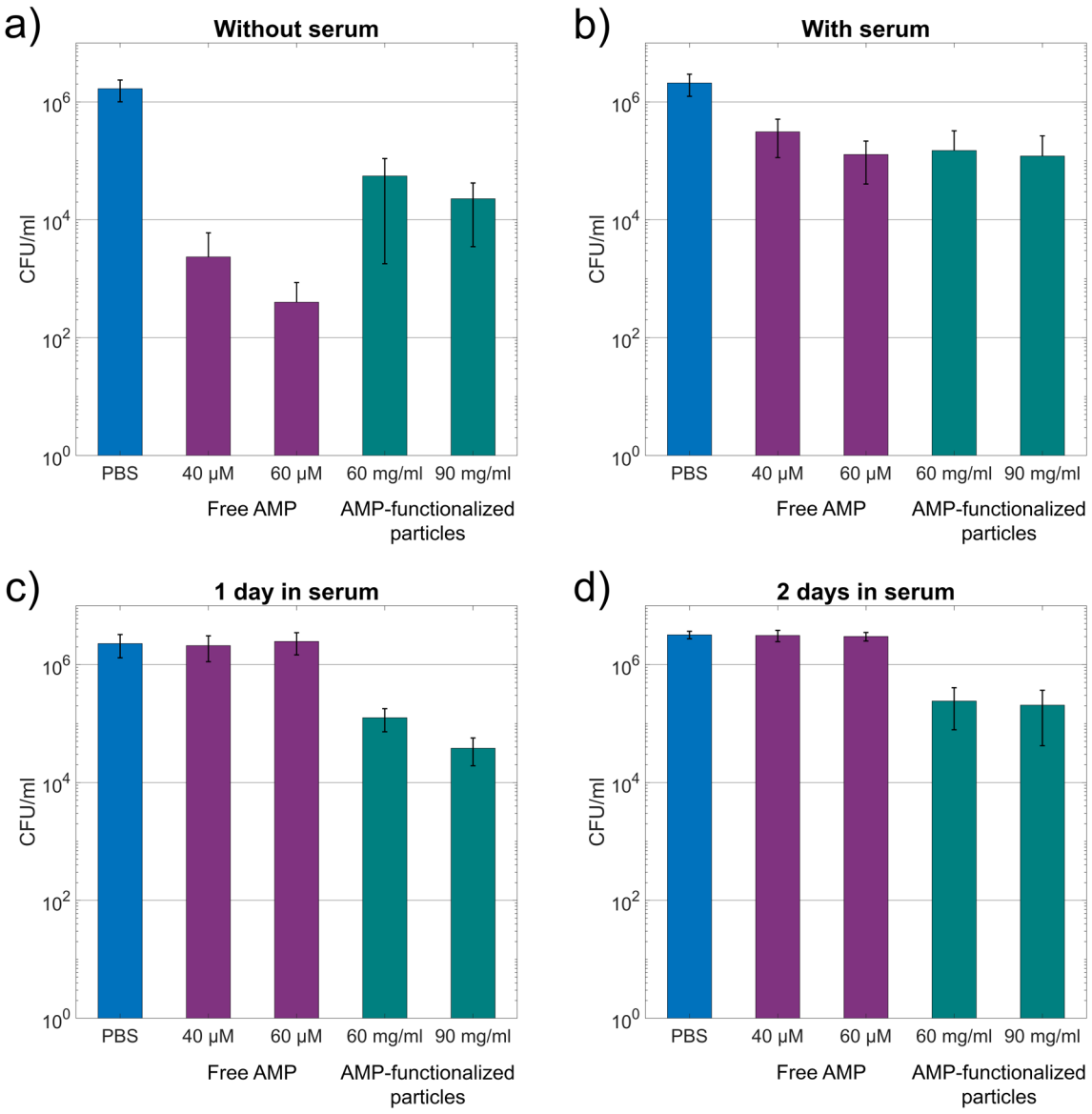

Figure 22. CFU of *S. epidermidis* in solutions of PBS, free AMP, and AMP-functionalized particles (a) without serum present, (b) with serum present, (c) after 1 day of incubation with serum prior to the addition of bacteria, and (d) after 2 days of incubation with serum prior to the addition of bacteria.

5.5 Cytotoxicity evaluation

In general, in the evaluation of medical devices that are designed to come in contact with our bodies, the most important feature is that it does not negatively affect our own cells and their functions. In **Paper I** and **II** this was in part evaluated for the hydrogel discs and particles by MTT assays on fibroblast. In **Paper I** this was done on primary human dermal fibroblasts and in **Paper II** this was done on the cell line L929, which is derived from mouse fibroblasts. As fibroblasts play a major role in the wound healing process, a cytotoxic potential against them in an *in vitro* setting could indicate a cause for concern regarding impeding the wound healing process. While fibroblasts are one of the more manageable cell types to cultivate *in vitro*, they will not grow on just any surface and the standard protocol is instead to expose the material to an extraction procedure and determine the cytotoxic potential of the extracts. The results from these studies are shown in Figure 23, where the viability of the cells cultivated with the media extracts from hydrogel discs are shown in (a) and cultivated with the media extracts from particles are shown in (b). For these types of tests, a substrate is deemed to show cytotoxicity if the cell viability is lower than 70% compared to the negative control.

For the hydrogel discs (a), there was no difference observed for the control hydrogels (no AMP) or the AMP-functionalized hydrogels compared to the negative control and the viability was significantly higher than 70%. This strongly indicates that the evaluated material did not show any cytotoxicity. However, it should be noted that the hydrogel discs evaluated roughly had a surface area of 0.5 cm^2 which in 1 ml of extraction vehicle is on the lower end of the recommended ratios. For the evaluation of the hydrogel particles, the surface area is very tricky to determine but ISO 10993-10 suggests a recommended extraction ratio for '*irregular shaped porous devices*' of 0.1 g/ml, which is what was used. The cell viability was evaluated after 1 and 2 days of cultivation with the fibroblasts (b) and while slightly reduced viability was observed, all values were above the 70% threshold. After three days in the media, it is fully possible that parts of the particles were broken down and that polymer (+ AMP) leached out in lower concentrations to the media. Another potential reason behind the slightly lower viability could be due to some form of dilution of the media, as swollen particles were used, and an exchange of internal and external components is to be expected. Since the control particles also saw a slight reduction in viability it is conceivable that the polymer or the material structure could affect the results. Regardless, the results indicate that the particles did not induce any considerable cytotoxic response.

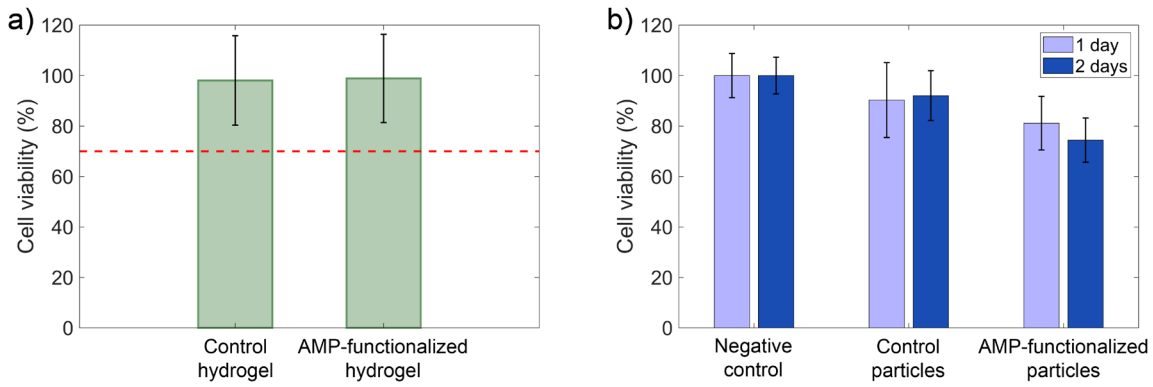

Figure 23. Cell viability measured by MTT assays, shown as percentage of the negative control. The viability of human dermal fibroblasts after culture with media after extraction of hydrogel disc, the bars are the average of n = 8 with the standard deviation as the error bars (a). The viability of L929 fibroblasts after 1 day (light blue) and 2 days (dark blue) of culture with media after extraction with control particles and AMP-functionalized particles, the bars are the average of n = 9 with standard deviation as the error bars.

Another cell type that a medical device or pharmaceutical is sure to come in contact with is erythrocytes, especially with wound care as the intended area of use. Another standard test to evaluate cytotoxicity is therefore to study the lysis of erythrocytes by analyzing the hemoglobin levels that leak out from damaged cells after incubation with the samples in a hemolysis assay. This is especially important to conduct on AMPs since the most common mode of action is just to permeabilize membranes. Hemolysis experiments were done for free AMPs and particles in **Paper II** and for the six different AMPs, free in solution and attached to the hydrogel surfaces in **Paper IV**. The results from these studies are presented in Figure 24 and Figure 25 where the level of hemolysis is shown on the y-axis as a percentage of the positive control (0.1% Triton X-100) which is set to 100% hemolysis. For the AMP R9W4 free in solution and attached to the particles, the hemolysis response is shown in Figure 24. The AMPs free in solution caused increasing hemolysis with an increase of concentration, and at 50 μM and higher more than 20% hemolysis was observed. Interestingly, the observed hemolysis for the AMP-functionalized particles was maintained at 5-10% hemolysis regardless of the particle concentration. For the 12.5 mg/ml sample, more AMPs are present in the solution than for the 200 μM of free AMP. This strongly indicates that the hemolytic properties of the AMPs are significantly reduced when they are covalently attached to the hydrogel surfaces. On the other hand, we also know from the MIC studies that the antibacterial effect of the AMPs gets somewhat hampered when attached to the hydrogel surfaces. However, with an increased serum stability and reduced chance to induce cytotoxic response, a reduced antibacterial effect in solution is a trade-off happily made. Considerations just need to be taken when deciding on the area of implementation.

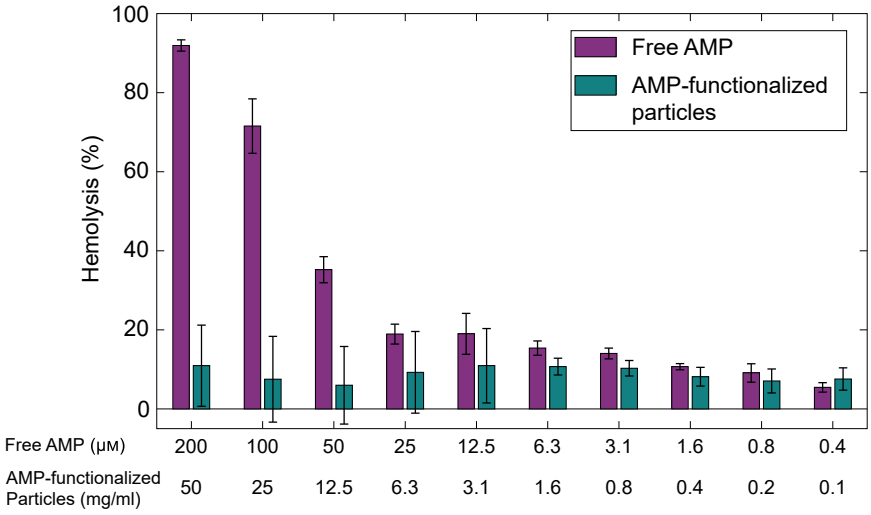

Figure 24. Hemolysis of erythrocytes from horse blood, shown as percentage of positive control on the y-axis. The substances tested were free AMPs and AMP-functionalized particles which concentrations are shown on the x-axis. The results are the average from three independent experiments with the standard deviation presented as error bars.

The balancing act of higher antibacterial effect and higher risk of cytotoxicity of AMPs is very evident when observing the hemolysis of the six free AMPs shown in Figure 25 (a). Changing the arginine amino acids in R9W4 to lysine might have reduced the antibacterial potency in the MIC tests, but a reduction in hemolysis was also observed for K9W4 and KR9W4. On the other hand, adding one extra tryptophan to the sequence, R9W5, showed a significant increase in the hemolysis rate. R9W4 crossed 20% hemolysis at 32 μM which reached 100% hemolysis for R9W5, 20% hemolysis was crossed at 8 μM. Omiganan showed among the lowest hemolysis rates, which is quite interesting since the antibacterial activity was considerably high in the MIC tests, hinting at an antibacterial effect that is not as susceptible to cause cytotoxicity towards mammalian cells. On the other side of things, Piscidin 1 might have had the undoubtably strongest antibacterial activity in solution, but it was also accompanied by the most potent hemolytic effect. 20% hemolysis was crossed at 4 μM, lower than MIC against *P. aeruginosa* and already at 16 μM 100% hemolysis was reached. This really emphasize the narrow therapeutic window that some AMPs are subject to in solution.

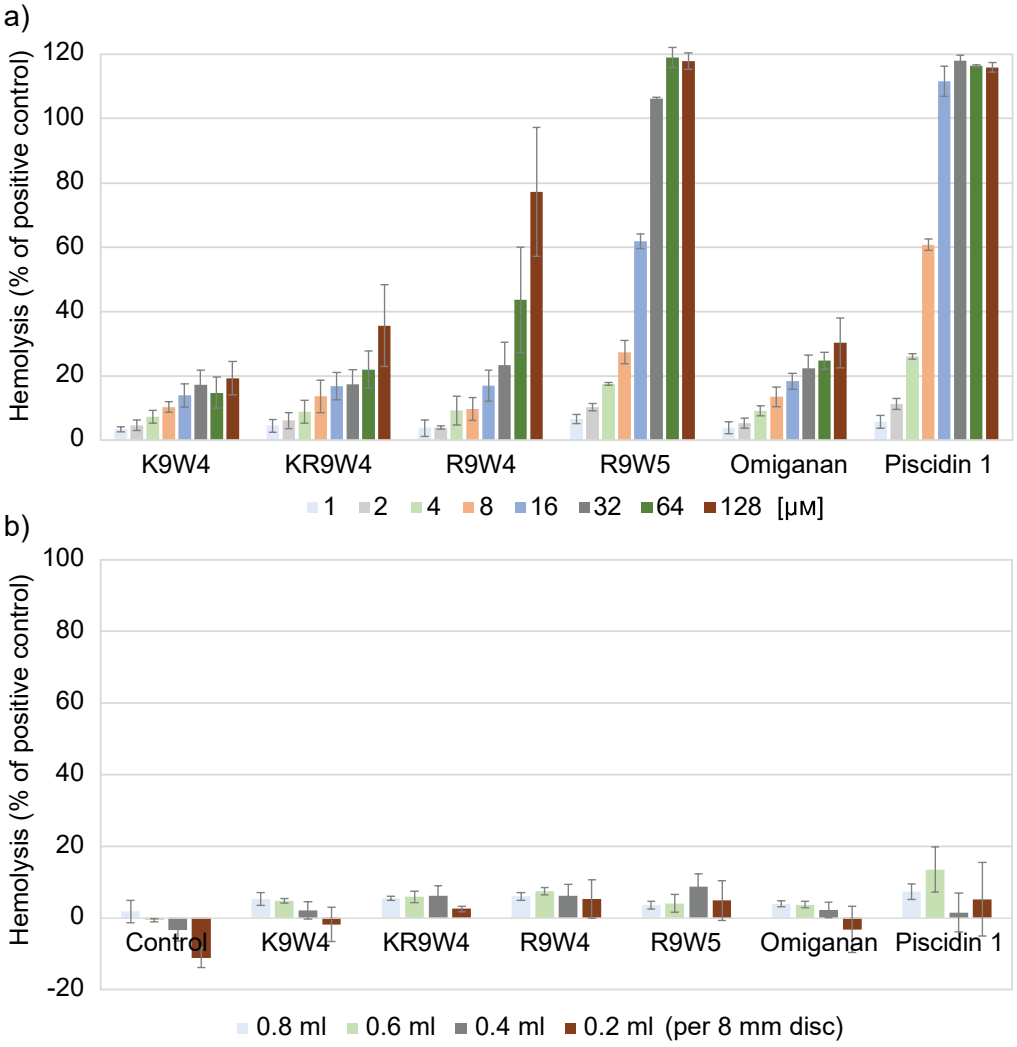

Figure 25. Hemolysis of 1 vol% erythrocytes compared to the positive control (0.1% Triton X-100). a) hemolysis from the free AMPs at different concentrations ranging from 1 μM to 128 μM. Each bar shows the average of three runs and the error bars are the standard deviation. b) hemolysis from the different AMPs when attached to the hydrogel surfaces, control hydrogels without any AMPs were also included. The hydrogel discs of the same size were placed in different volumes of the blood solution, 0.2 ml to 0.8 ml. Each bar is the average of three runs performed in duplicates, n = 6.

It was clear from the antibacterial experiments that the activity of the AMPs can change in relation to each other when the AMPs are covalently attached to the hydrogel substrate as compared to in solution. However, for the hemolysis experiments of the six AMPs when attached to the hydrogel discs, Figure 25 (b), no apparent difference between the six AMPs was found, as all AMPs barely showed any hemolysis at all. To investigate whether there was a concentration dependent effect, four different volumes were used to submerge hydrogels with a uniform size. If anything, this only appeared to have the effect of a few percent lower observed hemolytic effect for a higher surface area per volume. Since this was also the case for control hydrogels, this effect most likely comes from a dilution effect as there is an exchange between the PBS from the swollen hydrogels and the 1 vol% blood solution. Regardless, seeing

that neither R9W5 nor Piscidin 1, that showed such an immense hemolytic effect in solution, did not show any considerable hemolytic effect when attached to the surfaces hint at the significant reduction of their hemolytic potential.

5.6 Endotoxin interactions

As mentioned in earlier, AMPs of our immune system has roles beyond the immediate killing of bacteria. While some AMPs can upregulate other aspects of the immune system or work as chemokines which could be beneficial for wound healing, it is quite farfetched that this could work if they are covalently attached to the wound dressing. However, another feature that some naturally occurring AMPs provide is the binding and neutralization of endotoxins. Endotoxins, or lipopolysaccharides as they are also called, is what gives the gram-negative bacteria their negative charge and an affinity for AMPs. Since the AMP-functionalized hydrogel surfaces had shown an antibacterial effect against gram-negative strains, it was of interest if an endotoxin binding feature was also obtained. This was evaluated for the R9W4 AMP-functionalized hydrogel discs in **Paper V**. *P. aeruginosa* was cultivated in PBS, on absorbent pads (viscose/polyester nonwoven), on control hydrogels, and on AMP-functionalized hydrogels. The endotoxin levels after 3 and 24 h was then evaluated and presented in Figure 26.

It was clear that the endotoxin levels in the samples increased over time just by having bacteria present and proliferating, even in the lower nutritional media (5% TSB) used. In the PBS control, the endotoxin levels increased from roughly 450 EU/ml after 3 h of cultivation to over 20 000 EU/ml after 24 h of cultivation. Furthermore, it did not appear to be enough to absorb and contain the bacteria as no statistically significant difference was observed between the PBS and absorbent pad samples for either of the time points. For the control hydrogel no difference was observed after 3 h compared to the PBS and absorbent pad. However, a slight, but statistically significant difference was observed after 24 h. It is possible that the polyethylene oxide other shell limited growth on the surface slightly as it is used as an antifouling agent, though it should not have a significant impact on the viability of the bacteria. Another potential reason is that the endotoxins might interact with the repeating hydrophobic domains of the micelle cubic structure and did not get extracted in the same quantity as for the PBS and absorbent pad. Regardless of whether the hydrogel itself contributed anything to the lowering of endotoxins after 24 h of cultivation, the effect of adding AMPs to the hydrogel surface is clear. A significant reduction of endotoxin levels was observed compared to the other sample types for both time points with average endotoxin levels at 55 EU/ml after 3 h and 2 300 EU/ml after 24 h. While it is clear that the AMP-functionalized hydrogels reduced the endotoxin levels, it cannot be claimed whether the effect is due to the binding of free endotoxins released by the bacteria, or by killing bacteria, thereby reducing the number of endotoxins created. Most likely it is a combination of both. However, what is clear is that by exposing *P. aeruginosa* to the AMP-functionalized hydrogels, the endotoxin levels can be reduced. Meaning that pathogenic bacteria can be neutralized, while endotoxins levels are not being burst released, but in fact lowered, so that the immune system does not get over-activated.

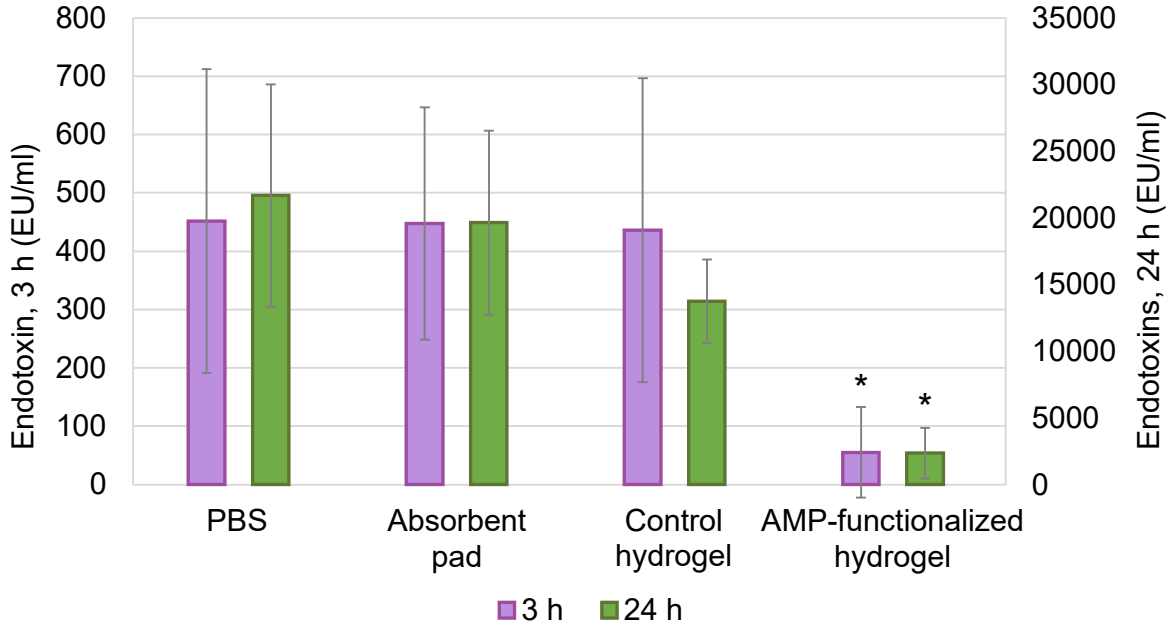

Figure 26. The measured endotoxin levels on/in the different sample types after 3 h (purple, left axis) or 24 h (green, right axis) of cultivation with *P. aeruginosa*. The bars are the average (n = 8), and the error bars are the observed standard deviation. '*' Indicates that the AMP-functionalized hydrogel was statistically lower than all other sample types within each time point, $p<0.05$, the control-hydrogel was statistically lower than PBS and the absorbent pad for 24 h, $p<0.05$.

5.7 Antibacterial evaluation *in vivo*

As one of the main motivations behind the covalent attachment of the AMPs to the hydrogels was to gain protection from biological environments, this is of course important to investigate. While the serum stability tests discussed in 5.4 showed very promising indications that a higher stability was obtained, the conditions evaluated are still less harsh than what is found in a more realistic setting. It is nothing wrong with that, since using 20% serum is a very standard and manageable method to gain insight into the system and the effect of enzymes and the like. However, serum is devoid of fibrinogen and all cell types, meaning that blood clotting and any other immune response has been removed. Since these factors of course can affect the interaction between a medical device and its surroundings significantly, an infected rat model was used to further evaluate the biostability of the AMP-functionalized hydrogels.

Pockets were made beneath the skin, on the backs of rats where hydrogel discs were placed with or without the addition of *S. aureus* followed by the pockets being sutured up. After 1 or 3 days, the pockets were opened, and the samples as well as the exudate in the pockets were collected. The CFU was determined for each case and is shown in Figure 27. While not a perfect model for a wound, information should still be obtained regarding the biostability of the AMPs. As evident from the results, the average of all observations is presented along with the individual average within the two sets. This is because the two sets behaved quite differently, at least for the CFU found on the sample surfaces (a). For the first set, no difference

was observed between the control and AMP-functionalized hydrogel for either of the time points. However, for the second set, almost a 2 log reduction was observed after 1 day as the bacterial concentration was reduced from 7.8×10^7 CFU/ml on the control hydrogel to 1.4×10^6 CFU/ml. After 3 days, the reduction was almost 5 logs as the bacterial concentration was reduced from 1.3×10^8 CFU/ml on the control hydrogels to 1.4×10^3 CFU/ml on the AMP functionalized hydrogels. The average for both sets show less than 1 log reduction as it will get skewed towards the higher values. In general, fewer bacteria was found in the exudate compared to on the control implants (b). Furthermore, a reduction of the bacterial concentration in the exudate was observed between the control and AMP functionalized hydrogels in all cases. However, a more prominent effect was observed after 3 days of implantation in the second set compared to the first.

There are several potential explanations behind the observed discrepancies between the two sets. The first being that there actually are issues with the biostability of the AMP attached to the hydrogels, and that the measured CFU is a result of this. If the results had been more evenly spread out in between the two sets this would have been more straight forward to accept. Other explanations could be that the samples were made from the ground up in between the two sets, which naturally leads to variations. Furthermore, the rats were from the same breeding group within each set, but different between the sets, and the bacteria used to inoculate with is from the same origin, but different cultivations were used for the two sets. Another potential reason is that there might have been issues with the AMP attachment in the first set. Since the CFU got reduced in the exudate but not on the implant, if the AMPs were released instead, they would reduce the CFU of the surroundings and leave a non-functionalized surface behind. Regardless, an overall antibacterial effect was observed, although with a questionable strength. This also means that the biostability question of the AMP functionalization still remains somewhat open. However, it should be added that the pilot study performed in **Paper I** also indicated that an antibacterial effect was maintained for the AMP-functionalized hydrogels compared to the controls. However, very few rats were included in the pilot study since the goal was mainly to set the parameters regarding bacterial concentration and time.

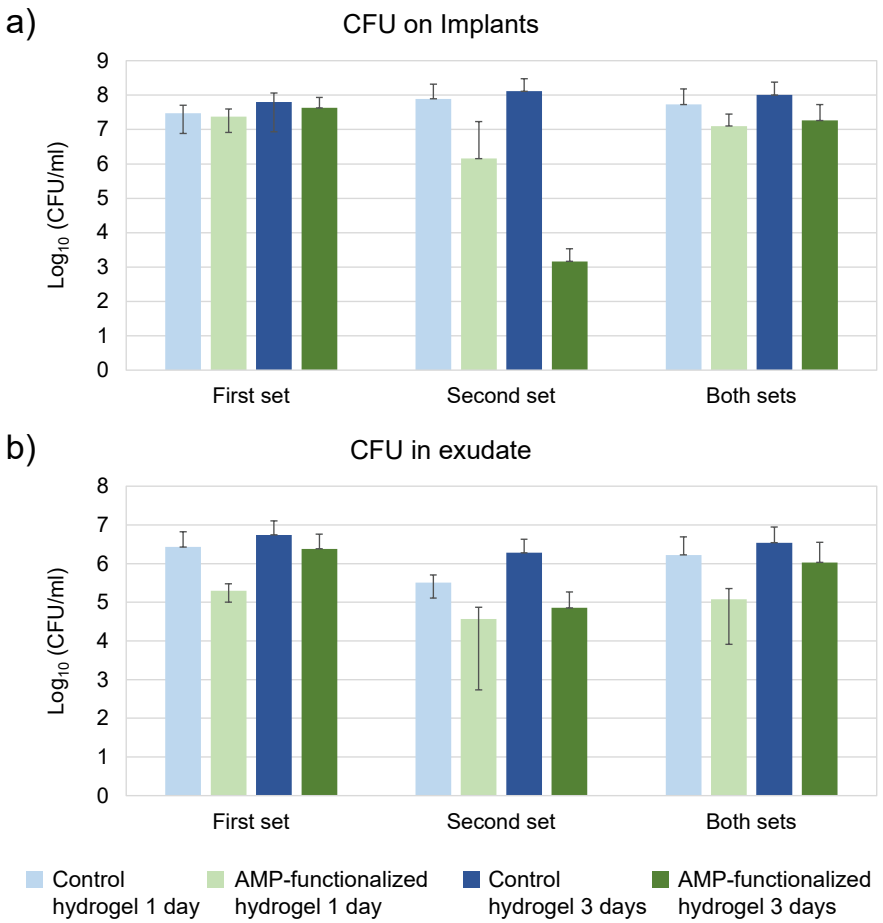

Figure 27. The CFU from an infected rat model after 1 and 3 day(s) of implantation, with the CFU on implants in (a) and CFU in the exudate in (b). The averages are shown for the first set of rats, second set of rats, and for all the rats. The y-axis is logaritmized (log_{10}) and the error bars are the standard deviation for n = 4 in the separated sets and n = 8 in the combined.

5.8 Clinical investigation

To get an indication whether the AMP-functionalized hydrogels would retain their antibacterial effect when used as a wound care device, an intact skin study was implemented. Hydrogel dressings were prepared and functionalized in a manner more easily adapted to an industry approach. The dressings were then evaluated by application on the skin of the forearms of healthy volunteers for 3 h after which the bacteria on the skin under the dressings was collected and counted. A control hydrogel without any AMPs were also included and evaluated together with a section of untreated skin. The results are presented in a box plot with whiskers shown in Figure 28. Looking just at the observed measurements for the untreated skin, the natural variance of our skin microbiota was very evident. The only restrictions the volunteers received before the inclusion in the study was to not apply any lotion on the day of testing. This means that while one volunteer might have washed their arms with soap in the shower just hours

before the test, others might not have washed their arms for days. However, this should also better reflect the variation of challenge a dressing applied to the skin might face.

The area beneath the control hydrogel did not show any statistical difference compared to the untreated skin. This was also evident from the total averages which for the untreated skin was 1266 CFU/cm^2 and for the control hydrogel was 1169 CFU/cm^2. This strongly indicates that the hydrogel itself does not affect the bioburden on the skin, at least not during the timeframe evaluated. When functionalized with AMPs, on the other hand, the dressings significantly reduced the bioburden compared to the untreated skin and control hydrogels. The box plots show that all quartiles have been shifted to 1 – 2 log lower concentrations. And the average bioburden under the AMP-functionalized hydrogels was 23 CFU/cm^2.

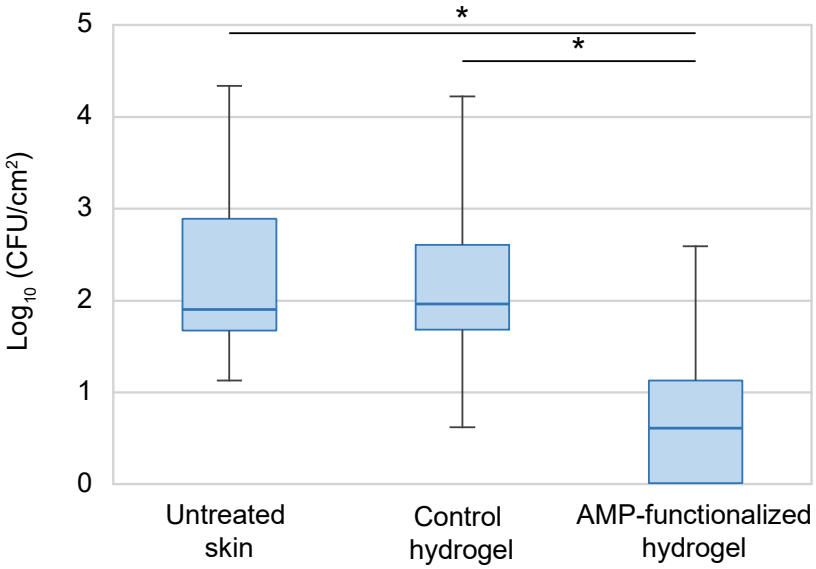

Figure 28. Box-whisker plots of the CFU (as Log_{10} per cm^2) measured on the skin of untreated skin, under control hydrogels, and under the AMP-functionalized hydrogels of 40 volunteers. The top and bottom box show the third and first quartiles, respectively, and the band within the show the median. The whiskers above and below the boxes indicate the highest and lowest microbial counts. The * indicates a statistically significant difference with a 95% confidence interval using Wilcoxon signed-rank test.

This clinical investigation showed that the AMP-functionalized hydrogels achieved an antimicrobial effect on the naturally present skin microbiota. This strongly indicates that the contact killing approach does not severely limit the utilization of the covalently attached AMPs as a wound dressing. However, while the intact skin model is good to evaluate the potential interference from hair, pores, pH, and moisture, it is still not a wound. On the other hand, when evaluating new technologies caution is crucial, and this model is a great first interaction with humans in a step wise approach. Since all wound dressings will come in contact with skin, and it is beneficial to reduce the microorganisms around the wound edges, if an antibacterial wound dressing does not reduce these bacteria, it has little hope of doing the same in the wound bed.

A natural next step would of course be to evaluate whether a reduction in infection rate is achieved when applying the AMP-functionalized hydrogels.

6 Conclusions and future perspectives

Our relationship with the microbes around us is complex and ever-changing. When found in places where they cause more harm than good, it is paramount that we have the right tools to fight the bacteria. While antibiotics have long been a staple in the way we deal with the bacteria in these cases, antibiotic resistance have threatened our way of life. The search for alternative methods to treat bacterial infections has therefore been of increasing interest. A substance that has shown potential in this is antimicrobial peptides (AMPs), as they are usually quite potent with broad-spectrum activity. They have also shown a lower probability of inducing resistance.

In this thesis the aim was to investigate the covalent attachment of AMPs to hydrogels prepared from amphiphilic polymers as a means to fight bacterial infection within wound care. The hydrogels were formulated in different ways, with hydrogel discs being the most convenient for laboratory evaluations. However, the material combination was also made into wound dressings and evaluated for a more applied verification. Furthermore, the material was also formulated as particles for potential future uses as a paste with better coverage, coating for functionalizing other materials, or potentially in solution. The importance of the choice of AMP was investigated, as AMPs with varied properties were attached to hydrogel discs. The antibacterial activity of the AMPs differed significantly in antibacterial and cytotoxic activity when free in solution. On the other hand, the AMPs showed no signs of cytotoxicity when attached to the material. Furthermore, the antibacterial activity was mostly maintained, although with a significant variation compared to their activity when free in solution.

The antibacterial activity of the materials was shown to be effective against a variety of clinically relevant strains, both gram-negative and gram-positive as well as some antibiotic resistant strains. The functionalization showed a contact killing mechanism which can be beneficial as a very local antibacterial effect can be obtained. This means that the bacteria that we are on good terms with does not have to be affected, something that will also lead to lower risk of inducing resistance. Furthermore, the AMP-functionalized hydrogels showed an ability to reduce the endotoxin levels released by *Pseudomonas aeruginosa*. Endotoxins can over-activate our immune system and, for instance, cause sepsis. By killing the bacteria present and controlling the endotoxin levels released, an infection can be treated or prevented while simultaneously reducing the chance for adverse effects.

A limitation with using AMPs as an antimicrobial substance is that they are quite sensitive to biological environments and are rapidly degraded by proteolytic enzymes. Since the covalent attachment is made to fight this, it was also evaluated, both *in vitro* using human serum but also *in vivo* in an infected rat model. These tests showed that the covalent attachment indeed provided the AMPs with protection, as an antibacterial effect was maintained for several days.

The work in this thesis does indeed show that the AMP-functionalization of amphiphilic hydrogels is a suitable material for applications within wound care. However, there is always more work to be done. For instance, a natural next step is to investigate whether the hydrogel formulated as a wound dressing has any effect on wound healing. A motivation behind the choice of material substrate is that hydrogels can provide a moist environment, which has been shown to increase wound healing. The impact of the AMPs on this process is also unknown. While AMPs of the innate immune system have been linked with the regulation of cells involved in wound healing it is not clear if the AMPs used in this work possess that effect. Furthermore, even if the AMPs have that effect when free in solution, it has been observed in this work that properties exhibited in solution do not always follow to when the AMPs are covalently attached to the hydrogel.

The intact skin study of this work is a great first step in the evaluation of the use of AMP-functionalized hydrogels in humans. While it showed clearly that the bacteria naturally present on our skin can be killed, the next step would be to show that the effect can also be achieved to bacteria present in a wound. The best way of doing this in humans is to perform a larger controlled study where the infection rate is determined for the same types of wounds. Furthermore, a limitation with the intact skin study was that a strain analysis of the bacteria was not available and is to be performed in future studies. Especially since this might provide information regarding whether the surviving bacteria is due to issues with the coverage of the hydrogel and the contact killing mechanism, or if there are strains present with a higher tolerance towards the AMP-functionalized hydrogels.

Regarding the particles prepared there are also several unanswered questions remaining, mainly regarding the size of the particles and the influence this has on the antibacterial and possibly cytotoxic activity. Depending on the application there might also be limitations on what size the particles are allowed to take before they end up in places where they are not desired. Furthermore, the degradation of the particles is of interest to study. Because if they are

to be used clinically, they will be much more difficult to remove than a whole dressing. The effect of any residual particles therefore needs to be studied to ensure that no adverse effects on our health is observed, as this is paramount.

Acknowledgements

First of all, I would like to thank my two fantastic supervisors. Martin, for showing me that science is meant to be fun and exciting. Your ability to only see opportunities where others see problems, and your seemingly endless supply of fresh ideas are truly an inspiration to admire. Anand, for all the support you have provided me with on this journey. I can always rely on becoming wiser after a discussion with you regarding all the walls that are inevitably hit when performing research.

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