Antibacterial hydrogels for managing wound infections

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**ANTIBACTERIAL HYDROGELS FOR
MANAGING WOUND INFECTIONS**

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NTU Institute for Health Technologies

ANTIBACTERIAL HYDROGELS FOR MANAGING WOUND INFECTIONS

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**Interdisciplinary Graduate School
NTU Institute for Health Technologies**

A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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A handwritten signature in blue ink that reads "Mary Bee Eng Chan". The signature is stylized with a long horizontal stroke at the end.

.....

Chan Bee Eng, Mary

Authorship Attribution Statement

This thesis contains material from one paper published in the following peer-reviewed journal where I was the first and/or corresponding author.

Chapter 2 is published as **Chun Kiat Yeo**, Yogesh Shankar Vihke, Peng Li, Zanru Guo, Peter Greenberg, Hongwei Duan, Nguan Soon Tan, and Mary B. Chan-Park, “Hydrogel Effects Rapid Biofilm Debridement with ex situ Contact-Kill to Eliminate Multidrug Resistant Bacteria in vivo”, *ACS Appl. Mater. Interfaces* 2018, 10(24), 20356-20367. DOI: 10.1021/acsami.8b06262.

The contributions of the co-authors are as follows:

- I carried out the syntheses of hydrogel polymers, formation of hydrogel wound dressings, *in vitro* and *in vivo* experiments.
- Dr. Vihke helped out with some syntheses and analysis of NMR characterizations.
- Dr. Li and Dr. Guo established the chemistry of hydrogel polymer syntheses and did early antimicrobial testing.
- Prof Greenberg advised on interpretations of the antimicrobial results.
- Assoc Prof Duan discussed the polymer synthesis and characterization.
- Assoc Prof Tan advised on the design and interpretation of *in vivo* experiments.
- Prof Mary Chan-Park advised on the design and interpretation of all experiments, and directed the overall project. Prof Mary Chan-Park and I did the main writing of the manuscript.

30 July 2019

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Date

Yeo Chun Kiat

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Abbreviations

AgNPs	Silver nanoparticles
AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
CR-AB	Carbapenem-resistant <i>Acinetobacter baumannii</i>
CR-PA	Carbapenem-resistant <i>Pseudomonas aeruginosa</i>
DESCK	Debridement followed by <i>ex-situ</i> contact-killing
DFU	Diabetic foot ulcer
DI	Deionized
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPS	Extracellular polymeric substances
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FE-SEM	Field emission-scanning electron microscopy
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
GSH	Glutathione
HBOT	Hyperbaric oxygen therapy
HDF	Human dermal fibroblast
IL	Interleukin
KGF	Keratinocyte growth factor

LB	Luria-Bertani
LPS	Lipopolysaccharide
MDR	Multi-drug resistant
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MMPs	Matrix metalloproteinases
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSCs	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NPWT	Negative pressure wound therapy
PA01	<i>Pseudomonas aeruginosa</i> 01
PBS	Phosphate-buffered saline
PCL	Polycaprolactone
PDGF	Platelet-derived growth factor
PEG	Poly(ethylene glycol)
PEGACA	Poly(ethylene glycol) acrylamide
PEGDACA	Poly(ethylene glycol) diacrylamide
PEGDMA	Poly(ethylene glycol) dimethacrylate
PEGMA	Poly(ethylene glycol) methacrylate
PEI	Polyethylenimine
PFA	Paraformaldehyde
PGA	Poly(glycolic acid)
PIM	Polyimidazolium
PLA	Poly(lactic acid)

PLGA	Poly(lactic-co-glycolic acid)
pNIPAM	Poly(N-isopropylacrylamide)
ROS	Reactive oxygen species
SDF-1	Stromal cell-derived factor-1
siRNA	Small interfering RNA
STZ	Streptozotocin
TGF	Transforming growth factor
TNF- α	Tumour necrosis factor- α
VEGF	Vascular endothelial growth factor
VLU	Venous leg ulcer
WHO	World Health Organization

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Summary

Chronic wound healing is a major concern worldwide. Chronic wounds are usually non-healing and typically caused by bacterial infection or underlying illnesses such as diabetes. This thesis deals with the pathophysiology of wound healing and the development of antibacterial hydrogels to treat infected wounds and diabetic wounds.

Thesis Abstract summarizes the intricacies of infected and diabetic wound healing and the strategies developed to treat these wounds.

Chapter 1 (Introduction) discusses the pathophysiology of acute and chronic wound healing, and introduces current therapeutic strategies for wound care in the literature.

Chapter 2 (Results) describes a novel antibacterial hydrogel that was developed by the UV-initiated crosslinking of polyethylenimine-graft-poly(ethylene glycol) methacrylate and poly(ethylene glycol) dimethacrylate. This hydrogel was able to achieve more than 99.9% killing of wound biofilms in a murine excisional wound infection model.

Chapter 3 (Results) presents an improved and more efficient way of making antibacterial hydrogels by thiol-maleimide Michael Addition reaction. This hydrogel was made by simply mixing their components (poly(ethylene glycol) tetra thiol, poly(ethylene glycol) tetra maleimide, polyimidazolium and N-acetylcysteine) in water. It is able to eradicate more than 99.9% of wound biofilms and has added antioxidative effects to accelerate wound healing in a murine diabetic wound infection model.

Appendix comprises data that are trivial and are not presented (but mentioned) in the main chapters.

Bibliography includes all references that are cited in this dissertation.

Miscellaneous provides other accomplishments that the Ph.D. candidate achieved during his candidature such as publications, patents and conferences presented.

Thesis Abstract

Thesis Abstract

Bacterial infection on wounds delay wound healing, and may even deteriorate the wound condition. Diabetic wound healing is even more problematic as patients suffer multiple conditions that prevent wounds from healing. Traditional dressings such as bandage, gauze or plasters are protective rather than proactive. Current antibacterial treatments are typically prophylactic and involve cytotoxic silver, and yet they do not remove bacterial debris. FDA-approved treatments for diabetic wounds also contain contraindications which limit their effectiveness. We have developed novel hydrogels to treat different types of wounds. First, a biocompatible, biofilm-debriding hydrogel was made by UV irradiation of poly(ethylene glycol) dimethacrylate and star cationic polyethylenimine (PEI). It is able to achieve more than 99.9% killing of wound biofilms such as methicillin-resistant *Staphylococcus aureus* (MRSA), and carbapenem-resistant *Pseudomonas aeruginosa* (CR-PA) and *Acinetobacter baumannii* (CR-AB) in a murine excisional wound infection model. Silver-based wound dressings (controls) showed almost no killing of MRSA and CR-PA biofilms. This debridement effect is largely due to the high water swellability and microporosity of the hydrogel, which harnesses hydrodynamic drag of the hydrogel and draws bacteria away from the wound site into the hydrogel. Bacteria will be contact-killed by the cationic pore walls of the hydrogel. The hydrogel also degrades in the presence of infection-related enzymes, releasing the star cationic PEI into the infection site to contact-kill bacteria there. A second-generation hydrogel was made with the same concept as the first but with simpler crosslinking and possesses more potent effects. This hydrogel was crosslinked by simply mixing the components together in water, which consisted of poly(ethylene glycol) tetra-thiol and poly(ethylene glycol) tetra-maleimide as the hydrogel network, and is

tethered with pendant antibacterial polyimidazolium and antioxidative N-acetylcysteine. This hydrogel was able to achieve the same bacterial killing as the first-generation hydrogel, with an even better wound healing on diabetic wounds. Overall, our hydrogels greatly reduce wound bioburden and its associated inflammations, and promote wound healing.

Chapter 1

Wound Healing Pathophysiology and Its Therapeutic Strategies

1.1. Introduction

The skin is the largest and outermost organ of our body. It serves as a barrier to protect the internal organs from microbial invasion and harmful UV radiation. Since it is in constant contact with the external environment, it is susceptible to physical, chemical and mechanical injuries resulting from accidental abrasions, cuts and burns, and also inevitably damaged by invasive surgeries. The skin has a natural ability to heal itself quickly upon damage by inducing inflammation, delivering wound healing factors to the site of injury, and accelerating the proliferation of fibroblasts and keratinocytes [1]. Superficial wound healing usually restores the skin back to its pre-injury state completely. However, deep and severe wounds may cause the development of scar tissues upon healing [2, 3]. Once healed, the skin is able to perform its functions as adeptly as its pre-injury state. Wounds are usually categorized as acute or chronic, depending on the efficiency of healing. Acute wounds generally show predictive and definite signs of healing within 4 weeks, or in more severe cases, a few months. Chronic wounds are typically non-predictable and do not heal in an orderly set of stages and usually remain non-healed. Chronic wounds can be caused by persistent infection, malnutrition, underlying skin problems, vascular defects or illnesses such as diabetes. These problems complicate the natural healing process by interfering with the delivery of nutrients to the wound site and preventing the migration of cells. An estimate of the economic cost of chronic non-healing wounds in the US alone is more than \$50 billion per year [4].

Numerous techniques have been developed throughout the years to tackle the intricacies of chronic wound healing or simply to accelerate wound healing in general. These techniques are not considered treatments as they are merely lab-based research that have not undergone clinical trials nor be translated as a commercial product.

However, there already exist a variety of commercial wound dressings on the market to aid wound healing. These dressings are mostly over-the-counter drugs and patches that treat common cuts or abrasions to the skin, and are usually protective rather than proactive. Most of these dressings are merely films or bandages that cover the wound to protect the injured site against foreign microbes or further perturbations to the injured tissues. A portion of these dressings have specific functions such as hydrating the wound, killing bacteria, or delivering growth factors. There also exist wound dressings that are only available via prescription, such as Regranex and Omnigraft, which are used specifically to treat diabetic foot ulcers. However, these dressings are limited by their contraindications and must be applied with close monitoring by physicians or healthcare professionals. Most dressings on the market only serve a single function (i.e. protection, hydration or antibacterial) and do not combine the components into a single treatment. This might be due to the difficulty of integrating these elements into a simple, single dressing, while the costs of manufacturing such dressings are also an issue. There is a need for truly multifunctional, bio-responsive and targeted treatments to tackle the problem of chronic wound healing, as the underlying challenges are not singular. In this chapter, we discuss the pathophysiology of wound healing, current methods to treat wounds, and future directions in wound care.

1.2. Pathophysiology of wound healing and its challenges

Wound healing consists of three overlapping stages: inflammation, proliferation, and maturation [5, 6] (Figure 1.1).

Figure 1.1. The three main overlapping stages of wound healing and their respective timelines from the onset.

A pre-inflammatory phase called haemostasis occurs immediately upon tissue injury to stop bleeding. In acute wound healing, these stages work in concert and progress in an orderly manner, resulting in complete healing within weeks (or months for severe and deep wounds). The early inflammatory response mobilizes local and systemic defence responses to the site of the wound [7, 8]. The inflammation stage usually lasts up to 7 days, with the peak of inflammation occurring at around 2 days post-injury [9, 10]. Inflammation is a crucial step in wound healing as neutrophils release reactive oxygen species (ROS) and proteases, and macrophages scavenge foreign microbes to prevent infection [11, 12]. Macrophages also secrete growth factors to recruit fibroblasts and keratinocytes to repair the damaged blood vessels, and form the platform for cell migration and proliferation which is the next stage in wound healing [13-15]. Cell proliferation starts at approximately 3 days post-injury and usually before the inflammation stage subsides [9, 10]. During this stage, neovascularization occurs to restore the vascular network that was damaged during injury. Initiators such as vascular

endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) are secreted and bind to the receptors on existing endothelial cells, activating an intracellular signalling cascade to sprout new blood vessels and repair damaged ones [16]. At the same time, re-epithelialization occurs to restore the epidermis [17-19]. Keratinocytes migrate from the free edges of the wound to cover the exposed area due to injury [17, 18]. Epidermal stem cells from hair follicles also differentiate into keratinocytes to increase the pool of cells to enclose the wound, and eventually form the new epidermis layer [20, 21]. Fibroblast migration and proliferation occur concurrently to produce new collagen and other extracellular matrix (ECM) components to reconstruct the connective tissues under the epidermis to strengthen the skin [22, 23]. Fibroblasts can also differentiate into a subpopulation of myofibroblasts to “pull” the wound edges in a process called wound contraction. Growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) are responsible for the migration and proliferation of the said cells. Ensuing the proliferative stage is the maturation phase. At this phase, the formation of granulation tissue stops through apoptosis of the cells. The wound matures by changing the components of the ECM. Collagen Type III, which were produced in the proliferative stage, are replaced by stronger collagen Type I [24]. Type I collagen are oriented in a parallel fashion and are different from the inter-weaving Type III collagen, hence forming scar tissues. Here, matrix-remodelling enzymes called matrix metalloproteinases (MMPs) interplay to determine the ECM composition and the extent of scar formation [25]. This process can take months or even years to complete. The completed wound healing process typically results in stronger tissues, scars, and a loss of sensation, hair follicles and sweat glands in the wounded area (Figure 1.2).

Figure 1.2. Sketch of the processes occurring during wound healing. Haemostasis: Platelets recruitment to clot the broken blood vessels to minimize bleeding. Inflammation: Neutrophils are quickly recruited to the wound site and secrete cytokines to recruit phagocytes and ROS to kill bacteria. Monocytes migrate into the wound site and differentiate into macrophages to engulf pathogens. Proliferation: Fibroblasts and keratinocytes migrate and proliferate to close the wound. Maturation: ECM is remodelled and collagen Type III are replaced by collagen Type I to strengthen the tissues [26]. Reprinted with permission from ref. 26.

Chronic wounds do not adhere to an orderly set of healing stages and are disoriented in terms of the cellular and molecular processes occurring during these phases. Chronic wounds usually come in the form of vascular ulcers, pressure ulcers and diabetic ulcers. Common characteristics of these wounds include prolonged inflammation, persistent infections, fibroblast senescence, impaired angiogenesis and elevated MMPs [27, 28]. Inflammation is prolonged in chronic wounds, and it is

believed that these wounds might be trapped in a chronic inflammatory state that fails to progress [29]. Specifically, recent investigations of chronic wound tissues and fluids indicate a continual competition between inflammatory and anti-inflammatory signals leading to an imbalanced environment for proper wound healing to occur [30, 31]. This locks the wound into a perpetual inflammatory state that hinders the proliferation stage of wound healing. Another hallmark of chronic wounds is the elevated level of ROS at the wound site. Due to persistent inflammation, macrophages dwell on the site indefinitely. These cells secrete ROS to fight microorganisms. However, in chronic wounds the concentration of ROS is constantly high and this has countereffects on the wound. High level of ROS damages cells, tissues, and the ECM, and leads to an enhanced stimulation of proteases (such as MMPs) and inflammatory cytokines, which further degrade the wound [32]. The endless cycles of high inflammation and ROS level cause the wound to be unable to escape the inflammatory phase. In severe cases, the cells undergo apoptosis due to the huge oxidative stress and this triggers a cascade of events that cause neighbouring cells to experience the same fate. This leads to necrotic wound tissues and steps such as tissue debridement or worse, amputation, need to be carried out to salvage the situation. Chronic wounds are usually heavily infected or occur in diabetic patients as diabetic foot ulcers (DFU). Most chronic wounds do not heal through regeneration but through fibrosis, forming excessive amounts of connective tissue. Increased collagen production during fibrosis causes the formation of fibrotic scar tissue, such as keloid or hypertrophic scars [33, 34].

1.2.1. Wound infections and healing

Bacterial colonization of wounds is common [35]. All wounds are colonized to a certain degree, and a major role of the inflammatory phase of wound healing is to bring

microbes down to steady-state and innocuous levels [11, 36]. Planktonic bacterial infections are generally less detrimental as the body's systemic defence easily overpowers them. An interplay of interleukins and proteases secreted by neutrophils, coupled with macrophages that scavenge foreign microorganisms, suppress the bacteria to a low and harmless level. However, if the immune response does not advance normally, or is hindered by underlying medical conditions, planktonic bacteria can outgrow the immune system and cause complications. At high numbers and given sufficient time and nutrients, planktonic bacteria progress to form biofilm on wounds [37]. Biofilm has the capability to seize nutrients in the ECM, such as carbon, nitrogen and phosphate, to supplement their own growth [38]. Once a full-fledged biofilm is formed, a layer of extracellular polymeric substances (EPS) surrounds and protects them against a vast of “predators” such as macrophages, antimicrobial peptides and antibiotics [39]. These “predators” fail to attack the biofilm because they cannot penetrate the protective EPS layer. Biofilm can stay on wound surfaces for as long as they are undisturbed by medical interventions. Biofilm infections on wounds are most commonly caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* invasion [40].

The biofilm produces waste products, free radicals, antigens, enzymes and toxins into the surrounding tissues. These molecules trigger an immune response and the body sends inflammatory cells to the wound site to clear them. However, due to the abundance of said molecules being released by biofilm bacteria indefinitely, coupled with the presence of seemingly invulnerable biofilm, inflammatory cells persist at the wound site. This endless loop of secretion by biofilm and immune response from the body sends the wound into a chronic state which fails to escape the inflammatory phase. Therefore, wound infection by biofilm is likely to be a huge contributing factor in prolonged inflammation and delayed wound healing. Furthermore, in these polymicrobial wound

communities, individual species may become more virulent and proliferate, which further impede wound repair [41]. Bacterial biofilm also inhibits wound healing by forming a barrier to re-epithelialization. This causes the cells to be unable to migrate or proliferate to close the wound. Complications due to wound infections include delayed wound closure [42], amputations [43] and even mortality [44]. Elimination of bacterial infection is a crucial step in wound healing as bacteria typically disrupt the natural healing process, and even worsen the condition of the wound [45, 46].

1.2.2. Diabetic wound healing

Diabetes mellitus is one of the most common chronic disease in the world. It is expected that the incidence of diabetes will rise to 552 million by 2030 [47]. This rising incidence of diabetes portend increasing cases of diabetic foot ulcers and problematic diabetic wound healing, as it is estimated that 15% of diabetic patients suffer from any form of chronic, non-healing ulcers [48]. DFU are also the leading cause of nontraumatic amputation.

Diabetic neuropathy and peripheral vascular diseases are the main culprits involved in DFU [49]. Denervation of peripheral limbs is a hallmark of diabetic neuropathy [50]. This results in a deficit of sensory neurons in those areas, therefore the patients are unable to respond to external stimuli such as pressure and heat. This leads to frequent injuries to limbs and may be overlooked for a long time since the patients do not feel pain. When they are finally noticed, these wounds may already be in a chronic state due to the lack of care during the early stages of wound healing, and require medical interventions in order to heal properly. For diabetic patients, sustained hyperglycaemia is known to increase vascular superoxide production, which inactivates nitric oxide and causes vascular dysfunction [51]. When the vascular network is poor

around the wound site, nutrients and signalling molecules do not enrich the wound enough for the proper molecular and cellular mechanisms to occur.

Apart from the pathologies of diabetes which indirectly affect wound healing, diabetes itself can delay wound healing directly. For example, high blood glucose level thickens the blood and retards blood flow. It also reduces the concentration of important nutrients and oxygen delivered to the injured site. Without these molecules, the immune system cannot function properly to defend against microbial invasion, and the wound gets infected easily. Molecular and cellular mechanisms in wound healing are also impeded by low concentration of nutrients and oxygen. Fibroblasts isolated from chronic diabetic ulcers are senescent with decreased responses to growth factors [52, 53]. Macrophages in diabetic wounds show decreased secretion of cytokines [54]. Excessive production of ROS from phagocytes caused by persistent infection damages cells and the ECM. Also, attachment and migration of keratinocytes are impaired in diabetic wounds due to altered ECM composition caused by ECM degradation. All these factors work in tandem to prevent diabetic wound from healing properly.

1.2.3. Reactive oxygen species and wound healing

Reactive oxygen species (ROS) exist in tissues and cells in a homeostatic balance. ROS are molecules containing O_2 which have been reduced to become a highly reactive and radical species. Examples of ROS are superoxides and peroxides, as well as hydroxyl radicals. ROS are produced during cellular mechanisms such as ATP production, where mitochondrial oxidative phosphorylation generates ROS as a by-product [55, 56]. In a homeostatic balance, ROS maintain normal cell functions and regulate vascular dilation and constriction [57, 58]. Low levels of ROS induce cell cycle arrest, while excessive ROS damage cells and trigger apoptosis [59, 60]. ROS levels are

controlled by antioxidants in cells. Antioxidants are a species that nullify the harmful effects of ROS by donating their own electrons, preventing the ROS from capturing electrons from important molecules such as DNA, proteins and lipids. Examples of antioxidants are glutathione, superoxide dismutase and catalase. In addition to naturally produced antioxidants, supplements such as vitamins and coenzyme Q also work to maintain a homeostatic ROS level.

ROS play an important role in the orchestration of normal wound healing, especially during the inflammatory stage. Their main purpose in wound healing is to kill foreign microorganisms [61, 62]. They act as secondary messengers to many immunocytes which are involved in the repair process. At a wound lesion, the production of ROS by neutrophils and macrophages recruits more phagocytes to the site of injury which engulf foreign microbes, and the ROS then destroy the engulfed pathogens. As the phagocytes destroy microbes, they also release H_2O_2 to inhibit the growth of invading microorganisms. ROS also possess the ability to regulate angiogenesis in the wound by upregulating the production of VEGF [63, 64] to allow perfusion of blood and nutrients to aid wound healing. Finally, ROS are also involved in re-epithelialization. H_2O_2 triggers the activation of receptors for EGF and keratinocyte growth factor (KGF), and induces the production of transforming growth factor- α (TGF- α) in fibroblasts [65]. The activation of these receptors and growth factors allows the migration and proliferation of epidermal cells to close the wound. After performing their functions, the ROS are quickly oxidized to their harmless form by antioxidants.

On the flip side, a high level of ROS leads to oxidative stress and is detrimental to wound healing. ROS-mediated transcription can cause elevated pro-inflammatory cytokine secretion and induction of MMPs which degrades the ECM [66-68]. Excessive

ROS can also directly degrade the ECM proteins and cause cellular malfunction. In addition, ROS can also inhibit the migration of fibroblasts and keratinocytes. In chronic wounds, the ROS are usually above homeostatic level due to persistent infections and cause cellular damage. Therefore, balancing the levels of ROS is crucial for wounds to heal properly.

1.3. Wound healing strategies

There is a myriad of strategies available to tackle the challenges of wound healing (Figure 1.3). Traditional wound dressing products include cotton wool, gauze, film, foam, plasters and bandages. They are used primarily to protect the wound against perturbations such as heat, harmful chemicals, microorganisms, and further contact injury, and do not serve any biomedical functions. Usually, these dressings require frequent changing to prevent maceration of healthy tissues, and they can be moistened from wound exudates and become adherent to wound tissues, causing problems during their removal. Generally, traditional dressings are designated for clean and dry wounds or used as secondary dressings. Since they fail to provide a conducive environment for wound healing, they are redundant in the treatment of severe wounds such as infected wounds, chronic wounds and diabetic ulcers. Fortunately, multifarious modern dressings with direct participation and benefits in the wound healing process have been developed.

Figure 1.3. Common modern strategies for wound healing. Tissue engineering is used to create skin substitutes and deliver stem cells to aid wound healing. Silver dressing is typically applied on infected wounds to kill bacteria. Hydrogel dressing is applied on wounds to keep them moist and can deliver substances such as growth factors. Negative pressure wound therapy is used for highly exuding wounds and can instil saline and antibiotics to cleanse the wound.

Due to the rapid progression of modern technologies, current approaches to wound healing are broad and advanced. Numerous strategies for wound healing revolve around the supplementation of deficient tissue components, such as growth factors [69, 70] and cell-based therapies [71, 72]. For example, topically applied recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) and granulocyte colony-stimulating factor (G-CSF) had positive effects on wound healing in small (20 patients), randomized, controlled studies involving venous leg ulcers (VLUs) and diabetic foot ulcers (DFUs) [41]. Over the past decade, stem cells from various sources have been investigated in numerous preclinical studies and a few pilot clinical studies. Clinical studies have shown that bone marrow- and adipose tissue-derived mesenchymal

stem cells (MSCs) can augment the repair process when applied locally to chronic skin wounds [73].

Hydrogel dressings are also vast in the clinical and research settings for wound care. Hydrogels are highly hydrophilic and insoluble materials that are usually made from the crosslinking of polymers such as polyesters, polyacrylamides, methacrylates, diacrylates, and alginates. Hydrogel encompasses many characteristics that are beneficial to wound healing. Examples of useful properties of hydrogel include high water content (~90%) and water retention, soft and conforms to the skin, high compressive strength, biocompatible, cooling, and serves as a huge reservoir for an assortment of bioactive substances. These properties allow hydrogels to be applied on wounds to provide a moist environment, protect injured tissues against mechanical perturbations, and deliver essential substances to aid wound healing. However, since hydrogels are porous and permeable to metabolites and even certain cells, debris such as wound exudates and dead bacteria may linger inside and create a foul environment. Therefore, frequent changing of hydrogel dressings is recommended.

Another approach to assist wound healing is untraditional and without the use of conventional dressings or delivering cells/factors. Such methods include debridement, negative pressure wound therapy (NPWT), hyperbaric oxygen therapy (HBOT) and electrostimulation [5, 74]. They are usually performed in a clinical setting by healthcare professionals. Debridement involves removing dead tissues, cells with altered phenotype, and bacteria. Such method goes against the conventional thinking that wounded tissues must be preserved and avoid further destruction in order to heal. Debridement is only practical in necrotic or heavily infected wounds where natural healing cannot occur due to obstruction of cell migration and the presence of moribund cells. After debridement, antibiotics are usually applied and dressings are still needed to

protect the delicate tissues and restoring their natural healing ability. NPWT has assumed a major role in the treatment of chronic [75], traumatic and surgical wounds [76]. NPWT are superior to standard therapy for these wounds as they typically produce a lot of exudates which conventional dressings cannot drain promptly. NPWT expedites wound drainage and newer devices even have the ability to instil antibiotics and saline to sterilize the wound [77]. HBOT involves delivering oxygen in a high-pressure chamber. Oxygen is required for wound healing and it is believed that delivering oxygen directly can speed up the process. Electrostimulation [78] is considered to induce the expression of genes involved in modulating the inflammatory stage of wound healing, as well as upregulating the production of nitric oxide and growth factors which increases angiogenesis and cell migration [79, 80]. However, such technology is still in its infant stage and the results are not compelling.

Given the current array of treatments for wound healing, there is still a lack of strategies to actually tackle the underlying problems of wound repair, such as infection, increased oxidative stress and inflammation, and reduced angiogenesis and fibroblast migration/proliferation. Modern research based on antibacterial or antioxidative dressings aim to solve these issues. N-acetylcysteine (NAC) has shown great promise as an antioxidant [81, 82]. It is a precursor to glutathione (GSH) which is the most abundant antioxidant in the body. NAC has been used clinically to treat a variety of conditions including acetaminophen toxicity, acquired immune deficiency syndrome, cystic fibrosis, chronic obstructive pulmonary disease, diabetes [83], and hearing loss [84]. However, studies on the effect of NAC on wound healing are rare and involve only the solution form [85-87].

1.3.1. Ideal properties of wound dressings

Natural skin is the perfect wound dressing because it is autogenic and does not consist of any foreign or synthetic components. However, it is impossible for us to produce what nature provides. Nevertheless, an ideal wound dressing should try to mimic its characteristics and properties. Traditionally, wound dressings were used to provide passive protection to the wound to allow natural healing to proceed unperturbed, and not engage in the wound healing process directly. However, in recent decades wound dressings were revolutionised by the discovery that moist wound dressings were able to accelerate wound healing. Furthermore, a moist wound environment is critical to induce biological activities such as the migration and proliferation of fibroblasts and keratinocytes, as well as to increase collagen synthesis, leading to accelerated wound healing and reduced scar formation [88, 89].

Besides providing a moist environment, it is also crucial that wound dressings: (i) allow for breathability and gaseous and fluid exchange; (ii) possess the ability to provide thermal insulation and mechanical protection; (iii) help in the drainage of wound exudates and debris removal so that tissues have a clean environment to grow and reconstruct; (iv) should not be cytotoxic and do not induce any immune or inflammatory responses from the body; (v) provide a barrier to protect the wound from foreign microorganisms; (vi) conform to the wound site and can be removed comfortably without causing pain and trauma [90] (Figure 1.4). Due to the specific characteristics of different types of wounds and their healing stages, and the chemical and architectural challenges to incorporate many ingredients together into a single dressing, it is arduous to design a wound dressing that encompasses all of the beneficial components discussed above. However, it is possible to develop and optimise the materials and compositions

of wound dressings such that they meet the general healing requirements of most common wounds.

Figure 1.4. Ideal properties of wound dressings.

1.3.2. Cell-based therapies

Cell-based therapies, ranging from tissue engineering to skin grafting, are probably the most common research area in the field of wound care. Skin grafting with autologous skin is a conceivable approach to treat deep wounds, since they originate from the same patient and bear no risk of rejection. Autologous skin tissues are usually obtained from the inner thighs or buttocks using a dermatome, and they contain the epidermis and a small portion of the dermis layer. The skin graft is then placed on the wound site and covered by secondary dressings such as films or bandages to protect the

area. The healing efficiency of the wound is best if a thicker layer of skin is obtained from the donor site; however, if the layer is too thick it will require shaving deeper into the donor site, which causes problems there. Donor sites will heal naturally and regain their capacity to donate again. Skin allografts are also used for treatment in such wounds but are more uncommon due to different genetic makeup of the donor tissues and the risk of rejection and immune response.

Another class of cell-based therapy is the use of stem cells. Stem cells are undifferentiated cells that possess the ability to self-renew and can be differentiated into specific cell types by molecular signals [91]. The three major types of stem cells are embryonic stem cells, adult stem cells and induced pluripotent stem cells. A huge class of adult stem cell are the mesenchymal stem cells (MSCs). MSCs are conveniently found in the bone marrow, but can also be isolated from other sources such as cord blood, peripheral blood, fallopian tube, and foetal liver and lung. MSCs circulate around the body in small amounts and can be homed by molecular signals such as SDF-1 [92-94]. MSCs can accelerate wound healing by increasing the migration of fibroblasts and keratinocytes [95, 96]. They also secrete VEGF, which enhances angiogenesis, and can also participate in vasculogenesis directly by differentiating into vascular endothelial cells [72, 95, 97]. A substantial number of studies have demonstrated that treatment with MSCs has significant immunomodulatory effects during tissue repair [98-101]. MSCs lowered the number of inflammatory cells and proinflammatory cytokines such as IL-1 and TNF- α with an increased level of IL-10 at the cutaneous wound bed in a rat model [102]. MSCs also improve proper ECM events during the healing process. Conditioned media from human umbilical cord blood MSCs have been demonstrated to inhibit the expression of MMP-1, which suggests that MSCs serve to preserve the ECM by suppressing their degradation by MMPs [103]. Traditionally, MSCs have been delivered

to wound sites via intradermal injection. However, cell retention at the targeted area is a problem with this method. More recently, three-dimensional matrices such as hydrogel and collagen allografts have been used to deliver the MSCs in a controlled manner and resulted in better healing [104, 105].

Tissue engineered skin substitutes have also been explored in the field of wound healing. These kinds of dressing contain non-organic parts (scaffold, sheet or fibre) and organic parts (sterile tissues from donors). The major role of these human skin equivalents is to secrete or stimulate the secretion of growth factors to enhance epithelialization. 3D printing of a cell construct via laser-assisted bioprinting was able to form a multi-layered epidermis with differentiation into stratum corneum, and blood vessels could be found growing from the wound bed and the wound edges in the direction of the printed cells [106]. 3D blended scaffolds of silk fibroin and human hair-derived keratin were fabricated by freeze-drying and showed significant enhancements in cell adhesion and cell proliferation as compared to controls [107]. An example of an artificial skin product is Alloderm, which is commonly used in plastic surgeries.

Cell-based therapies and tissue engineering are prevalent in modern treatments to chronic wound healing because of their similarities to the skin. Furthermore, they work in tandem with the patients' own tissues to heal the wound. However, if the cells are obtained from allogenic sources, there exist a risk of eliciting an immune response from the body which can cause serious complications to wound healing and the general health of the patient. Also, stem cells are difficult to isolate from the body and the ethical issues of using them for research and treatments are still very much debatable.

1.3.3. Bioactive materials and delivery systems for wound healing

A multitude of systems have been explored to deliver factors or substances in various biomedical applications. Delivery systems for wound healing is one such area of research. Many delivery systems in literature are based on highly organised scaffolds or platforms such as hydrogels, nanofibers, nano and microparticles, 3D printed and electrospun materials, or biomacromolecular platforms. In the past, any application of drugs or substances to the wound are usually done topically or directly through hypodermic injections or rinsing. Since the introduction of biomaterials and delivery systems, these compounds can be released onto the injured site in a controlled manner to exert a longer lasting effect over a period of time. This reduces the frequency of application and the overall quantity of compounds needed in a full course of treatment, since their release is optimised to prevent overloading and wastage. Materials used in delivery systems should be immunocompatible and non-degradable, so that they do not cause any allergenic responses from the body. Dressings delivering drugs and healing factors should preserve the activity of the compounds and release them at a desired and controlled rate.

Developing systems that can respond to their environment and alter their state is attractive for wound healing. In a wound environment, properties such as temperature and pH deviate from a steady-state physiological condition. Wound tissues have a higher temperature and lower pH than their healthy counterparts due to the molecular processes that are occurring during their repair. Hydrogels that can swell or change their properties in response to external factors have been developed. Poly(N-isopropylacrylamide) (pNIPAM)-based polymers have been widely used for engineering thermo-responsive drug delivery systems. The critical temperature of pNIPAM is around 32 °C, which is close to physiological skin temperature. pNIPAM is hydrophilic below that temperature

and hydrophobic above that, so aqueous solutions of hydrophilic drugs will precipitate at higher temperature and be released from the system. Tran et al. designed such a thermo-responsive system by electrospinning pNIPAM and polycaprolactone (PCL) to create nanofibers with a high surface area-to-volume ratio [108]. The composite nanofibers showed a reduced burst release as well as a controlled release profile over 4 h compared to fibers made of only pNIPAM at 37 °C. Other thermo-responsive polymers such as Pluronic F-127 and chitosan have been successfully explored to engineer a responsive system for wound healing applications [109-112].

Non-responsive and biodegradable vessels have found their way in wound healing as well. In polymeric systems, the release of substances is determined by their molecular weight, glass transition temperature, crystallinity, solubility and degradation rate. The degradation rate of these systems can be controlled by varying their crosslinking density and molecular weights of the polymers. Polyesters such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and poly(lactic-co-glycolic acid) (PLGA) have been studied rigorously for drug delivery applications. PLGA particles are mainly considered as bulk-eroding system, providing an initial burst release followed by a zero-order release. Growth factors have been encapsulated in PLGA particles for wound treatment. VEGF was encapsulated in 10 – 60 µm PLGA particles and showed an initial burst release followed by 2 weeks of sustained release and enhanced endothelial cells migration and proliferation [113]. *In vivo* studies on diabetic mice showed that EGF encapsulated PLGA nanoparticles significantly increased cell migration as compared to the control and soluble EGF treated groups [114].

A negative outcome of delivering drugs or particles topically is their poor retention rate on the skin, as well as inactivation of the substances before reaching their target site. As such, transdermal delivery systems have been developed to alleviate these

problems. These tools consist of micro or nano carriers that can pass through the skin barrier and the stratum corneum, or microneedles that can be painlessly applied on the skin to deliver substances transdermally. Microneedles are arrays of short needles which can penetrate the stratum corneum but not hit the nerves underneath the skin. Yeo et al. fabricated a microneedle patch with FDA-approved liquid crystalline polymer and discovered that the treatment prevented dermis tissue thickening in 83.33% of wounds in a rabbit ear hypertrophic scar model [115]. A transdermal drug delivery system was explored with oil body-linked oleosin-recombinant EGF particles in the range of 700 – 1000 nm and was found to accelerate wound healing in rats and increased the expression of TGF- β 1, bFGF and VEGF [116].

Bioactive dressings solve many issues that traditional dressings cannot. Bioactive dressings participate in the wound healing process by delivering a wealth of substances such as drugs, antibiotics, growth factors, peptides, and particles. Their properties such as release and degradation rate can be tuned to suit the application. Furthermore, they are also biocompatible as they are typically made from synthetic polymers which are inert to the body. Disadvantages of bioactive dressings consist of the difficulty in passing the skin barrier and poor retention rate of the drug molecules and substances. Transdermal patches can solve these problems but they usually cause skin irritation and itchiness.

1.3.4. Antibacterial dressings

Antibacterial dressings are a huge field of study in wound care. As discussed previously, all wounds are colonized to a certain degree, with the most severe being multi-drug resistant (MDR) biofilm infection. Biofilm bacteria are typically difficult to treat with current antibiotics since these are designed to treat metabolically active

planktonic bacteria [117-122]. Various strategies other than antibiotics, such as silver-related formulations and contact-active cationic polymers, have been investigated to address the challenge of eradication of biofilms of MDR bacteria. Silver-derived formulations have been extensively investigated in wound dressings, and majority of the antibacterial wound dressings on the market consist of some form of silver.

Typically, debridement is the first step to treat infected wounds. Debridement removes majority of bacteria as well as non-viable cells at the wound edge. Although it enlarges the wound initially, it is a necessary step to stimulate the healing of non-healing and heavily infected wounds. After debridement, the wound is usually rinsed with disinfectants to kill the remaining bacteria. However, should colonies of drug-resistant bacteria persist inside the wound, or there remain pathogens deep inside the wound that are unable to be excavated, they may regrow and eventually form back the biofilm and disrupt the healing process again.

The most common antibacterial dressings are topical antimicrobials. Topical antimicrobials include antibiotics and antiseptics such as chlorhexidine and silver sulfadiazine. They typically come in the form of a cream or a solution that is applied directly on wounds. Topical antimicrobials are only effective to the extent of the potency of the drugs, and may not kill drug-resistant bacteria. Furthermore, they may contribute to the formation of resistant strains if those colonies “escape” from the effects of the antimicrobials.

Antimicrobial peptides (AMPs) are known to exist in all living organisms. Despite their different amino acid sequence, the vast majority of AMPs share a cationic character due to the presence of basic residues, and an amphipathic structure in membrane-mimicking environments [123]. Their mechanism of killing is mainly due to electrostatic attraction with the anionic bacterial cell membrane, followed by

perturbation of the membrane which leads to cell lysis and death [123-125]. This mode of action makes them less susceptible to induce resistance in bacteria than antibiotics, as the bacteria have to “redesign” their phospholipid composition in order to escape the effects of AMPs. AMPs also inhibit nucleic acid and protein biosynthesis and metabolism in bacteria [124-126]. While applying their antimicrobial effects, AMPs can also aid the wound healing process by neutralizing the pro-inflammatory lipopolysaccharide (LPS) [126], modulating cytokine production, and inducing immune cell chemoattraction and cell proliferation [125]. AMPs occur naturally within human skin and are synthesized in different cells. Keratinocytes synthesize and store AMPs such as RNase 5 and RNase 7 within lamellar bodies in healthy skin [127]. During an infection, other AMP families with a broad spectrum of antimicrobial activity such as defensins and cathelicidins are additionally expressed by keratinocytes [127]. Besides keratinocytes, sebocytes and immune cells can generate AMPs as well to increase their pool. AMPs can also be delivered to wounds via topical application or through a vessel such as hydrogel. AMP was synthesized and self-assembled in response to a pH shift to form a hydrogel and chemically functionalized to incorporate a NO-donor moiety on lysine residues. This AMP hydrogel showed up to 9 log reduction of bacteria *in vitro* and was able to increase collagen production by human dermal fibroblasts [128]. Song et al. immobilized LL37 onto electrospun silk fibroin nanofiber membranes using NHS/EDC and thiol-maleimide click chemistry for wound care purposes. The membrane exhibited antimicrobial activity against *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa* without biofilm formation on the membrane surface. It also promoted the proliferation of fibroblasts and keratinocytes, and suppressed TNF- α expression of monocytes [129].

Silver is the most common compound in current antibacterial wound dressings on the market. It is an ancient technology which dates back centuries ago when the ancient Greeks and Romans used it as a disinfectant. Silver ions are the active ingredient in silver-based dressings, as they are positively charged and interact with the anionic bacterial membrane, causing disruption and eventual cell death. Silver ions also bind to bacterial enzymes and DNA, preventing cellular mechanisms from operating properly [130]. In a study, a comparison between silver sulfadiazine and silver nanoparticles (AgNPs) showed that AgNPs implicated a faster wound healing than silver sulfadiazine and is less toxic against fibroblasts, while displaying similar bactericidal property [131]. AgNPs were also doped into collagen-alginate composite which demonstrated low toxicity at low AgNPs concentration and high antimicrobial activity [132]. Despite the potency of silver in eradicating bacteria, repeated or high doses of silver are toxic and carcinogenic to the cells. Hence, silver dressings may only be a short-term solution to remove infection on wounds, while eventually being replaced by more biocompatible dressings after the infection is eradicated.

Modern antibacterial technologies for wound care revolve around non-tissue products, triggered or controlled release of substances, and bioactive materials. Cationic polymers and polypeptides such as polyethyleneimine (PEI) and polylysine have found their ways to remove pathogens on wounds [133-136]. Chitosan is another popular polymer that is widely studied for killing a broad spectrum of bacteria and can be formulated as a particle, coating or hydrogel [137-139]. Hydrogels form a huge part of modern antimicrobial technologies in wound care as they possess very desirable properties such as hydrophilicity, biocompatibility, excellent mechanical strength, and breathability. They can be formulated to contain certain characteristics such as charge density, high swellability, degradability, and controlled release of substances. Due to

the adaptable nature of hydrogels, targeted therapy can be achieved. It was discovered that antibacterial hydrogels made from cationic polymers kill bacteria by first absorbing the bacteria through hydrodynamic drag force created by the evaporation of water in the hydrogel and its subsequent rehydration. Once the pathogens are inside the pores of the hydrogel, cationic polymers on the pore walls perturb bacterial membrane and cause cell lysis [140]. An antibacterial and anti-oxidant electroactive injectable hydrogel was made to target cutaneous wound healing. The hydrogel was crosslinked from quaternized chitosan-g-polyaniline and poly(ethylene glycol)-co-poly(glycerol sebacate). It is able to self-heal, showed good antibacterial activity and biocompatibility, adhesiveness, swelling ratio and free radical scavenging ability. The hydrogel possessed haemostatic effect and accelerated wound closure in a mouse model [141]. As chronic wound healing is made complex by MDR bacteria, modern techniques that do not require delivery of antibiotics or similar substances will be critical. Fortunately, there is a myriad of methods that are inherently antibacterial and kill bacteria by a different mode such as contact-killing which is difficult to gain resistance.

Antibacterial dressings on the market include Aquacel Ag, Allevyn Ag, Acticoat, Tegaderm Ag, Durafiber Ag and Iodofoam. A vast majority of these dressings contain some form of silver, with a few incorporating iodine as the antimicrobial agent. Silver is effective in eradicating wound infections but for prolonged usage they are toxic to the body and alters the skin colour and composition. There is a shortage of non-silver related antibacterial dressings on the market. There has been no real breakthrough in translating modern antibacterial technologies from the lab to the wound care market. Patients are stuck with using silver dressings as the only solution to treat infected wounds. There is a dire need to translate modern technologies such as antimicrobial

hydrogels and peptides to the wound care market to complement or replace silver dressings.

1.4. Current outlook of wound healing

Treatment of chronic wounds remain a huge challenge. Chronicity of wounds depend on the extent of deviation from the normal timeline and processes in acute wound healing. Chronic wounds are usually caused by underlying problems such as persistent infection, malnutrition, vascular and skin defects, or illnesses such as diabetes. Various technologies have been explored to treat chronic wounds. A vast majority of these treatments aim to supplement the deficient components of non-healing wounds, instead of tackling the underlying complications such as infection or vascular defects. Recently, more focus has been put to tackle these problems, such as the development of antibacterial dressings to remove infection and stem cell therapies to induce the proliferation and migration of fibroblasts and keratinocytes. The advantages and disadvantages of common strategies to treat wounds are highlighted in Table 1.1. However, majority of these methods might be stuck at the lab stage and fail to translate to an actual product in the market.

Table 1.1. Wound healing strategies and their advantages and disadvantages.

Wound healing strategy	Advantages	Disadvantages
Traditional methods (e.g. plasters, bandages, films, cotton wools)	Inexpensive, simple to manufacture, protect wounds	No bioactive functions, do not accelerate wound healing beyond natural means
Debridement	Removes bacteria and necrotic tissues, provides a clean wound bed for healing	Initial enlargement of wound, bacteria can repopulate the wound
Tissue engineering	Forms a scaffold for fibroblasts and keratinocytes to migrate	Might elicit immune response
Stem cell therapy	Induces cellular mechanisms and recruits growth factors	Ethical issues, difficult to obtain autologous stem cells
Hyperbaric oxygen therapy	Painless, induces a vast of beneficial cellular mechanisms due to elevated oxygen level	Time consuming, cumbersome, expensive
Negative pressure wound therapy	Drains wound exudate efficiently, keeps wound clean	Cumbersome, needs frequent monitoring of wound
Silver	Eradicates and prevents infection	At high doses might be toxic and carcinogenic
Antimicrobial peptides (AMPs)	Eradicate infection, modulate cytokine production and cell proliferation	Difficulty of isolating AMPs, instability of AMPs
Hydrogel	Retains moisture on wound, delivers a vast of substances to aid wound healing, biocompatible	Might turn foul when wound exudates and cellular debris enter the hydrogel, frequent changing needed
Transdermal patches	Deliver a vast of substances to aid wound healing, controlled release of substances	Irritation/itchiness of skin, skin barrier is different for every individual

For timely wound healing, early detection or diagnosis of wounds are crucial, especially in diabetic patients. These patients usually fail to notice wounds that develop at the limbs because of a loss of sensation due to neuropathy at these regions. Smart diagnostic devices should be of paramount importance to help these group of people to

detect skin defects early. In general, diagnosis should work hand in hand with treatments as a complete course of action to treat non-healing wounds successfully. Modern techniques such as bioactive materials can be very effective in treating chronic wounds, so the outlook of chronic wound treatment is bright. However, the usual hurdles of clinical trials and FDA approval need to be crossed in order to translate these techniques from the lab to the market. All in all, more focus has to be put to translate modern dressings into clinics as the basic science of wound healing and its therapeutic strategies are well established.

Chapter 2

Antibacterial Hydrogel Based on Cationic Polyethylenimine Show Rapid Biofilm Debridement on Excisional Wounds

Note:

This chapter is also published as **Chun Kiat Yeo**, Yogesh Shankar Vihke, Peng Li, Zhanru Guo, Peter Greenberg, Hongwei Duan, Nguan Soon Tan, and Mary B. Chan-Park, “Hydrogel Effects Rapid Biofilm Debridement with ex situ Contact-Kill to Eliminate Multidrug Resistant Bacteria in vivo”, *ACS Appl. Mater. Interfaces* 2018, 10(24), 20356-20367. DOI: 10.1021/acsami.8b06262.

2.1. Introduction

Bacterial infections begin with colonization by planktonic pathogens but by the time of diagnosis, they usually have progressed beyond colonization to formation of biofilm. Biofilm bacteria are typically difficult to treat with current antibiotics since these are designed to treat metabolically active planktonic bacteria [117-122]. Also, biofilm bacteria are protected by a matrix of extracellular polymeric substances (EPS) which retard the diffusion of antimicrobial agents [142]. The antibiotic concentration required to kill bacteria in biofilm can be as much as 1000x that required to kill planktonic bacteria [122, 143, 144]. This problem is compounded by the inevitable, and now widespread, emergence of resistance to conventional antibiotics. Infections with multi-drug resistant (MDR) bacteria in biofilm form are difficult to treat, particularly in hospitalized patients who may be immunocompromised.

Various strategies other than antibiotics, such as silver and metal-derived formulations, and contact-active cationic polymers, have been investigated to address the challenge of eradication of biofilms of MDR bacteria. These strategies typically prevent biofilm formation but do not treat established infection. Silver-derived formulations have been extensively investigated in wound dressings, catheters, coatings, etc. [145-149]. However, various studies have indicated that silver eradicates planktonic but not biofilm bacteria [150-152]. Non-silver-based treatments have been studied but they are rather prophylactic [153]. An alternative bactericidal mechanism, such as contact-killing by cationic hydrogel, is desirable from the standpoint of avoiding harm to tissue from released toxic materials. However, hitherto, contact-active hydrogels suffer from the drawback that bacteria within the infected tissue or protected in the biofilm may not come into contact with the cationic polymer network. Various antibacterial contact-active hydrogel coatings have been reported [154, 155] but these

are typically for prophylactic treatment of bacteria and do not eradicate biofilm bacteria. Debridement (i.e. removal of bacteria and endotoxin from the infection site) by enzymes has been reported [156, 157] but not by non-leaching mechanisms such as diffusion or advection in fluid flow as reported here. Debridement can reduce inflammation and is critically important for the healing of chronic wounds [28, 158]. Debridement resulting in bacterial translocation away from the infection site followed by *ex-situ* killing is a novel concept for anti-biofilm contact-active hydrogel.

2.2. Antibacterial hydrogel based on polyethylenimine and poly(ethylene glycol)

We describe herein the first demonstration of a hydrogel that eradicates biofilm bacteria by non-leaching-based **debridement** followed by ***ex-situ* contact-killing** (DESCK) away from the infection site. The hydrogel network is made from a star polyethylenimine(PEI)-derived methacrylated copolymer (Scheme 2.1a) and a poly(ethylene glycol) dimethacrylate (PEGDMA) crosslinker (Scheme 2.1b).

Scheme 2.1. Chemical structures of (a) PEI-PEGMA and (b) PEGDMA.

The PEI copolymer (molecular weight 25 kDa) has low grafting density of methacrylate group, about 1 per PEI molecule and would dangle from the hydrophilic crosslinked PEGDMA network. The hydrogels are highly swellable and have pores of 10 – 20 μm in diameter which are larger than bacteria to minimize clogging. The DESCK hydrogel causes debridement -- it absorbs biofilm bacteria from the wound into itself to a depth of 10 – 20 μm -- probably because of diffusion and slow fluid flow into the hydrogel. Bacteria absorbed into the hydrogel are *ex-situ* contact-killed by the cationic pore walls (Scheme 2.2 – mode 1). Further, the infection also triggers the release of the bactericidal cationic PEI polymer from the hydrogel network to kill bacteria at the infection site which is distant from the hydrogel (Scheme 2.2 – mode 2). This new hydrogel effectively treats and prevents bacterial biofilm infections in a mouse wound model of carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* – which are declared critical by the World Health Organization (WHO) [159], as well as of methicillin-resistant *Staphylococcus aureus* (MRSA). In both its prophylactic and treatment modes, the hydrogel reduces bacterial load by several orders of magnitude, which bettered silver-based wound dressings (controls).

Table 2.1. Characteristics of different formulations of PEI hydrogels.

Hydrogel series	Cationic polymer (10% w/v)	Designed grafting ratio	Actual grafting ratio	Crosslinker (10% w/v)
1a	PEI(25K)-PEGMA (1:1)	1	1.25	PEGDMA
1b	PEI(25K)-PEGMA (1:2)	2	1.83	PEGDMA
1c	PEI(25K)-PEGMA (1:3)	3	3.02	PEGDMA
1d	PEI(25K)-PEGMA (1:4)	4	4.24	PEGDMA
1e	PEI(25K)-PEGMA (1:6)	6	5.96	PEGDMA
2	PEI(800)-PEGMA (1:1)	1	0.90	PEGDMA
3	PEI(750K)-PEGMA (1:6)	6	6.50	PEGDMA
4	PEI(25K)-decane-PEGMA (1:10:2)	2	2.08	PEGDMA

Scheme 2.3. The synthesis strategy for PEI-PEGMA.

For Series 2 and 3, we used a lower, as well as a higher, molecular weight PEI (800 and 750K Daltons) to obtain (2) PEI(800)-PEGMA (1:1) and (3) PEI(750K)-PEGMA (1:6) respectively; the respective titrated grafting ratios (mole/mole) were measured to be 0.9 and 6.50 (Table 2.1). To study the effect of hydrophobicity, we also added a second graft of decane to make the 4th series: PEI(25K)-decane-PEGMA

(1:10:2) using a similar chemistry. ^1H NMR spectrum of this graft copolymer in Figure 2.2 confirms the chemical structure of this copolymer. The titrated PEGMA grafting per PEI ratio (mole/mole) is 2.08 (Table 2.1). The final hydrogel is synthesized by UV-initiated crosslinking and composed of (10% w/v) PEI-PEGMA or PEI-decane-PEGMA mixed with (10% w/v) PEGDMA.

a

C

Figure 2.2. Representative NMR spectra; (a) PEI-decane in D₂O and (b) PEI-decane-PEGMA in D₂O.

2.4. *In vitro* antibacterial activity of hydrogels

The *in vitro* contact killing efficacies of the hydrogels were measured against various multi-drug resistant Gram-negative and Gram-positive bacteria (Table 2.2). The bacterial log reduction results show that Series 1a (with lowest PEGMA grafting ratio), Series 3 and Series 4 totally eradicated the various bacteria loaded onto the hydrogel discs; the bacteria eradicated include ESKAP (i.e. *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumannii* (*A. baumannii*, AB) and *Pseudomonas aeruginosa* (*P. aeruginosa*, PA)), including methicillin-resistant *S. aureus* (MRSA USA300) and carbapenem-resistant Gram-negative AB and PA strains (CR-AB and CR-PA), which are pathogens of great concern worldwide [32, 159]. The other hydrogels (1b - 1e and Series 2) do not completely eradicate the bacteria.

Table 2.2. Bacterial log reductions of different formulations of PEI hydrogels against eight strains of bacteria.

Hydrogel	Bacterial log reduction							
	PA01	CR-PA	A. <i>baumannii</i> 19606	CR-AB	<i>E. coli</i> 8739	K. <i>pneumoniae</i> 13883	S. <i>aureus</i> 29213	MRSA USA300
PEI(25K)-PEGMA (1:1)	7.31*	7.63*	7.55*	7.33*	7.12*	7.13*	7.35*	7.52*
PEI(25K)-PEGMA (1:2)	6.37	6.33	6.10	6.23	6.72	6.35	7.35*	7.52*
PEI(25K)-PEGMA (1:3)	5.55	5.12	5.60	5.84	5.52	5.55	7.35*	7.52*
PEI(25K)-PEGMA (1:4)	4.49	4.90	4.73	5.31	4.34	4.24	5.48	5.39
PEI(25K)-PEGMA (1:6)	3.39	3.87	3.38	4.39	3.93	3.34	4.28	4.27
PEI(800)-PEGMA (1:1)	0.23	0.87	0.68	0.82	1.45	0.77	2.56	0.45
PEI(750K)-PEGMA (1:6)	7.31*	7.63*	7.55*	7.33*	7.12*	7.13*	7.35*	7.52*
PEI(25K)-decane-PEGMA (1:10:2)	7.31*	7.63*	7.55*	7.33*	7.12*	7.13*	7.35*	7.52*

* denotes that no bacterial colonies were observed on the agar plate after incubation for 16 h. The initial bacterial inoculum was approximately 1×10^7 CFU per sample.

An agar diffusion test performed with *P. aeruginosa* PA01 using Series 1a and Series 4 (together with a control - PEGDMA) hydrogels proved that bacteria were killed by contact with the gels instead of leaching of residual uncured cationic polymers as no zones of inhibition (Figure 2.3) were observed.

Figure 2.3. Agar diffusion test. No zones of inhibition were observed for all of the hydrogels. Control = PEGDMA hydrogel, PEI = PEI(25K)-PEGMA (1:1) hydrogel, PDP = PEI(25K)-decane-PEGMA (1:10:2) hydrogel.

2.5. *In vitro* biocompatibility and characterizations of hydrogels

The two hydrogels (both with 25 kDa PEI) that showed the highest *in vitro* bacterial killing were chosen for further characterizations and comparisons; Series 1a and Series 4 are hereinafter respectively denoted as PEI(1a) and PDP. These hydrogels are transparent (Figure 2.4a) and swell significantly and rapidly in water: PEI(1a) and PDP hydrogels swelled by 11.7x and 11.3x (final mass/initial mass) respectively within 15 min of immersion into water (Figure 2.4b). Also, the water-equilibrated PEI(1a) and PDP hydrogels have relatively good compressive strengths at 50% strain of 119 ± 6.9 kPa and 12.3 ± 1.1 kPa respectively and also good ultimate compression strains of more than

50% (Figure 2.4c). The *in vitro* viabilities of human dermal fibroblasts (HDF) when tested via Transwell and contact MTT assays were close to 100% and 90% respectively for both formulations (Figure 2.4d), indicating low acute toxicity of these two hydrogels. This good biocompatibility is due to negligible leaching (Figures 2.4e, f) and high hydration of the charged hydrogel.

Figure 2.4. *In vitro* characterizations of hydrogels. (a) Photos of 6 mm circular disc of PEI(1a) and PDP hydrogel with scale reference. (b) Swelling ratio (final mass/initial mass) against time of PEI(1a) and PDP hydrogels (n=3). (c) Compressive strength of hydrogels (n=4). (d) Cell viability of human dermal fibroblasts (HDF) when incubated with PEI(1a) and PDP hydrogels for 24 h with Transwell and contact MTT assays (n=3). The leachability of (e) PEI(1a) hydrogel and (f) PDP hydrogel in water when compared against low concentrations of their respective raw polymers.

The contact angles of water on PEI(1a) hydrogel after 0 min and 2 min of water droplet deposition were measured to be $21.8^{\circ} \pm 2.1^{\circ}$ and $11.4^{\circ} \pm 1.2^{\circ}$ respectively while those of PDP hydrogel were higher ($50.6^{\circ} \pm 3.1^{\circ}$ and $30.6^{\circ} \pm 2.4^{\circ}$ respectively, Figure 2.5), corroborating that the decane graft increases the hydrophobicity of the PDP hydrogel.

Figure 2.5. Contact angles of water on PEI(1a) and PDP hydrogels at 0 min and 2 min.

Using the LIVE/DEAD assay, we show that all bacteria inoculated on the PEI(1a) and PDP hydrogels were dead and stained red (Figure 2.6), indicating permeabilized membranes. On the control hydrogel (PEGDMA), all bacteria were stained green, indicating live bacteria (Figure 2.6).

Figure 2.6. LIVE/DEAD assay on bacteria inoculated on hydrogels. Confocal images of MRSA USA300 on (a) PEGDMA control hydrogel, (b) PEI(1a) hydrogel and (c) PDP hydrogel. Confocal images of PA01 on (d) PEGDMA control hydrogel, (e) PEI(1a) hydrogel and (f) PDP hydrogel. Incubation time for bacteria on hydrogel is 1 h. Green colour indicates viable bacteria while red colour indicates dead bacteria.

Freeze-dried hydrogels were examined by field emission-scanning electron microscopy (FE-SEM). The PEI(1a) and PDP hydrogels are microporous with pores larger than 10 μm (Figure 2.7). FE-SEM images of PEI(1a) and PDP hydrogels inoculated with MRSA USA300 and PA01 show that the bacteria are attached to the pore walls and experience severe membrane perturbation (Figure 2.7). Bacterial debris can be seen sticking to the hydrogel wall (Figure 2.7, arrows), likely because of cell lysis and release of cellular contents. The control hydrogel (PEGDMA) is also microporous and does not appear to affect the morphology or membrane integrity of the bacteria loaded (Figure 2.7). Hence, the LIVE/DEAD assay and FE-SEM results show that PEI(1a) and PDP hydrogels are contact-active and bactericidal.

Figure 2.7. Morphology of cross-section of (a) PEGDMA control hydrogel, (b) PEI(1a) hydrogel and (c) PDP hydrogel using FE-SEM. Morphology of MRSA USA300 on cross-section of (d) PEGDMA control hydrogel, (e) PEI(1a) hydrogel and (f) PDP hydrogel using FE-SEM. Morphology of PA01 on cross-section of (g) PEGDMA control hydrogel, (h) PEI(1a) hydrogel and (i) PDP hydrogel using FE-SEM. Insets show magnified morphology (scale bar = 1 µm). White arrows represent bacterial debris.

2.6. *In vivo* bactericidal activity of hydrogels

The *in vivo* bactericidal activities of PEI(1a) and PDP hydrogels against Gram-negative and Gram-positive bacteria were tested with a murine excisional wound infection model using MRSA USA300, CR-AB, CR-PA and PA01 and were compared with commercial silver-based antimicrobial wound dressings (specifically Allevyn Ag and Algisite Ag from Smith & Nephew; both employ silver as antimicrobial agent). After wound creation and infection, the wounds were treated by application of a wound

dressing for one day starting either almost immediately for the prophylactic treatment model or 24 h post-infection for the anti-biofilm treatment model.

In the anti-biofilm treatment model, the PEI(1a) hydrogel showed greater than 3 log reduction (>99.9%) for all four tested bacteria (MRSA, CR-AB, CR-PA and PA01) (Figures 2.8a – e). This is superior to the generally ineffective treatment (<1.0 log reduction) with Allevyn Ag (Figures 2.8a – e); Algisite Ag was also ineffective (<1.0 log reduction) against PA01, CR-PA and MRSA (Figures 2.8a, c – e) though it moderately suppressed CR-AB (with 2.1 log reduction, Figures 2.8b, e). Hence, the PEI(1a) hydrogel is broad spectrum and significantly kills (>3 log reduction) the MDR biofilm bacteria tested, which the Ag-based wound dressings did not. PDP hydrogel also generally outperformed the Ag-based wound dressings with more than 3 log reduction for MRSA and more than 2 log reduction for all the Gram-negative strains (Figures 2.8a – e). We also studied the dynamics of biofilm bacteria (MRSA USA300) reduction at the wound site over a period of seven-day treatment with the different dressings. Most of the reduction in wound bacteria occurred during the first day of treatment, after which bacterial counts remained almost constant (Figure 2.8f).

Figure 2.8. Mouse *in vivo* wound infection model with 24 h post-infection treatment. Bacterial counts of (a) MRSA USA300, (b) CR-AB, (c) CR-PA and (d) PA01 on various treated and untreated control wounds after one day (n=6). * denotes $P < 0.05$ and ** denotes $P < 0.01$. (e) Table summarizing the log reduction data from Figures 2.8a – d. (f) Bacterial counts of MRSA USA300 on various treated and untreated control wounds on days 0, 1, 3, 5 and 7 (n=6).

We also investigated the bactericidal effect with an *in vivo* prophylactic model, i.e. the wound dressing was applied almost immediately (10 min or 0⁺ h) after bacterial inoculation. In the prophylactic model, the PEI(1a) hydrogel achieved >4.0 log reduction (>99.99%) for all 4 tested bacteria (Figures 2.9a – e). These bactericidal effects were also better than those of the Ag-based Allevyn Ag and Algisite Ag dressings (by about 2 log and 1 log respectively). We also studied the dynamics of planktonic bacteria (MRSA USA300) reduction at the wound site over a period of seven-day treatment with the different dressings. Most of the reduction in wound bacteria occurred during the first to third days of treatment, after which bacterial counts remained almost constant (Figure 2.9f). Hence, it appears that the Ag-based wound dressings work fairly well for prophylactic treatment involving planktonic bacteria (Figure 2.9e) but do not generally kill biofilm bacteria (Figure 2.8e), corroborating published results [152].

Figure 2.9. Mouse *in vivo* wound infection model with 0⁺ h post-infection treatment. Bacterial counts of (a) MRSA USA300, (b) CR-AB, (c) CR-PA and (d) PA01 on various treated and untreated control wounds after one day (n=6). ** denotes P < 0.01, *** denotes P < 0.001 and **** denotes P < 0.0001. (e) Table summarizing the log reduction data from Figures 2.9a – d. (f) Bacterial counts of MRSA USA300 on various treated and untreated control wounds on days 0, 1, 3, 5 and 7 (n=6).

2.7. *In vivo* wound healing and inflammatory response

We also studied the healing of MRSA USA300 infected wounds over a 2-week period using the prophylactic treatment with PEI(1a) hydrogel (Figure 2.10a). At days 7, 9, 12 and 14 post-infection, the sizes (normalized to the initial wound size) of the PEI(1a) hydrogel treated wounds were much smaller than the untreated control wounds (Figure 2.10b). Moreover, over the entire duration, the PEI(1a) hydrogel treated wounds were cleaner than the control wounds; much more pus were observed on the control wounds (Figure 2.10a). Furthermore, secondary wound sites were seen on the control wounds only; these were most likely caused by the spread of infection from the wound site to nearby skin areas (Figure 2.10a, arrows). A comparison among the six different untreated control and PEI(1a) hydrogel treated wounds on day 7 showed that five out of six control wounds have secondary wound sites while none of the PEI(1a) hydrogel treated wounds have secondary wound sites (Appendix Figure A1.1, arrows). Histological analysis of skin tissues beside the wound bed on day 3 showed reduced inflammation for the PEI(1a) hydrogel treated wound as compared with untreated infected wound (Figure 2.10c).

Figure 2.10. Full wound healing study for the *in vivo* prophylactic model. (a) Wound pictures of untreated control and PEI(1a) hydrogel treated wounds on various days. Scale bar = 5 mm. Black arrows indicate secondary infection sites. (b) Wound sizes of untreated control and PEI(1a) hydrogel treated wounds on various days as a percentage of the initial wound size (n=6). * denotes $P < 0.05$ and ** denotes $P < 0.01$. (c) H&E stains of the tissues beside the wound bed showing the extent of inflammation in wounds of untreated control and PEI(1a) hydrogel treated wounds on day 3. Black arrows signify inflamed areas as indicated by dark spots. Scale bar = 300 μm .

Further analysis by fluorescence-activated cell sorting (FACS) showed that the percentage of CD11b^+ cells (i.e. leukocytes, which include monocytes, neutrophils, granulocytes and macrophages) increased in untreated infected (by MRSA USA300 and PA01) wounds (Figure 2.11). However, infected wounds treated with PEI(1a) hydrogel did not show any excess inflammatory (CD11b^+) cells over the levels present in uninfected wounds for both strains of bacteria (Figure 2.11), indicating that PEI(1a) hydrogel wound dressing likely reduces the inflammation due to infection by killing and

removing bacteria from the wound site. PDP hydrogel, however, did not modulate the number of CD11b⁺ cells after 3 days (Figure 2.11).

Figure 2.11. Percentage of CD11b⁺ cells on wounds after treatment for 3 days with MRSA USA300 and PA01 infected mice (n=6). The percentage of CD11b⁺ cells is directly proportional to the extent of inflammation in the skin. * denotes P < 0.05 and ** denotes P < 0.01.

2.8. Antibacterial killing mechanism of hydrogels

To investigate the mechanism of the *in vivo* in-wound bacterial count reduction of PEI(1a) hydrogel, we repeated the mouse anti-biofilm wound experiment using fluorescently labelled bacteria, specifically mCherry labelled MRSA USA300 and green fluorescent protein (GFP) labelled PA01. After a 24 h treatment period (the interval during which most of the bacterial count reduction takes place, Figure 2.8f), the hydrogels were examined by confocal microscopy (Figure 2.12a). Numerous fluorescent bacteria were observed in the hydrogels up to 25 μ m and 15 μ m deep for MRSA USA300 and PA01 respectively (as measured from the wound contact surface

of the hydrogel) (Figures 2.12b, c). The bacteria are absorbed into the pore spaces of the hydrogel wound dressing as depicted in Scheme 2.2 – mode 1.

Figure 2.12. Bacterial translocation into hydrogel. (a) Schematic showing the imaged angle of the hydrogel for confocal microscopy. 3D and side views of the (b) mCherry MRSA USA300 and (c) GFP PA01 trapped in the bottom (wound contact) surface of the PEI(1a) hydrogel.

To examine the influence of the ester linkage degradation on bactericidal effects (Scheme 2.2 – mode 2), we tested a modified PEI hydrogel in which the ester linkages were replaced with acrylamide (aca) linkages. A new copolymer was synthesized from PEI by linking it to poly(ethylene glycol) acrylamide (PEGACA) instead of PEGMA (Scheme 2.4); the new copolymer is PEI(25K)-PEGACA (1:1) (hereinafter called

PEI(aca) polymer). Also, the crosslinker used was poly(ethylene glycol) diacrylamide (PEGDACA) instead of PEGDMA in the new hydrogel formulation.

Scheme 2.4. The synthesis strategy for PEI-PEGACA.

Using the 24 h post-infection/24 h treatment model, we observed that PEI(aca) hydrogel achieved 3.1 and 2.2 log reduction of MRSA and PA01 instead, which is significantly less than the PEI(1a) hydrogel (with 3.9 and 3.1 log reduction respectively) (Figure 2.13a). Incubation of the PEI(1a) and PEI(aca) hydrogels with wound fluid shows that PEI(aca) is more stable and releases less of the rhodamine-tagged PEI polymer (Figure 2.13b) than PEI(1a). No signals were observed for the control fluid (PBS). The less stable PEI(1a) due to degradation of ester linkages by bacterial lipases and leukocyte esterases probably releases the PEI polymer into the wound site to contribute towards the bactericidal effect; on the other hand, the acrylamide bonds in the less bactericidal PEI(aca) hydrogel are degradable only by neutrophil elastase [160] so that less PEI is released from PEI(aca) than from PEI(1a) hydrogel. An *in vitro* experiment conducted by incubating the hydrogel in the presence of bacteria and leukocytes, which are present at the wound sites, also confirmed the release of PEI (Figure 2.13c). MRSA USA300 led to more (0.33 mg) release of PEI from the hydrogel than PA01 (0.18 mg) after 24 h. The THP-1 macrophage also caused substantial release of PEI (0.35 mg) after 24 h (Figure 2.13c). The longer duration shows higher release.

Also, MRSA USA300 caused more PEI polymer to be released into the system as compared to PA01, which explained the higher order killing of MRSA USA300 in the *in vivo* experiments. This release of PEI is non-cytotoxic when incubated with HDFs for 24 h when tested with an MTT assay (Figure 2.13d).

Figure 2.13. (a) The bacterial counts of MRSA USA300 and PA01 on PEI(1a) and PEI(aca) hydrogel treated wounds after one day in a 24 h post-infection treatment model (n=6). (b) The fluorescence intensities of 1 mL of extracted wound fluid from MRSA USA300 and PA01 infected wounds immersed with rhodamine B labelled PEI(1a) and PEI(aca) hydrogel. Control was done by immersing hydrogels in PBS. The amount of fluorescent PEI released into the solution was calculated based on a standard curve (Appendix Figure A2.1) measured independently and is indicated above each bar (n=3). ** denotes $P < 0.01$ and *** denotes $P < 0.001$. (c) The amount of PEI polymer released into the system as a function of fluorescence intensity when incubated with different cells (n=3). (d) Cell viability of human dermal fibroblasts (HDF) when incubated with PEI(1a) and PEI(aca) polymers for 24 h (n=3).

2.9. Discussion

Rapid debridement of biofilm bacteria is a unique property of this series of hydrophilic and loosely crosslinked hydrogels. The PEI(1a) hydrogel is rapidly and highly swellable, and possesses pores that are much larger (10 – 20 μm diameter) than bacterial size so that they do not easily clog. The PEI(1a) hydrogel becomes fully hydrated relatively quickly (in 15 minutes) as compared to the much slower swelling kinetics of many other hydrogels [161, 162] which may take hours or even days to reach equilibrium swelling. The rapid swelling of these hydrogels is attributable to the hydrophilicity of both PEG and the star PEI polymer which has high density of protonated amines ($-\text{NH}_3^+$), the low crosslink density of the hydrogel network and the high porosity of the hydrogel.

We hypothesize that the combination of large, accessible pores and rapid rehydration promotes absorption of bacteria into the superficial pore spaces of the hydrogel. Non self-motile bacteria such as the species studied here can move by Brownian motion and hydrodynamic drag due to evaporation-driven re-swelling of the hydrogel. The thermal scale height ($k_B T / m_b g$) [163] is of the order of 10 microns (where k_B is the Boltzmann constant, T is temperature, m_b is buoyant mass (i.e. volume of particle $\times$ (density of particle – density of body serum)), g is gravitational acceleration) [163]. This scale height (10 μm) is large enough to permit slow vertical migration of bacteria from biofilm into the pore space of a hydrogel pressed into conformal contact with the infected wound. Also, evaporation of wound fluid/exudate from the top surface of the hydrogel probably causes fluid flow into the hydrogel from below, which causes upward hydrodynamic drag on the bacteria, contributing to bacterial translocation from wound to hydrogel. Water evaporates at the rate of 0.722 $\text{mg}/\text{min}\cdot\text{cm}^2$ at 25 $^\circ\text{C}$ (about 500 $\mu\text{m}/\text{h}$ of water film thickness) when uncovered [164]. The fluid flow rate necessary

to lift bacteria against gravity by itself may be estimated by Stokes' law to be about 200 $\mu\text{m/h}$ (Appendix A3), which is significantly smaller than the flow rate into the hydrogel if it evaporates from its upper surface at a rate comparable to that of uncovered water (Appendix A3). The high degree of hydration of the hydrogel also keeps the wound area moist, which is crucial in the wound healing process [165-167]. Translocation of bacteria from wound to hydrogel is also promoted by dispersal processes intrinsic to mature biofilms, such as surfactant production in *S. aureus* biofilms that normally promotes cell and biofilm fragment detachment and spread of infection from the site of a mature biofilm [168, 169]. Importantly, the pore size of our hydrogels is about 10 – 20 μm in diameter which makes the interior pore space highly accessible. Smaller pore sizes may retard the upward motion of bacteria due to clogging at the hydrogel surface, while larger pores may reduce the accessible internal surface area of hydrogel within easy vertical diffusion distance, retarding bacterial absorption. That PEI(1a) hydrogel has better anti-biofilm killing than alkylated PDP hydrogel (Figure 2.8) corroborates the hypothesis that hydrophilicity and hence swelling contribute to bacterial absorption out of the wound environment into the hydrogel. The various translocation processes and the destruction of bacteria within the hydrogel significantly reduces bacterial load on the wound site.

The high positive charge of the pore walls due to cationic PEI helps to hold bacteria in place once they diffuse into contact and over time also kills them through membrane perturbation. The Debye length in physiological fluids is of nanometer length scale so that the interaction between anionic bacterial membrane and cationic pore wall is essentially a contact interaction. Our hydrogel absorbs bacteria into its network and then contact-kills the bacteria away from the infection site (i.e. *ex-situ*) to leave the site fairly clean.

The second kill mode (“triggered release”) acts against both bacteria trapped in the hydrogel pore spaces and bacteria that remain in the wound. Infection-triggered degradation of the hydrogel network results in release of the bacteria-toxic cationic PEI into the aqueous environment where it can interact with bacteria both in the dressing and in the wound site. While triggered release of cationic agents is a subsidiary kill mode for the PEI(1a) hydrogel, it is not insignificant; the difference in efficacy of PEI(1a) vs. PEI(aca) *in vivo*, which is attributable to differences in the amount of PEI released, suggests that released PEI contributes about 1 log reduction.

Our PEI(1a) hydrogel is more bactericidal to biofilm bacteria than the two commercial silver-based wound dressings tested, even to CR-PA and CR-AB which urgently need new antibacterial therapies [159]. The hydrogels effectively cause debridement of the bacteria and kill them *ex-situ* in the hydrogel. Treatment with the PEI(1a) hydrogel left a clean wound, unlike the untreated control wounds that had pus and even secondary infection sites near the infected wound. These hydrogel dressings also improve healing quality by accelerating wound closure and suppressing inflammation. Debridement (Figures 2.12b, c) followed by *ex-situ* killing is advantageous over conventional *in-situ* destruction of bacteria as removal of bacteria and endotoxin from the wound would be expected to promote healing.

In conclusion, we present a hydrogel which demonstrates a new mechanism for effective biofilm removal; i.e. non-leaching-based debridement followed by *ex-situ* contact-killing within itself. The high water swellability and microporosity of the hydrogel probably contribute towards its biofilm debridement capability while the dangling high molecular weight (25 kDa) cationic PEI effectively contact-kills bacteria. The hydrogel also kills bacteria in the wound through triggered release of PEI. The PEI(a) hydrogel leaves a cleaner wound throughout the healing process, reduces

inflammation and also accelerates wound closure. The DESCK hydrogel effectively eradicates more than 99.9% of multi-drug resistant Gram-positive and Gram-negative biofilm bacteria tested in a mouse excisional wound infection model. The biofilm bacteria reduction is substantially greater than that achieved with silver dressings which can prevent but not treat biofilm PA and MRSA infections. Our PEI(1a) hydrogel also has outstanding *in vivo* prophylactic ability – it eradicates more than 99.99% of planktonic bacteria tested. With minor modifications, this material may also find useful application in coatings on medical devices such as indwelling catheters or implants.

2.10. Materials and methods

2.10.1. Chemicals

Branched polyethylenimine (PEI, Sigma-Aldrich, $M_w = 800, 25,000$ and $750,000$) was lyophilized to dryness before use. 1-Bromodecane, sodium hydroxide, potassium carbonate, isopropanol, poly(ethylene glycol) methacrylate (PEGMA, $M_n = 360$) and 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) were purchased from Sigma-Aldrich and used as received. Chloroacetyl chloride, absolute ethanol, toluene and methylene chloride were purchased from Merck Pte Ltd (Singapore) and used without further purification. Poly(ethylene glycol) dimethacrylate (PEGDMA, $M_n = 1000$) was purchased from Polysciences, Inc. Amine-terminated poly(ethylene glycol) acrylamide (PEGACA, $M_n = 400$) and poly(ethylene glycol) diacrylamide (PEGDACA, $M_n = 1000$) were purchased from Biochempeg.

2.10.2. Synthesis of chloro-functionalized poly(ethylene glycol) methacrylate (Cl-PEGMA)

Poly(ethylene glycol) methacrylate (PEGMA, $M_n = 360$) (11.8 mL, 35.3 mmol) was first dissolved in toluene (100 mL), then chloroacetyl chloride (11.25 mL, 141.2 mmol) was added into the solution at room temperature. The solution was stirred and refluxed for 24 h, then cooled and toluene was evaporated on a rotary evaporator. The gum was dissolved in methylene chloride (100 mL), then potassium carbonate (1 g) was added to the solution and the mixture was stirred for 10 min. Then, the solid potassium carbonate was removed by filtration and solvent evaporation afforded the desired Cl-PEGMA (Scheme 2.3). The product was confirmed by ^1H NMR (300MHz) (Figure 2.1b) in CDCl_3 at 25 °C: δ_{H} (ppm) 6.06 (m, 1H, methylene), 5.50 (m, 1H, methylene), 4.29-4.21 (m, 2H $-\text{CH}_2-$), 4.03 (d, 2H), 3.69-3.67 (m, 2H), 3.66-3.57 (m, ethylene protons), 1.89-1.87 (m, 3H methacrylate $-\text{CH}_3-$).

2.10.3. Synthesis of polyethylenimine grafted with PEGMA (PEI-PEGMA)

Polyethylenimine (PEI) of molecular weights 800, 25,000 and 750,000 were grafted with various ratios of PEGMA. Here, we use PEI(25K)-PEGMA (1:1) as a representative example (Scheme 2.3). PEI (1 g) was first dissolved in DI water (10 mL), and NaOH (0.4 mL, 1 M) solution was added at room temperature. Then, Cl-PEGMA (0.2 g) in isopropanol (1 mL) was added dropwise into the solution. The mixture was stirred for 3 h at room temperature and then subjected to dialysis (MWCO 12000 – 14000) in DI water for three days. The product was obtained via lyophilization. ^1H NMR (300MHz) (Figure 2.1c) in D_2O at 25 °C: δ_{H} (ppm) 5.54 (d, 1H, methylene), 5.24 (d, 1H, methylene), 3.59-3.11 (m, ethylene glycol protons), 2.80-2.57 (m, PEI ethylene protons), 1.79-1.75 (m, 3H methacrylate $-\text{CH}_3-$), 1.03 (m, PEI NH/NH_2 protons).

2.10.4. Synthesis of alkylated polyethylenimine (PEI-decane)

Polyethylenimine-decane ($M_w = 25,000$) was prepared through alkylation reaction. PEI (2.0 g, 0.2 mmol) was dissolved in absolute ethanol (50 mL) and 1-Bromodecane (0.442 g, 2 mmol) was added at room temperature. The solution was stirred and refluxed for 24 h, then the generated HBr was neutralized with sodium hydroxide (0.2 g) under the same conditions for an additional 24 h. After removal of the solvent on rotary evaporator, the resulting residue was dissolved in DI water and dialyzed (MWCO 12000 – 14000) against DI water for three days. The polymer PEI-decane was obtained via lyophilization. ^1H NMR (300MHz) (Figure 2.2a) in D_2O at 25 °C: δ_{H} (ppm) 3.05 (t, methylene -NH-CH₂- protons), 2.64-2.56 (m, PEI ethylene protons), 1.21 (m, PEI NH/NH₂ protons), 1.40-0.81 (m, decyl -CH₂-CH₃- protons).

2.10.5. Synthesis of PEI-decane grafted with PEGMA (PEI-decane-PEGMA)

PEI-decane (1 g) was first dissolved in DI water (10 mL) and NaOH solution (0.4 mL, 1 M) was added at room temperature. Then, Cl-PEGMA (0.64 g) in isopropanol (1 mL) was added dropwise into the solution. The mixture was stirred for 3 h at room temperature and then subjected to dialysis (MWCO 12000 – 14000) in DI water for three days. The polymer PEI-decane-PEGMA was obtained via lyophilization. ^1H NMR (300MHz) (Figure 2.2b) in D_2O , 25 °C: δ_{H} (ppm) 5.56 (d, 1H, methylene), 5.27 (d, 1H, methylene), 3.60-3.11 (m, ethylene glycol protons), 3.01-2.58 (m, PEI ethylene protons), 1.79-1.75 (m, 3H methacrylate -CH₃-), 1.20 (m, PEI NH/NH₂ protons), 1.50-0.80 (m, decyl -CH₂-CH₃- protons).

2.10.6. Determination of double bond content

Double bond content was characterized according to a previous protocol with slight modifications [154]. To a solution of PEI derivative (0.06 g) in DI water (2 mL), mercaptoethanol solution (1 mL, 3%) and NaOH solution (0.2 mL, 2 M) were added at room temperature. After stirring for 20 min and the sequential addition of HCl (0.5 mL, 1 M) and three drops of starch indicator, the solution was then titrated with iodine solution (0.05 M) until a blue coloration was observed.

The content of double bond was derived from the relation (Equation 2.1):

$$\text{Double bond amount (mmol/g)} = \frac{(V_1 - V_2) \times 0.05}{W} \quad (2.1)$$

where W refers to the weight in grams of the dried methacrylated PEI derivatives, V_1 refers to the volume in mL of iodine used in titration without PEI derivative and V_2 refers to the volume in mL of iodine used for sample titration. 0.05 refers to the iodine concentration.

2.10.7. Formation of hydrogels

Hydrogels were formed by UV irradiation of the hydrogel precursor solutions. Irgacure 2959, the UV initiator, was first dissolved in ethanol to make a 10% w/v stock solution. Hydrogel solutions containing the PEI polymer (10% w/v), crosslinker (PEGDMA, 10% w/v) and UV initiator (Irgacure 2959, 0.1% w/v) were mixed and dissolved completely in DI water in a 1.5 mL microtube. Hydrogel solution (50 μ L) was transferred to each well of a 96-well plate. The solutions were then irradiated with UV light (365 nm, 18 mW/cm²) for 10 min to crosslink the precursor solutions into hydrogels. The hydrogels were washed in ethanol three times and in DI water three times with sonication to remove all unreacted precursors.

2.10.8. *In vitro* antimicrobial assay of hydrogels

(1) Preparation of bacterial suspensions

Bacteria (*E. coli* 8739, *S. aureus* 29213, *K. pneumoniae* 13883, *A. baumannii* 19606, MRSA USA300, PA01, CR-AB and CR-PA) were inoculated and dispersed in Mueller-Hinton broth (MHB, 4 mL) at 37 °C with continuous shaking at 220 rpm to mid log phase. Bacterial suspension (1 mL) was added into a sterile microtube and MHB was removed by centrifugation, followed by decanting of the supernatant. Bacteria were washed with phosphate buffered saline (PBS, 1 mL) thrice and the final bacteria suspensions (1×10^9 CFU/mL) were prepared with PBS (1 mL).

(2) Inoculation of bacteria on hydrogels

Bacterial suspension in PBS (10 μ L), containing approximately 1×10^7 CFU was inoculated and spread evenly onto the surface of hydrogels, which were placed on a small petri dish. A control was prepared by inoculating bacteria on a small petri dish with no hydrogel. The hydrogels were incubated at 37 °C for 1 h with 90% relative humidity.

(3) Bacterial counts

Hydrogels were immersed in PBS (1 mL) and vortexed to release bacteria. Then, a series of ten-fold dilutions of the bacterial suspensions were prepared in a 24-well plate and dilutions were plated onto Luria-Bertani (LB) agar. The plates were incubated at 37 °C for 16 h and bacterial colonies were counted.

Results were evaluated as follows (Equation 2.2):

$$\text{Log reduction} = \text{Log (total CFU of control)} - \text{Log (total CFU on hydrogels)} \quad (2.2)$$

2.10.9. Agar diffusion test

A suspension of bacteria (PA01, 1×10^9 CFU/mL) in PBS was prepared followed by spreading (100 μ L) on an LB agar plate, after which they were incubated with PEGDMA (control), PEI(25K)-PEGMA (1:1) and PEI(25K)-decane-PEGMA (1:10:2) hydrogels at 37 °C for 16 h. An image was taken after the incubation.

2.10.10. Swelling kinetics of hydrogels

Hydrogels were washed thoroughly and lyophilized to dryness. The masses of fully dried hydrogels were weighed at time zero. Then, copious amount of DI water was added to the hydrogels to induce swelling. The masses of the swelled hydrogels were taken at 5, 10, 15, 20, 25 and 30 min after drying with filter paper. Swelling ratio was calculated using the formula (Equation 2.3):

$$\text{Swelling ratio} = \frac{\text{mass of hydrogel at } n^{\text{th}} \text{ min} - \text{initial mass of hydrogel}}{\text{initial mass of hydrogel}} \quad (2.3)$$

2.10.11. Compression test

The mechanical properties of the hydrogels were characterized by compressive stress–strain measurements which were performed on water-equilibrated hydrogels using an Instron 5543 Single Column Testing System. The cylindrical gel sample, 6 mm in diameter and 2 mm in thickness, was put on the lower plate and compressed by the upper plate, which was connected to a load cell, at a strain rate of 0.1 mm/min. Four parallel samples per measurement were performed, and the obtained values were averaged and plotted in a graph.

2.10.12. *In vitro* biocompatibility assay of hydrogels and polymers

Biocompatibility studies were carried out on human dermal fibroblasts (NHDF-Ad-Der Fibroblasts, CC2511, Lonza). Fully supplemented DMEM, consisting of foetal bovine serum (FBS, 10%) and antibiotics (penicillin-streptomycin, 1%) was used as cell culture medium.

(1) *Transwell MTT assay*

HDF cells were cultured in 24-well plates from an initial density of 5×10^4 cells in each well, and incubated in a CO₂ incubator at 37 °C for 24 h for cell attachment. Hydrogels were placed in transwell inserts (Falcon, 1 µm pores) before incubation with HDF cells at 37 °C for 24 h. Then, the hydrogels were removed and the culture media were replaced with MTT solution (1 mg/mL in DMEM) and incubated at 37 °C for 4 h to stain viable cells. MTT solution was discarded, dimethyl sulfoxide (DMSO) was added and mixed well. The cell viability was calculated based on the absorbance of each well at 570 nm against the cell only control wells which served as the 100% cell viability control.

Results were evaluated as follows (Equation 2.4):

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of hydrogel treated cells}}{\text{Absorbance of control cells}} \times 100\% \quad (2.4)$$

(2) *Contact MTT assay*

The procedures are same as Transwell MTT assay, but hydrogels were immersed in the cell cultures directly and removed prior to MTT addition, instead of using transwell inserts.

(3) *PEI polymer MTT assay*

The procedures are the same as above, but instead of hydrogels PEI polymers (200 and 400 µg/mL) were immersed in the cell cultures.

2.10.13. Hydrogel leaching tests

Hydrogels were washed thoroughly with ethanol and water. The washed hydrogels were then immersed in DI water (5 mL) for 24 h before measuring the UV-Vis absorbance of the solution using Thermo Evolution 600 BB UV-Vis spectrophotometer. Control was done by measuring the UV-Vis absorbance of the respective polymers at 100, 10 and 1 $\mu\text{g/mL}$ concentration. Wavelengths measured were 190 – 300 nm.

2.10.14. Contact angle measurements

Contact angles of water on hydrogels were taken with FTA200 Contact Angle Analyzer. Briefly, water was injected (20 $\mu\text{L/min}$) onto the surface of fully swollen hydrogels, until a drop of water sits on the surface of the hydrogels. The images were taken with a camera and the contact angle was measured by the accompanying software.

2.10.15. LIVE/DEAD staining to examine bacterial viability and membrane permeabilization

Suspensions of bacteria (MRSA USA300 and PA01, 1×10^9 CFU/mL) in PBS were prepared followed by inoculation (10 μL , bacterial count = 1×10^7 CFU) on the hydrogels, after which they were incubated at 37 °C for 1 h. Then, the hydrogels were stained with BacLight bacterial viability kit L13152 reagents (Invitrogen) for 15 min at room temperature. The hydrogels were then imaged at surfaces inoculated with bacteria with confocal microscopy (ZEISS LSM 800). A control was prepared by staining live bacteria on PEGDMA hydrogel.

2.10.16. Scanning electron microscopy to visualize hydrogel-bacteria interactions

Suspensions of bacteria (MRSA USA300 and PA01, 1×10^9 CFU/mL) in PBS were prepared followed by inoculation (10 μ L, bacteria count = 1×10^7 CFU) on the hydrogels, after which they were incubated at 37 °C for 1 h. Then, the bacteria were fixed on the hydrogels by dripping a small amount of glutaraldehyde. The hydrogels were then freeze-dried overnight before sectioning and FE-SEM imaging using a JEOL JSM-6701 FE-SEM. A control was prepared using PEGDMA hydrogel.

2.10.17. Mouse *in vivo* wound infection model

All animal studies were approved and performed in compliance with the regulations of the Institutional Animal Care and Use Committee of Nanyang Technological University.

(1) Wounding experiment and FACS analysis

Eight-week old female C57BL/6 mice were used. The mice were anaesthetized, depilated and two 6 mm diameter full-thickness excisional wounds were inflicted on the dorsal skin and the underlying panniculus carnosus as previously described [170]. Next, bacteria (MRSA USA300 and PA01, 1×10^6 CFU in 20 μ L of PBS) were topically inoculated onto the wounds and left to settle for 10 min prior to application of the dressing to simulate an infection that is promptly treated. Hydrogels were applied on the wounds and secured with Tegaderm (3M) transparent dressing. Untreated infected and uninfected wounds served as controls. At 3 days post injury and treatment, the wounds including 5 mm of the peripheral region were excised. Single-cell suspensions from wound samples were obtained using gentleMACS Dissociator according to the manufacturer's protocol (Miltenyi Biotec). Cells were immuno-labelled with CD11b and Ly6G (Biolegends) and flow cytometry was carried out using an Accuri C6 flow

cytometer (BD Biosciences). Data analysis was performed using Flowjo software version 7.6.5 (Tree Star). The mean percentage values (n = 6) were plotted for each treatment, $\pm$ SEM. A two-tailed Student's t test was used for comparisons.

(2) Infection models and enumeration of bacterial load on wounded skin

Wound creation and infection procedures are the same as above. Bacteria tested were MRSA USA300, CR-AB, CR-PA and PA01. For the anti-biofilm treatment model, treatments were applied 24 h post-infection. For the prophylactic treatment model, treatments were applied 10 min post-infection. In the 24 h treatment study, dressings were removed after 24 h and the wounds, including 5 mm of the peripheral region, were excised. In the 7-day study, wounds were harvested on days 1, 3, 5 and 7. Each wound was homogenized in PBS (900 μ L) to release bacteria (n = 6). Then, a series of ten-fold dilutions of bacterial suspension was done in PBS and plated on LB agar. The plates were incubated at 37 °C for 16 h and bacterial colonies were counted. A two-tailed Student's t test was used for comparisons.

(3) Full wound healing study

PEI(1a) hydrogel and MRSA USA300 bacteria were used for this study. At day 0, mice were wounded and infected with bacteria. The wounding and infection procedures are the same as above. Untreated wounds were secured with Tegaderm and served as controls, while PEI(1a) hydrogels were applied to the treated wounds and were secured with Tegaderm. Photographs of the wounds were taken before dressing application and on days 1, 3, 5, 7, 9, 12 and 14, and at these points the dressings were replaced with fresh hydrogels. The wound size at each time point was determined using ImageJ software (n = 6). A two-tailed Student's t test was used for comparisons. Wound sizes were calculated using the formula (Equation 2.5):

$$Wound\ size\ (\%) = \frac{wound\ area\ on\ n^{th}\ day}{wound\ area\ on\ day\ 0} \times 100\% \quad (2.5)$$

(4) Histological analysis

Wound biopsies were fixed in paraformaldehyde-PBS (PFA, 4%) overnight at 4 °C. For paraffin embedding procedure, the tissues were dehydrated over a graded series of increasing concentrations of ethanol followed by xylene. Dehydrated tissues were submerged in molten paraffin wax prior to paraffin embedding using the tissue embedding system Leica EG1160 (Leica Microsystems, USA). Paraffin sections of 5 µm thickness were used for basic Haematoxylin and Eosin staining. Images of stained sections were captured using an Axioscan Z1 (Carl Zeiss).

(5) Confocal imaging of hydrogel after treatment

mCherry tagged MRSA USA300 and GFP tagged PA01 and the 24 h post-infection treatment model were used. PEI(1a) hydrogels were retrieved after one day of treatment. The wound contact surfaces of the hydrogels were imaged by 3D confocal microscopy (ZEISS LSM800).

(6) Wound fluid incubation of fluorescent hydrogels

Wound fluid was extracted by vortexing one infected wound tissue sample in PBS (1 mL) for 10 min, followed by centrifugation to discard the residue. PEI(1a) hydrogel was compared with acrylamide-bonded PEI(aca) hydrogel. The PEI component of the hydrogels were tagged with rhodamine B before incubation with extracted wound fluid for 24 h. PBS was used as the control fluid. The fluorescence intensities of the solutions were measured with LS 55 fluorescence spectrometer (PerkinElmer) at excitation wavelength of 553 nm and emission wavelength of 576 nm, and subtracted with the intensity of blank wound fluid. The quantities of PEI polymer released into the system were calculated based on a standard curve done independently (Appendix A2.1). A two-tailed Student's t test was used for comparison.

2.10.18. Degradability of hydrogels in the presence of bacteria and macrophages

PEI(1a) hydrogels were tagged with rhodamine B and immersed in 1 mL of PBS (control), MHB media containing 1×10^7 CFU of either MRSA USA300 or PA01, or RPMI media containing THP-1 activated macrophages for 12 h and 24 h. The fluorescence intensities of the solutions were measured with LS 55 fluorescence spectrometer (PerkinElmer) at excitation wavelength of 553 nm and emission wavelength of 576 nm, and subtracted with blank MHB and RPMI media respectively. The quantities of PEI polymer released into the system were calculated based on a standard curve (Appendix Figure A2.1).

Chapter 3

Biofunctional Hydrogel Reduces Bioburden and Oxidative Stress to Accelerate Diabetic Wound Healing

3.1. Introduction

Wound healing consists of three main stages: inflammation, proliferation, and maturation [5, 6]. The early inflammatory response mobilizes local and systemic defence responses to the site of the wound [7, 8]. Inflammation is prolonged in chronic wounds, and it is believed that these wounds might be trapped in a chronic inflammatory state that fails to progress [29]. Specifically, recent investigations of chronic wound tissue and fluid indicate a continual competition between inflammatory and anti-inflammatory signals leading to an imbalanced environment for proper wound healing to occur [30, 31].

Bacterial colonization of wounds is common [35]. Wound infection is likely to be a contributing factor in prolonged inflammation and delayed wound healing. All wounds are colonized to some degree, and a major role of the inflammatory phase of wound healing is to bring microbes down to steady-state and innocuous levels [11, 36]. Furthermore, in these polymicrobial wound communities, individual species may become more virulent and proliferate to form biofilm, which further impedes wound repair [41]. Complications due to wound infections include delayed wound closure [42], amputations [43] and even mortality [44]. An estimate of the economic cost of chronic non-healing wounds in the US alone is more than \$50 billion per year [4]. Elimination of bacterial infection is a crucial step in wound healing as bacteria typically disrupt the natural healing process, and even worsen the condition of the wound [45, 46]. For diabetic patients, sustained hyperglycaemia is known to increase vascular superoxide production, which inactivates nitric oxide and causes vascular dysfunction [51], hence delaying wound healing. To accelerate chronic or diabetic wound healing there is a need to both eliminate bacteria and reduce oxidative stress.

Current strategies for wound healing revolve around the supplementation of deficient tissue components, such as growth factors [69, 70] and cell-based therapies [71, 72]. Clinical studies have shown that bone marrow- and adipose tissue-derived mesenchymal stem cells (MSCs) can augment the repair process when applied locally to chronic skin wounds [73]. Dressings that contain small interfering RNA (siRNA) silenced MMP-9 expression and improved diabetic wound healing *in vivo* [171]. However, there is a lack of strategies to actually tackle the underlying problems of wound repair, such as infection, increased oxidative stress and inflammation, and reduced angiogenesis and fibroblast migration/proliferation.

N-acetylcysteine (NAC) has shown great promise as an antioxidant [81, 82]. It is a precursor to glutathione (GSH) which is the most abundant antioxidant in the body. NAC has been used clinically to treat a variety of conditions including acetaminophen toxicity, acquired immune deficiency syndrome, cystic fibrosis, chronic obstructive pulmonary disease, diabetes [83], and hearing loss [84]. However, studies on the effect of NAC on wound healing are rare and involve only the solution form [85-87].

3.2. Antibacterial and antioxidative hydrogel based on poly(ethylene glycol), polyimidazolium and N-acetylcysteine

We herein construct an antibacterial and antioxidative hydrogel through thiol-maleimide Michael Addition reaction. The hydrogel network is formed by the crosslinking of poly(ethylene glycol) tetra thiol (PEG-4SH) and poly(ethylene glycol) tetra maleimide (PEG-4mal). Thiol-maleimide crosslinking occurs selectively and spontaneously at near neutral pH (7.2 – 7.6) [172, 173], and the aforementioned components pre-dissolved in DI water form a hydrogel when mixed at near physiological pH. We further added a cationic polyimidazolium-maleimide (PIM-mal)

as the antibacterial component which is easily synthesized via a one-pot reaction and has properties, such as molecular weight and charge, readily tuned by adjusting the reaction conditions [174]. The antibacterial (PIM-mal) and antioxidative (NAC) components are also tethered in tandem with the crosslinking of the hydrogel network via the same thiol-maleimide Michael Addition reaction. This hydrogel is hereinafter referred to as **PEG-PIM-NAC** (PPN) hydrogel. PPN hydrogel effectively treats biofilm infections in a diabetic mouse excisional wound infection model of multi-drug resistant (MDR) bacteria. The hydrogel is able to kill >99.9% of biofilm bacteria on wounds and greatly accelerates wound closure.

3.3. Polyimidazolium syntheses and characterizations

Polyimidazolium-maleimide (PIM-mal) was used as the antibacterial agent in this hydrogel. Main-chain PIM-mal was synthesized via the reaction of 1,4-diaminobutane, formaldehyde and glyoxal (Scheme 3.1a) followed by end-functionalization with maleimide (Scheme 3.1b). The ^1H NMR characterizations of the chemical structures of these polymers are presented in Figures 3.1 and 3.2. The molecular weights of the synthesized polymers were measured by gel permeation chromatography and the M_n were found to be 2617 Da for PIM and 2766 Da for PIM-mal (Figures 3.3 and 3.4).

Scheme 3.1. Synthesis of polyimidazolium polymers. (a) Synthesis of amine-terminated polyimidazolium (PIM). (b) Synthesis of maleimide-terminated polyimidazolium (PIM-mal).

Figure 3.1. NMR spectrum of PIM in DMSO- d_6 .

Figure 3.2. NMR spectrum of PIM-mal in DMSO-d₆.

Figure 3.3. Molecular weight of PIM using gel permeation chromatography.

Figure 3.4. Molecular weight of PIM-mal using gel permeation chromatography.

The minimum inhibitory concentration (MIC) of PIM-mal varies from 2 to 8 $\mu\text{g/mL}$ (Table 3.1) when tested against various ESKAPE strains of bacteria (*E. faecium* 19434, methicillin-resistant *S. aureus* (MRSA BAA-40 and MRSA USA300), *K. pneumoniae* 13883, carbapenem-resistant *A. baumannii* (CR-AB), *P. aeruginosa* PA01, carbapenem-resistant *P. aeruginosa* (CR-PA) and *E. aerogenes* 13047), indicating that the polymer is potent in inhibiting the growth of a wide strain of bacteria.

Table 3.1. Minimum inhibitory concentration (MIC) of PIM-mal against various ESKAPE bacteria.

Polymer	MIC ($\mu\text{g/mL}$)			
	<i>E. faecium</i> 19434	MRSA BAA- 40	MRSA USA300	<i>K. pneumoniae</i> 13883
PIM-mal	4	2	4	8
	CR-AB	PA01	CR-PA	<i>E. aerogenes</i> 13047
	8	4	8	4

3.4. Hydrogel formulations and their *in vitro* antibacterial activities

A series of hydrogel formulations were prepared with poly(ethylene glycol) tetra thiol (PEG-4SH, 5% w/v), poly(ethylene glycol) tetra maleimide (PEG-4mal, 5% w/v), PIM-mal (0.1, 1 or 10 mg/mL) and N-acetylcysteine (NAC, 1 mM) in DI water (Table 3.2). The hydrogel crosslinks via specific and efficient thiol-maleimide Michael Addition reaction at near neutral pH (7.2 – 7.6) [172, 173]. We found that the hydrogel precursors were able to crosslink in DI water in less than one minute. The hydrogel network is mainly made of PEG-4SH and PEG-4mal, while NAC and a portion of the PIM-mal are tethered to the network as pendant molecules. These hydrogels are termed **PEG-PIM-NAC (PPN)** and suffixed according to the PIM-mal concentration of 0.1, 1 and 10 mg/mL as PPN0.1, PPN1 and PPN10 respectively. A gel control was made with mixing just PEG-4SH (5% w/v) and PEG-4mal (5% w/v) in DI water without the active components (labelled as PPcontrol) for use as a comparison. The hydrogels were washed thoroughly in ethanol followed by DI water with sonication.

Table 3.2. PPN hydrogel formulations.

Hydrogel formulation	PEG-4SH	PEG-4mal	PIM-mal	NAC
PPcontrol	5%	5%	-	-
PPN0.1	5%	5%	0.1 mg/mL	1 mM
PPN1	5%	5%	1 mg/mL	1 mM
PPN10	5%	5%	10 mg/mL	1 mM

The *in vitro* contact killing efficacies of the hydrogels were measured against various multi-drug resistant (MDR) Gram-negative and Gram-positive bacteria, specifically strains that are relevant in wound infections (*S. aureus*, *P. aeruginosa* and *A. baumannii*) [40]. PPN1 and PPN10 totally eradicated the various bacteria loaded onto

the hydrogel discs in just 1 h (Table 3.3); the bacteria eradicated include methicillin-resistant *S. aureus* (MRSA USA300), *P. aeruginosa* PA01, and carbapenem-resistant Gram-negative *P. aeruginosa* and *A. baumannii* strains (CR-PA and CR-AB), which are pathogens of great concern worldwide [32, 159]. PPN0.1 hydrogel did not completely eradicate the bacteria (Table 3.3), probably due to the lower concentration of the active antibacterial PIM-mal tethered to the hydrogel. PPcontrol did not exhibit bactericidal properties.

Table 3.3. *In vitro* bacterial log reductions of the PPN hydrogels against various clinically relevant bacteria strains.

Hydrogel	Log reduction			
	MRSA USA300	CR-AB	PA01	CR-PA
PPcontrol	0.05	0.03	0.04	0.02
PPN0.1	3.15	2.97	2.76	3.09
PPN1	7.20*	7.37*	7.06*	7.19*
PPN10	7.20*	7.37*	7.06*	7.19*

* denotes that no bacterial colonies were observed on the agar plate after incubation for 16 h. The initial bacterial inoculum was approximately 1×10^7 CFU per sample.

3.5. *In vitro* biocompatibility and characterizations of hydrogels

The *in vitro* biocompatibility of the materials was studied by challenging human dermal fibroblasts (HDF) with PIM-mal polymer solution and PPN hydrogel extract, and with hydrogel contact MTT assays; HDF cell viabilities in all tests were excellent. The PIM-mal polymer is relatively non-cytotoxic even at high concentration of 10 mg/mL as the viability of HDF was above 81% (Figure 3.5a). The cell viability of HDF was 100% for all the hydrogel extracts (Figure 3.5b). For the hydrogel contact MTT assay, the cell viabilities were 97%, 94% and 89 % for PPN0.1, PPN1 and PPN10 respectively (Figure 3.5b), indicating low acute toxicity of these hydrogels. This good

biocompatibility is due to biocompatible hydrogel precursors, efficient thiol-maleimide cross-linking, extensive washing and high hydration of the charged hydrogel. The PPN1 and PPN10 hydrogels showed similarly high *in vitro* bacterial killing and good biocompatibility. PPN1 was chosen for further characterizations as it contains a lower concentration of PIM-mal.

Figure 3.5. *In vitro* biocompatibility of PIM-mal and PPN hydrogels. (a) Cell viability of human dermal fibroblasts (HDF) when incubated with different concentrations of PIM-mal for 24 h (n=3). (b) Cell viability of HDF when incubated for 24 h with various PPN hydrogels using hydrogel extract and contact MTT assays (n=3).

The PPN1 hydrogel is transparent (Figure 3.6a) and swells significantly and rapidly in water: PPN1 hydrogel swelled to an equilibrium of 10.9x its dry weight within 50 min of immersion into water (Figure 3.6b).

Figure 3.6. (a) Visual appearance and size of PPN1 hydrogels fabricated in (i) 96-well plate and (ii) 24-well plate. (b) Swelling ratio (mass increase/initial mass) against time of PPN1 hydrogel (n=3).

3.6. Stability and degradability of hydrogels

PPN1 hydrogels were stable when incubated with bacterial extracts of MRSA USA300 and CR-PA for 2 and 7 days, and showed almost constant mass throughout these time periods (Figures 3.7a, b). The slight variation (but non-significant) of its mass was due to dynamic swelling of hydrogel which is affected by temperature, pH and chemical potential of the solution [175-177]. This proved that PPN1 hydrogel is resistant to degradation by bacterial extracts. It is also stable in *in vivo* testing as the hydrogel remained intact throughout 2 days treatment of infected wounds (Figure 3.7c). The hydrogel was transparent initially. It turned reddish-brown after 2 days of treatment due to the absorption of wound fluid and dead bacteria. The hydrogels also showed no degradation when incubated with infected wound fluids (Figure 3.7d). This proved that PPN1 hydrogel is also resistant to degradation by infected wound fluids.

Figure 3.7. Mass of swollen PPN1 hydrogels when incubated with extracts of (a) MRSA USA300 and (b) CR-PA for 2 and 7 days (n=3). (c) PPN1 hydrogel images before (left) and after (right) 2 days of treatment on MRSA USA300 infected wound. (d) Mass of swollen PPN1 hydrogels when incubated with wound fluids of MRSA USA300 and CR-PA infected wounds for 2 and 7 days (n=3).

3.7. *In vivo* bactericidal activity of hydrogels

The *in vivo* bactericidal activities of PPN1 hydrogel against clinically relevant Gram-negative and Gram-positive bacteria were tested with a murine excisional diabetic wound infection model using MRSA USA300, CR-AB, PA01 and CR-PA and were compared with commercial silver-based antimicrobial wound dressing (Allevyn Ag from Smith & Nephew). The mice were first induced with diabetes by streptozotocin (STZ) treatment. Following wound creation and infection on diabetic mice, the wounds were treated by application of a wound dressing for one day starting 24 h post-infection to simulate an anti-biofilm treatment.

In the anti-biofilm treatment model, PPN1 hydrogel showed greater than 3 log reduction (>99.9%) for all tested bacterial strains (Figures 3.8a – d). This is superior to the generally ineffective treatments (0.1 – 0.3 log reduction) with Allevyn Ag and the PPcontrol (Figures 3.8a – d). Hence, the PPN1 hydrogel is broad spectrum and significantly kills (>3 log reduction) the MDR biofilm bacteria tested, which the Ag-based Allevyn dressing did not.

Figure 3.8. Mouse *in vivo* diabetic wound infection model with 24 h post-infection treatment. Bacterial counts of (a) MRSA USA300, (b) CR-AB, (c) PA01 and (d) CR-PA on various treated and untreated control wounds after one day (n=6). * denotes $P < 0.05$, *** denotes $P < 0.001$ and **** denotes $P < 0.0001$.

3.8. *In vivo* wound healing and bacterial reduction over 2 weeks

We studied the dynamics of biofilm bacteria (MRSA USA300) reduction at the wound site over a period of 2-weeks treatment with the different dressings (Figure 3.9a). Most of the reduction in wound bacteria occurred during the first three days of treatment with PPN1 hydrogel, after which bacterial counts remained almost constant at about 10⁴ of the initial count (Figure 3.9a). Allevyn Ag and PPcontrol removed much less bacteria and the untreated control barely had any reduction of bacteria over 2 weeks (Figure 3.9a).

We also studied the healing of MRSA USA300 infected diabetic wounds over a 2-week period with PPN1 hydrogel, PPcontrol and Allevyn Ag (Figure 3.9b). The wounds that were treated with PPN1 hydrogel were cleaner and smaller than the untreated control, PPcontrol and Allevyn Ag treated wounds at all time points (Figure 3.9c). The wounds also fully closed at day 12 for the PPN1 treated group but did not close after 2 weeks for the other groups (Figure 3.9b, c). Much more pus was observed on the untreated control wounds over the duration of the study, indicating biofilm formation and high inflammation (Figure 3.9c). Furthermore, the untreated control wounds deteriorated and showed erratic healing (Figure 3.9c, Appendix Figure A4.1). These were most likely caused by the spread of infection from the wound site to the neighbouring skin, and delayed wound healing.

Figure 3.9. Full wound healing study. (a) Bacterial counts of MRSA USA300 on various treated and untreated control wounds on days 0, 1, 3, 5, 7, 9, 12 and 14 (n=6). (b) Wound sizes of untreated control, Allevyn Ag, PPcontrol and PPN1 hydrogel treated wounds on various days as a percentage of the initial wound size (n=6). (c) Visual appearance of representative untreated control, Allevyn Ag, PPcontrol and PPN1 hydrogel treated wounds between dressing changes. Scale bar = 5 mm.

3.9. Inflammatory response and ELISA on wound healing factors

Further analysis by fluorescence-activated cell sorting (FACS) showed that the percentage of CD11b⁺ cells (i.e. leukocytes, which include monocytes, neutrophils,

granulocytes and macrophages) increased in untreated infected (by MRSA USA300) wounds (Figure 3.10a). However, infected wounds treated with PPN1 hydrogel did not show any excess inflammatory (CD11b⁺) cells over the levels present in uninfected wounds (Figure 3.10a). The reduction in wound inflammation produced by PPN1 hydrogel is likely due to its killing and removal of bacteria from the wound site (see discussion below). Allevyn Ag, however, did not modulate the number of CD11b⁺ cells after 2 days of treatment (Figure 3.10a).

ELISA was used to determine the concentration of wound healing related factors that were present in the wounds at day 3. The concentration of pro-MMP9 (which is a precursor to MMP9 and is detrimental to wound healing [178]) was high for the untreated control wounds, whereas PPN1 hydrogel significantly reduced the level of pro-MMP9 in the wounds (Figure 3.10b). PPcontrol and Allevyn Ag also significantly reduced pro-MMP9 concentrations (Figure 3.10b), though to a lesser degree than PPN1. The concentrations of other wound healing factors (VEGF-A, PDGF-BB, FGF-2 and EFG) were also measured by ELISA and found to be significantly higher in PPN1 treated wounds than untreated control, PPcontrol and Allevyn Ag treated wounds (Figures 3.10c – f).

Figure 3.10. Characterizations of MRSA USA300 infected wound tissues of diabetic mice made 2 days post-treatment (n=6). (a) Percentage of CD11b⁺ cells in wounds. The percentage of CD11b⁺ cells is directly proportional to the extent of inflammation in the skin. (b) Concentration of pro-MMP9 in wounds. Concentrations of wound healing factors (c) VEGF-A, (d) PDGF-BB, (e) FGF-2 and (f) EGF in wounds. ** denotes $P < 0.01$, *** denotes $P < 0.001$ and **** denotes $P < 0.0001$.

3.10. Discussion

Previously, we discovered that hydrogels made with cationic polymers kill bacteria by first absorbing them into their pore spaces via the hydrodynamic drag force generated by the evaporation of water from the hydrogel and its subsequent rehydration, followed by contact killing of the bacteria by the cationic polymers on the pore walls [140]. In this study, we applied a similar concept but with a more efficient and quicker crosslinking chemistry by thiol-maleimide Michael Addition reaction of the hydrogel polymers. The hydrogel network is made of highly biocompatible and hydrophilic PEG and tethered with antibacterial polyimidazolium-maleimide and antioxidative N-acetylcysteine. This method of fabricating the hydrogel allows for different concentrations of the active components (PIM-mal and NAC) to be grafted onto the hydrogel to treat different severity of infected wounds.

Diabetic wounds on patients are usually chronically inflamed due to infection, and they suffer from poor blood circulation and impaired immune function [179]. These factors lead to decreased production and repair of new blood vessels, reduced production and delivery of wound healing factors and huge bioburden on wounds, which delay wound healing. Both active components of our hydrogel are important to treat infected diabetic wounds. First, the hydrogel absorbs bacteria into its pore spaces due to hydrodynamic drag force, followed by contact killing of the bacteria, away from the wound site, on the cationic hydrogel pore walls. Then, thiol substitution by free thiols (mainly glutathione, GSH) present in wound tissues causes NAC and singly-attached PIM-mal to dissociate away from the hydrogel network into the wound site. Solution PIM-mal can exert its antibacterial effects to kill more bacteria in the wound site. NAC, which is a precursor to GSH, can replenish the GSH level in the tissues. GSH is the body's main antioxidant which neutralizes free radicals, reduces oxidative stress and

inflammation, and boosts the immune system [180]. This further helps by suppressing infection in the wound and accelerates wound healing by allowing new blood vessels to form and promoting delivery of wound healing factors as shown by the elevated wound healing factors in the wound tissues (Figures 3.10c – f). PPN hydrogel is not detectably degraded by bacterial extracts (Figures 3.7a, b) or wound fluids (Figure 3.7d) over 7 days, or on wound (Figure 3.7c) over 2 days. Singly-attached (pendant) PIM-mal and NAC are released from the gel into the wound; while there is presumably also some network breakage (conversion of quadruply-attached PEG or doubly-attached PIM to singly-attached), it is insufficient to damage the hydrogel's overall structural integrity before the dressing is likely to be changed, or the wound fully healed.

Our PPN1 hydrogel is more bactericidal to biofilm bacteria than the commercial silver-based wound dressing Allevyn Ag, even to CR-PA and CR-AB which urgently need new antibacterial therapies [159]. Treatment with the PPN1 hydrogel left a clean wound and also improved healing quality by accelerating wound closure and suppressing inflammation. The results proved that PIM and NAC are required to remove biofilm and accelerate wound healing as treatment by the gel controls (without PIM and NAC) did not have significant killing of bacteria and the wounds healed slower. Many research on wound dressings only focus on one aspect of wound healing or on one type of wound (i.e. infected wound or diabetic wound) [181-184] but our hydrogel is dual-function.

In conclusion, we present a hydrogel which demonstrates effective biofilm removal and accelerates diabetic wound healing. This technology may alleviate the growing problem of diabetic wound healing as current treatments are limited by their contraindications. The fact that this hydrogel can crosslink simply by mixing in water without the need for any chemical initiator or external sources of energy such as UV or

heat makes it possible for *in situ* applications. The hydrogel can also be made via electrospinning or 3D printing to easily create patterns or shapes that fit the application. Finally, this hydrogel can also be used in other biomedical applications such as coatings for biomedical devices.

3.11. Materials and methods

3.11.1. Chemicals

1,4-diaminobutane, 37% formaldehyde solution, 40% glyoxal solution, glacial acetic acid, maleic anhydride, sodium bicarbonate and N-acetylcysteine (NAC) were purchased from Sigma-Aldrich and used as received. Poly(ethylene glycol) tetra thiol (PEG-4SH, $M_n = 20,000$) and poly(ethylene glycol) tetra maleimide (PEG-4mal, $M_n = 20,000$) were purchased from Biochempeg.

3.11.2. Synthesis of maleimide-terminated polyimidazolium (PIM-mal)

1,4-diaminobutane (2.974 g, 33.7 mmol) was dissolved in glacial acetic acid (75 mL) in a round-bottom flask and cooled in an ice bath. 37% formaldehyde solution (2.735 g, 33.7 mmol) and 40% glyoxal solution (4.895 g, 33.7 mmol) were first dissolved in DI water (37.5 mL), then added to the round-bottom flask in a dropwise manner. The solution was stirred at room temperature for 24 h, and the solvents were evaporated completely on a rotary evaporator. The produced polyimidazolium (PIM) was dissolved in DI water and then subjected to dialysis (MWCO 2000) in DI water for three days. The final product was obtained via lyophilization. PIM (2 g, 0.7 mmol) was then dissolved in glacial acetic acid (50 mL) in a round-bottom flask. Maleic anhydride (0.27 g, 2.8 mmol) and sodium bicarbonate (0.24 g, 2.8 mmol) were then added to the round-bottom flask and stirred to dissolve. The solution was stirred at 100 °C for 24 h, and the

solvent was then evaporated completely on a rotary evaporator. The produced PIM-mal was dissolved in DI water and then subjected to dialysis (MWCO 2000) in DI water for three days. The final product was obtained via lyophilization.

3.11.3. Minimum inhibitory concentration of PIM-mal

Bacteria (*E. faecium* 19434, MRSA BAA-40, MRSA USA300, *K. pneumoniae* 13883, CR-AB, PA01, CR-PA and *E. aerogenes* 13047) were inoculated and dispersed in Mueller-Hinton broth (MHB, 4 mL) at 37 °C with continuous shaking at 220 rpm to mid log phase. A two-fold serial dilution of PIM-mal was made (1024 µg/mL to 2 µg/mL in 50 µL MHB) on a 96-well plate. Then, bacterial suspension (50 µL) was added to all the wells that contained PIM-mal to a final concentration of 5×10^5 CFU/mL. The plate was then incubated in a shaker at 37 °C for 18 h and the optical densities of the wells were measured to determine MICs.

3.11.4. Formation of hydrogels

Hydrogels were formed by simply mixing the precursor solutions. First, two component solutions were prepared. One solution contained poly(ethylene glycol) tetra thiol (PEG-4SH, 10% w/v) and N-acetylcysteine (NAC, 2 mM) in DI water; the other solution contained poly(ethylene glycol) tetra maleimide (PEG-4mal, 10% w/v) and PIM-mal (0.2, 2 or 20 mg/mL) in DI water. These solutions were mixed at equal volume in a 1.5 mL microtube and 50 µL of the hydrogel solution was quickly transferred to each well of a 96-well plate (the final hydrogel solution contained 5% PEG-4SH, 5% PEG-4mal, 1 mM NAC and 0.1, 1 or 10 mg/mL PIM-mal). The solutions were then left on the bench for 5 min to gel. DI water was added to the wells to swell the hydrogels. The hydrogels were then washed in ethanol three times and in DI water three times with sonication to

remove all unreacted precursors. A gel control was made with just PEG-4SH (5% w/v) and PEG-4mal (5% w/v).

3.11.5. *In vitro* antimicrobial assay of hydrogels

(1) Preparation of bacterial suspensions

Bacteria (MRSA USA300, CR-AB, PA01 and CR-PA) were inoculated and dispersed in Mueller-Hinton broth (MHB, 4 mL) at 37 °C with continuous shaking at 220 rpm to mid log phase. MHB was removed by centrifugation, followed by decanting of the supernatant. Bacteria were washed with phosphate buffered saline (PBS) thrice and the final bacteria suspensions were prepared with PBS (1 mL, 1×10^9 CFU/mL).

(2) Inoculation of bacteria on hydrogels

Bacterial suspension in PBS (10 µL), containing approximately 1×10^7 CFU was inoculated and spread evenly onto the surface of hydrogels, which were placed on a small petri dish. A control was prepared by inoculating bacteria on a small petri dish with no hydrogel. The hydrogels were incubated at 37 °C for 1 h with 90% relative humidity.

(3) Bacterial counts

Hydrogels were immersed in PBS (1 mL) and vortexed to release bacteria. Then, a series of ten-fold dilutions of the bacterial suspensions were prepared in a 96-well plate and dilutions were plated onto Luria-Bertani (LB) agar. The plates were incubated at 37 °C for 16 h and bacterial colonies were counted.

Results were evaluated as follows (Equation 3.1):

$$\text{Log reduction} = \text{Log (total CFU of control)} - \text{Log (total CFU on hydrogels)} \quad (3.1)$$

3.11.6. *In vitro* biocompatibility assay of PIM-mal and hydrogels

Biocompatibility studies were carried out on human dermal fibroblasts (NHDF-Ad-Der Fibroblasts, CC2511, Lonza). Fully supplemented DMEM, consisting of foetal bovine serum (FBS, 10%) and antibiotics (penicillin-streptomycin, 1%) was used as cell culture medium.

(1) PIM-mal MTT assay

HDF cells were cultured in 96-well plates from an initial density of 1×10^4 cells in each well, and incubated in a CO₂ incubator at 37 °C for 24 h for cell attachment. Different concentrations of PIM-mal (0.1, 0.5, 1, 5 and 10 mg/mL) were prepared in DMEM and added to the wells and incubated at 37 °C for a further 24 h. Then, the culture media were replaced with MTT solution (1 mg/mL in DMEM) and incubated at 37 °C for 4 h to stain viable cells. MTT solution was discarded, dimethyl sulfoxide (DMSO) was added and mixed well. The cell viability was calculated based on the absorbance of each well at 570 nm against the cell-only control wells which served as the 100% cell viability control.

(2) Hydrogel extract MTT assay

HDF cells were cultured in 24-well plates from an initial density of 5×10^4 cells in each well, and incubated in a CO₂ incubator at 37 °C for 24 h for cell attachment. Concurrently, hydrogels were placed in each well of a 24-well plate with 1 mL of DMEM and incubated at 37 °C for 24 h to collect the extracts, before transferring the extracts to incubate with HDF cells at 37 °C for 24 h. Then, the culture media were replaced with MTT solution and subsequent steps are the same as above.

(3) Contact MTT assay

The procedures are same as the above, but instead of collecting hydrogel extracts the hydrogels were directly immersed in the cell cultures and removed before addition of MTT.

Results were evaluated as follows (Equation 3.2):

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of polymer/hydrogel treated cells}}{\text{Absorbance of control cells}} \times 100\% \quad (3.2)$$

3.11.7. Swelling kinetics of hydrogels

Hydrogels were washed thoroughly and lyophilized to dryness. The masses of fully dried hydrogels were weighed at time zero. Then, a copious amount of DI water was added to the hydrogels to induce swelling. At 5 min intervals until equilibrium swelling, hydrogels were removed from the water, dried with filter paper, and their masses measured. Swelling ratio was calculated using the formula (Equation 3.3):

$$\text{Swelling ratio} = \frac{\text{mass of hydrogel at } n^{\text{th}} \text{ min} - \text{initial mass of hydrogel}}{\text{initial mass of hydrogel}} \quad (3.3)$$

3.11.8. Hydrogel stability in bacterial extracts

Bacterial extracts were prepared by shaking different concentrations of bacteria in PBS at 37 °C for 24 h. Extracts were collected after centrifugation and removal of the bacteria. PPN1 hydrogels were placed in a 24-well plate and equilibrated by soaking in PBS for 24 h. Their initial masses were measured. Each hydrogel was then incubated with bacterial extract (1 mL) at 37 °C for 2 and 7 days and their masses were measured at the respective time points.

3.11.9. Hydrogel stability in wound fluids

Wound fluids of MRSA USA300 and CR-PA infected wounds were prepared by homogenizing tissue from one wound in 900 μ L of PBS, followed by 10x dilution in PBS (total volume = 10 mL). The wound fluid supernatants were collected after centrifugation and removal of the tissues. PPN1 hydrogels were placed in a 24-well plate and equilibrated by soaking in PBS for 24 h. Their initial masses were measured. Each hydrogel was then incubated with wound fluid (1 mL) at 37 °C for 2 and 7 days and their masses were measured at the respective time points.

3.11.10. Mouse *in vivo* diabetic wound infection model

All animal studies were approved and performed in compliance with the regulations of the Institutional Animal Care and Use Committee of Nanyang Technological University.

(1) Induction of diabetic mice using streptozotocin

Eight-week old male C57BL/6 mice were used. Streptozotocin (STZ) was prepared by dissolving the powder in 50 mM sodium citrate buffer to 4 mg/mL. Mice were fasted for 4 h before injecting STZ intraperitoneally at 40 mg/kg daily for five consecutive days. 10% sucrose water was provided during the injection days and changed to regular water on day 6. Blood glucose levels of the mice were measured three weeks after STZ injection and mice were deemed to be diabetic if their blood glucose level exceeded 11.1 mmol/L.

(2) Wound infection models and enumeration of bacterial load on wounded skin

Mice were anaesthetized, depilated and 6 mm diameter full-thickness excisional wounds were inflicted on the dorsal skin and the underlying panniculus carnosus as previously described [170]. Next, bacteria (MRSA USA300, CR-AB, PA01 and CR-PA, 1×10^6

CFU in 10 µL of PBS) were topically inoculated onto the wounds and left to settle for 10 min prior to securing with Tegaderm (3M) transparent dressing. The bacteria were left untreated on the wounds for 24 h to form biofilm. The wounds were then treated with PPcontrol, PPN1 hydrogel and Allevyn Ag. Untreated wounds served as controls. In the 24 h treatment study, dressings were removed after 24 h and the wounds, including 5 mm of the peripheral region, were excised. In the 2-weeks study, wounds were harvested on days 1, 3, 5, 7, 9, 12 and 14. Each wound was homogenized in PBS (900 µL) to release bacteria (n = 6). Then, a series of ten-fold dilutions of bacterial suspension was done in PBS and plated on LB agar. The plates were incubated at 37 °C for 16 h and bacterial colonies were counted. A two-tailed Student's t test was used for comparisons.

(3) Wound healing study

At day 0, mice were wounded and infected with bacteria (MRSA USA300). The wounding and infection procedures were the same as above. Untreated wounds were secured with Tegaderm and served as controls, while PPcontrol, PPN1 hydrogel and Allevyn Ag were applied to the treated wounds and were secured with Tegaderm. Photographs of the wounds were taken before dressing application and on days 1, 3, 5, 7, 9, 12 and 14, and at these points the dressings were replaced with fresh ones. The wound size at each time point was determined using ImageJ software (n = 6). A two-tailed Student's t test was used for comparisons. Wound sizes were calculated using the formula (Equation 3.4):

$$\text{Wound size (\%)} = \frac{\text{wound area on } n^{\text{th}} \text{ day}}{\text{wound area on day 0}} \times 100\% \quad (3.4)$$

(4) FACS analysis of inflammatory cells

Wound infection procedures were the same as above, using MRSA USA300 as the test pathogen. The wounds were then treated with PPN1 hydrogel and Allevyn Ag.

Untreated infected and uninfected wounds served as controls. Two days post treatment, the wounds including 5 mm of the peripheral region were excised. Single-cell suspensions from wound samples were obtained using gentleMACS Dissociator according to the manufacturer's protocol (Miltenyi Biotec). Cells were immuno-labelled with CD11b and Ly6G (Biolegends) and flow cytometry was carried out using an Accuri C6 flow cytometer (BD Biosciences). Data analysis was performed using Flowjo software version 7.6.5 (Tree Star). The mean percentage values ($n = 6$) were plotted for each treatment, $\pm$ SEM. A two-tailed Student's t test was used for comparisons.

(5) ELISA for wound healing factors

Wound infection procedures were the same as above, using MRSA USA300 as the test pathogen. The wounds were then treated with PPcontrol, PPN1 hydrogel and Allevyn Ag. Untreated infected wounds served as controls. Two days post treatment, the wounds including 5 mm of the peripheral region were excised. Each wound was homogenized in PBS (900 μ L) and centrifuged to remove tissues and bacteria. The supernatants were collected and tested with ELISA kits according to the manufacturer's protocol (pro-MMP9, VEGF-A, PDGF-BB, FGF-2 and EGF, Lonza). The mean concentration values ($n = 6$) were plotted for each treatment, $\pm$ SEM. A two-tailed Student's t test was used for comparisons.

Appendix

A1: PEI hydrogel wound healing study (full data)

Figure A1.1. Visual appearance of untreated control wounds between dressing changes over 2 weeks. Scale bar = 5 mm. Black arrows indicate secondary infection sites.

Figure A1.2. Visual appearance of PEI(1a) treated wounds between dressing changes over 2 weeks. Scale bar = 5 mm.

A2: Standard curve to determine fluorescent hydrogel polymer content

Rhodamine B labelled PEI polymers were measured for their fluorescence intensity at concentrations of 1, 0.8, 0.6, 0.4, 0.2, 0.1, 0.08, 0.06, 0.04 and 0.02 mg/mL with LS 55 fluorescence spectrometer (PerkinElmer) at excitation wavelength of 553 nm and emission wavelength of 576 nm. A standard curve was plotted based on the relationship of fluorescence intensity against concentration of polymer (Figure A2.1).

Figure A2.1. Standard curve of fluorescence intensity against concentration of PEI polymer.

A3: Hydrodynamic drag calculation

Reynolds number

The Reynolds number is $Re = \frac{V_F L}{\eta}$. Given that L is micron scale (10^{-4} cm) and V_F is of order of 10^{-4} cm/s or less and η , the dynamic viscosity, is 0.01 in the same unit system, the Reynolds number is of order 10^{-6} or less, the fluid flow is extremely laminar. The Stokes drag formula is thus highly appropriate.

Stokes Drag Formula

At low Reynolds number, the Stokes Drag Formula is appropriate:

Stoke's equation:

$$F_D = 6\pi\eta V_F R_P \quad (A1)$$

where η is the dynamic viscosity of the fluid, V_F the fluid speed with respect to the particle and R_P the spherical particle radius.

To determine the value of the fluid speed that is required to suspend a bacterium against gravity, we set the drag force equal to the differential gravitational force on the particle and its fluid environment (*i.e.*, the particle buoyant weight).

$$F_D = 6\pi\eta V_F R_P = F_G = g M_B = g \frac{4\pi R_P^3 \rho_B}{3} \quad (A2)$$

where g is the gravitational acceleration constant ($g \sim 980$ cm/s²), M_B is the buoyant mass of the particle and ρ_B is the buoyant density of the particle, *i.e.* the difference between the mass density of the particle and the mass density of the fluid environment. It is assumed that the particle is at least slightly denser than the fluid, otherwise it would float to the fluid surface due to buoyant forces and there would be no need for fluid drag to lift the particle against gravity.

This equation may be solved for the fluid speed in terms of the other parameters of the problem:

$$V_F = \frac{2}{9} \frac{g R_P^2 \rho_B}{\eta} \quad (\text{A3})$$

The dynamic viscosity of water at room temperature is ~ 1 cP (centi-Poise, $1 \text{ cP} = 0.01 \text{ g/cm/s}$) so this gives a required fluid flow of

$$V_F = 1.1 \times 10^{-5} \text{ cm/s} \left(\frac{R_P}{0.5 \mu\text{m}} \right)^2 \left(\frac{\rho_B}{0.2 \text{ g/cm}^3} \right) \quad (\text{A4})$$

The diameter of MSRA from the SEM images is slightly less than $1 \mu\text{m}$ and the buoyant density of bacteria is around 0.1 g cm^{-3} [185], hence the fluid flow speed required to lift the bacteria against gravity (calculated from Equation A4) is 50 nm/s , which is about $200 \mu\text{m/h}$. Since water evaporates at the rate of $0.722 \text{ mg/min.cm}^2$ at 25°C when uncovered [164], it can be estimated that the evaporation rate from our hydrogel (volume is 50 uL , radius is 0.32 cm) is $500 \mu\text{m/h}$, which is higher than the flow required to lift bacteria against gravity.

A4: PPN hydrogel wound healing study (full data)

Figure A4.1. Visual appearance of untreated control wounds between dressing changes over 2 weeks. Scale bar = 5 mm.

Figure A4.2. Visual appearance of Allevyn treated wounds between dressing changes over 2 weeks. Scale bar = 5 mm.

Figure A4.3. Visual appearance of PPcontrol treated wounds between dressing changes over 2 weeks. Scale bar = 5 mm.

Figure A4.4. Visual appearance of PPN1 treated wounds between dressing changes over 2 weeks. Scale bar = 5 mm.

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Miscellaneous

Publications

Chun Kiat Yeo, Surendra H. Mahadevegowda, David Leavesley, Nguan Soon Tan, and Mary B. Chan-Park, “Biofunctional Hydrogel Reduces Bioburden and Oxidative Stress to Accelerate Diabetic Wound Healing”, pending submission.

Chun Kiat Yeo and Mary B. Chan-Park, “Wound Healing: Biomaterials and Biologics to the Rescue”, pending submission.

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Patents

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Conference Presentations

3rd Bioengineering & Translational Medicine Conference

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Oral Presentation: Hydrogel Effects Rapid Biofilm Debridement with ex situ Contact-Kill to Eliminate Multidrug Resistant Bacteria in vivo

The 5th International Conference on Cellular and Molecular Bioengineering (ICCMB5)

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Poster Presentation: Inherently Antibacterial Hydrogel for Wound Care

Student Helper