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ENZYMES OF THE IMMUNE SYSTEM FUNCTION AS BIOMARKERS FOR INFECTION DETECTION IN CHRONIC WOUNDS

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Preamble

This cumulative thesis is divided into 9 chapters. The general introduction focuses on the infection triggered events in the human body undergoing an immunological reaction. It is furthermore explained how chronic wounds arise and how an infection a chronic wound can be diagnosed. As this thesis is dealing with enzymes of the polymorphonuclear neutrophils, a sub-chapter is dedicated to their mechanisms and some particular enzymes that are stored within the cells. Chapter two highlights the motivation of the work and is followed by an extensive review comparing different possible biomarkers for infection detection and new innovative methods. Chapter four deals with the infection detection of chronic wounds based on three enzymes that are derived from the neutrophils and chapter five presents an innovative electrochemical sensor for the detection of one of these enzymes. In chapter six the focus is set on enzyme responsive biomaterials, reviewing popular enzyme responsive polymers and their application in microbe infection detection. Chapters seven eight and nine exemplify possible applications using biopolymers or polymers in terms of infection detection in wounds. These chapters are followed by the conclusion and the list of tables, figures and abbreviations. The end of the thesis includes a list of publications and a CV.

Abstract

Timely accurate detection of wound infection in chronic wounds is, and has always been challenging for health care employees. An overseen infection has a huge impact on the patient, including prolonged hospitalization and pain. The focus of this Ph.D. thesis was set on the development of new diagnostic methods, reviewing the state of the art applications in wound diagnostic, searching for new biomarkers and developing promising devices for wound infection detection. A chronic wound is usually judged by its external signs of infection, or by microbiological investigations. No time saving and accurate devices that are easy to handle are available on the market. In this thesis the focus is therefore set on enzymes that are derived from the neutrophils, as they are the first cells present in an infection. The wound fluid harbours all the information of the current wound status, therefore it was our aim to develop new methods and assays to display an infected wound fluid. Preliminary work was done during the European project LIDWINE, selecting the most promising enzymes displaying an infection. Here we extended this work finding new approaches for lysozyme, myeloperoxidase, human neutrophil elastase and matrix metalloproteinases. As a first step, the applicability of these enzymes was verified in a study comparing the outcomes of the biochemical experiments to microbiological investigations. The results of the enzyme activity measurements were in accordance to the output of the silver standard methods. Based on these results, an electrochemical sensor was developed, displaying high levels of myeloperoxidase upon hydrogen peroxide consumption and subsequent current loss. The second focus of this work was set on the use of natural or synthetic polymers as enzyme responsive matrices. Peptidoglycan, a substrate for lysozyme, was therefore labelled with a blue dye and implemented in a precursor lateral flow device. Upon incubation with infected wound fluids, the peptidoglycan was hydrolysed, and only oligomers were able to pass a size exclusion membrane. Those blue dyed fragments accumulated in a subsequent second layer indicating an infection in wound fluids. Gelatine based beads with incorporated dyes were used as enzyme responsive tools for displaying high levels of gelatinolytic enzymes in the wound fluid. A colour release was observed and enabled the distinction between infected and non-infected wound fluids upon colour development. A new substrate for myeloperoxidase was synthesized, suitable for immobilization on various surfaces, like silica plates. Incubation with infected wound fluid led to a blue colour formation directly on the surface. We invested several different new strategies for wound infection determination with the focus on fast and easy reachable results avoiding complicated handling steps or machineries. These new methods could be implemented in bandage materials or be further developed in hand-held devices for infection detection.

Keywords: Chronic wounds, Infection detection, lysozyme, elastase, myeloperoxidase, MMP

Kurzfassung

Die zeitnahe Erkennung von Wundinfektionen in chronischen Wunden ist nach wie vor ein herausforderndes Thema. Längere Krankenhausaufhalte und schmerzhafte Behandlungen der Wunden sind nur einige Auswirkungen die der Patient zu tragen hat. Das Ziel dieser Doktorarbeit war es daher, neue Methoden der vorzeitigen Infektionsdetektion zu erforschen.
In der klinischen Prxis beläuft sich die Beurteilung chronischer Wunden meist über die Begutachtung der äußeren Faktoren wie Schwellung, Rötung, vermehrte Schmerzempfindlichkeit und erhöhte Wärmeentwicklung. Die mikrobiologische Untersuchung gilt als Silberstandard, kann aber auch nicht immer ausreichende Informationen liefern. Nach ausreichender Recherche konnte auch festgestellt werden, daß sich derzeit kein Produkt auf dem Markt befindet, welches sich eignet schnelle und auch sichere Auskünfte über den Wundstatus zu liefern. Diese Arbeit beschäftigt sich mit den Enzymen der neutrophilen Granulozyten, da es erwiesen ist, daß diese sehr früh und in hoher Zahl in der infizierten Wunde anzutreffen sind. Der Nachweis der Enzyme, die von den neutrophilen Zellen direkt in die Wundflüssigkeit abgegeben werden, liefert einen genauen Status der Wunde. Die Idee dazu wurde in dem europäschen Projekt LIDWINE geboren, welches es sich zur Aufgabe machte Strategien zur besseren Wundinfektions-Detektion zu entwickeln. Darauf aufbauend, wurden in dieser Arbeit neue Strategien für die Detektion von Lysozym, Myeloperoxidase, neutrophiler Elastase und Matrixmetalloproteinasen erarbeitet.
Eine wissenschaftliche Studie konnte belegen, dass sich die Resultate der biochemischen Studien der Enzymaktivitäten in den Wundsekreten mit den Ergebnissen des momentanen Silberstandards decken. Als Alternative zu herkömmlichen Enzymassays wurde ein elektrochemischer Sensor zum Nachweis von Myeloperoxidase in infizierten Wundflüssigkeiten entwickelt. Der Sensorstrom wurde hierfür mit einer gerichteten Konzentration von Wasserstoffperoxid eingestellt. Wird dieses durch Myeloperoxidase verbraucht, sinkt auch der Strom proportional zum Verbrauch ab, eine Infektion kann detektiert werden. Ein weiterer Fokus dieser Arbeit lag auf der Entwicklung von Oberflächen oder Partikeln aus Polymeren, die auf vermehrtes Enzymaufkommen ansprechen. Für das Enzym Lysozym wurde eine Vorstufe eines „lateral flow devices“ gefertigt. Peptidoglykan wurde blau gefärbt und mit infiziertem Wundfluid inkubiert. Diese Lösung wurde mit einer Größenausschluß Membran in Verbindung gebracht - nur bereits hydrolysierte Fragmente konnten von dort aus in eine zweite Membran wandern und sich gut sichtbar akkumulieren. Gefärbte Kugeln aus Gelatine zeigten signifkante Unterschiede in der Ausschüttung der inkoporierten Farbe wenn sie mit infiziertem Wundfluid inkubiert wurden. Auch hier war eine Unterscheidung von infizierten und nicht infizierten Wundsekrets aufgrund eines erhöhten

Aufkommens von Matrixmetalloproteiniasen nachweisbar. Ein neues Substrat für Myeloperoxidase wurde auf verschiedenen Oberflächen wie Silika Platten immobilisiert. Das Enzym konnte das immobilisierte Substtrat binnen weniger Minuten umsetzten, was zu einer Blaufärbung der ganzen Oberfläche führte. Verschiedenste Ansätze zur Detektion von Wundinfektion wurden während dieser Arbeit erforscht. Ziel wird in Zukunft sein, diese leicht zu handhabenden Methoden zukünftig in schon bestehende Materialien, wie Verbände einzuarbeiten, oder eigenständige diagnostische Systeme zu entwickeln, die den klinischen Alltag erleichtern.

Schlüsselwörter: Chronische Wunden, Detektion von Wundinfektion, Lysozym, Matrixmetalloproteinase, Elastase, Myeloperoxidase

Content

1 Introduction

1.1 The innate immune defence and the immune system

The human body has to strike against numerous invaders and injuries every day. During the course of evolution, two cooperating defence mechanisms were developed to fight against the endangerment of the body. The *innate immune defence* is not specific; it cannot distinguish between different infectious particles, whereas the *immune system* is developed to strike against specific pathogens and is always adapted throughout our lifetime [1].
The innate immune defence can be separated in a first and a second line of defence. A penetrating debris or pathogen has to overcome the first barrier of the body- the skin.

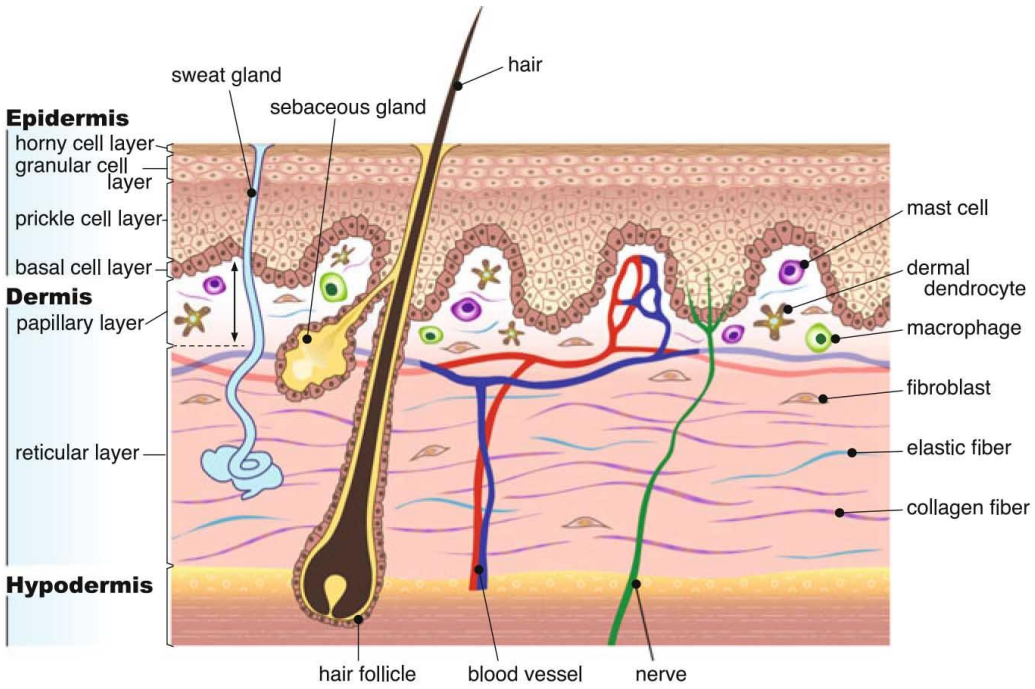

Figure 1 The adult human skin is a layered organ consisting of an epidermis and a dermis. The epidermis is composed of four distinct layers: horny, granular, prickle, and basal cell layers (from top to bottom). The dermis is a highly elastic, tough, and flexible tissue made up of a meshwork of collagenous, reticular, and elastic fibres. It is divided into two functional layers, the papillary dermis and reticular dermis. Fibroblasts, the main cells in the dermis, are essentially located in the papillary layer and are found only in very small numbers in the reticular dermis. They play an important part in production of the extracellular matrix. The skin also contains hair follicles, glands and nerve endings responsible for the sense of touch and pain (Permission obtained by Springer) [2].

The skin has a common pH range from 3 to 5, distracting many microorganisms from colonization. It is composed of two layers, the epidermis and dermis. In addition to these two

layers, the hypodermis, which is sometimes considered a layer of the skin, lies beneath the dermis and is composed mainly of adipose tissue [3]. The epidermis, which is a terminally differentiated and stratified squamous epithelium, is the most superficial layer of the skin and is classified into several layers, including the basal layer at the bottom [4]. The dermis consists of two layers, the papillary and reticular layer. The papillary layer includes the cellular component as the fibroblasts, mast cells, macrophages and dermal dendrocytes as well as the extracellular matrix (ECM) components, collagen and elastic fibres and matrix components as glycoproteins and proteoglycans. The ECM of the thicker reticular layer contains a network of collagen and elastic fibres, blood vessels and nerves are embedded in these layers [2].

Saliva, tears and mucus contain bactericide proteins that support the “cleaning” of the epithelial layer. Once a foreign particle enters this barrier, the innate immune defence is activated. It is composed of mono- and granulocytes, macrophages and natural killer cells, the complement system, acute phase proteins, cytokines and interferons [5].

A specific and adaptive immune response of the immune system is developed after an infection. It comprises the antibody producing B-cells, as the humoral immune system, the T-helper cells as well as the cytotoxic T-lymphocytes, both together representing the cellular immune defence. Selected pathogens (or subtypes) can be recognised and eliminated. A part of the stimulated lymphocytes is converted to memory cells. These long lived cells represent the immunologic memory and explain why exposure to a particular pathogen or vaccination against a pathogen, results in protection in the form of antibodies. Cytokines and interferons play a major role for the communication of the innate immune defence and the specific immune response. A stimulus for the immune system is an antigen, represented by the pathogen itself, or e.g. a foreign protein or saccharide component [6].

1.2 Wound healing

In case of an injury, the normal healing process begins in the moment the tissue is broken. As a normal tissue response, four distinct overlapping phases in the wound healing are known, haemostasis, inflammation, proliferation and remodelling [7]. This process usually takes 3 to 14 days to complete [8,9].

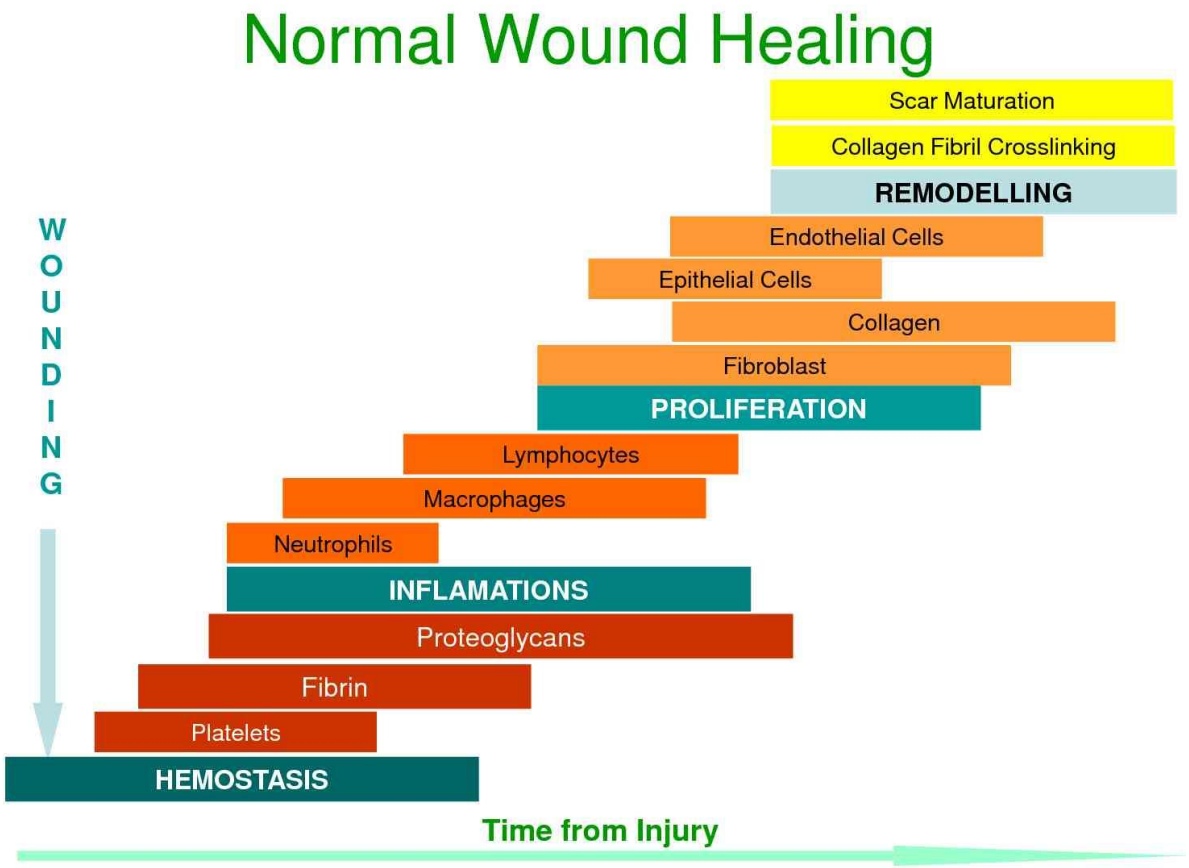

Figure 2: The sequence of events during normal wound healing and the participant factors (Permission obtained by Frontiers in Bioscience) [10].

Due to the injured tissue, disrupted blood vessels and lymphatics expose platelets to fibrin and collagen. The activated platelets start to adhere to the collagen and to each other and furthermore release clotting factors [10]. They also provide a cascade of chemical signs known as cytokines or growth factors. The platelet derived growth factor (PDGF) and the transforming growth factor beta (TGF- β), are the two most important ones in this cascade. PDGF initiates the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts [11]. Neutrophils arrive in the first 24 hours and have the function to prepare the wound bed by killing bacteria and removing devitalized tissue as well as the recruiting of the fibroblasts [7] . In the early phase of inflammation neutrophils predominate, they kill bacteria

and remove foreign material from the wound by releasing enzymes and by phagocytosis. In a process called “respiratory burst” free radicals are released, triggering the killing of the bacteria. Later in the inflammatory phase neutrophils reduce in number, undergo apoptosis and are engulfed and replaced by macrophages [12,13]. Monocytes, recruited from the blood become activated and differentiate into macrophages after migration to the wound site. Besides preparing the wound bed, they play a role in the healing process by the synthesis of numerous growth factors including platelet derived growth factor, tumour necrosis factor , transforming growth factor and insulin growth factor 1 [14]. The inflammation of the wound bed brings a change in the micro-environment of the wound and sets the stage for proliferation. The proliferation phase overlaps with the inflammatory phase and starts on the second or third day of injury, fibroblasts appear in the wound. They play the key role in the production of the building blocks of the extracellular matrix. Influenced by growth factors they proliferate and synthesise proteoglycans, elastins, fibronectin and collagen. Production of extracellular matrix is seen clinically as formation of granulation tissue [15]. While new matrix is synthesised, existing matrix in and around the wound margin is degraded by several enzyme systems such as matrix metalloproteinases and plasminogen activators. The effect of these enzymes is regulated by tissue inhibitors, which are important in healing by preventing excessive matrix degradation [16]. Endothelial cells are essential to scar formation. They detach from the basement membrane to migrate across the wound surface, stimulated by the vascular endothelial growth factor (VEGF). They produce degradation agents to destroy the fibrin clot once the new granulation tissue is laid down [17]. In the epithelisation stage, undamaged epithelial cells migrate from the wound margins. This process continues until the migrating cells from opposing sides of the wound touch each other. Once closure of the wound has been achieved, remodelling of the resulting scar takes places over months or years, with a reduction of both cell content and blood flow in the scar tissue [18].

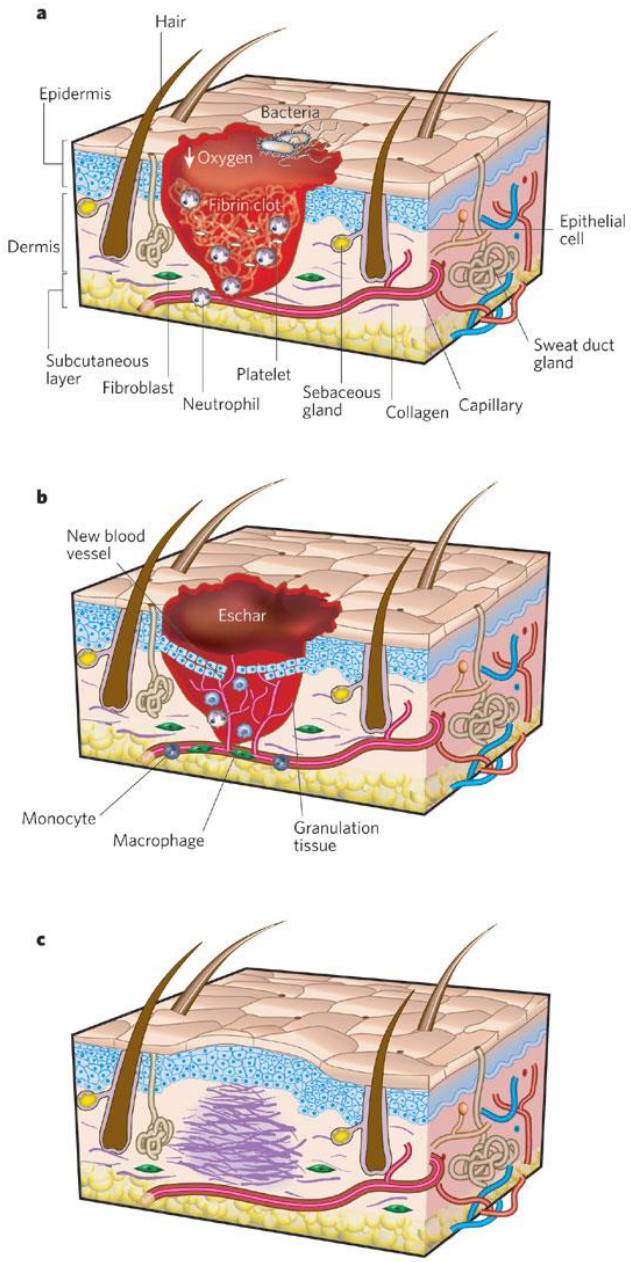

Figure 3: The classic stages of wound repair: haemostasis, inflammation (a), new tissue formation (b) and remodelling (c). The inflammation state lasts until about 48 h after injury. The wound is characterized by a hypoxic (ischaemic) environment in which a fibrin clot has formed. Bacteria, neutrophils and platelets are abundant in the wound. Normal skin appendages (such as hair follicles and sweat duct glands) are still present in the skin outside the wound. This stage of new tissue formation occurs about 2–10 days after injury. An eschar (scab) has formed on the surface of the wound. Most cells from the previous stage of repair have migrated from the wound, and new blood vessels now populate the area. The migration of epithelial cells can be observed under the eschar. During remodelling, disorganized collagen has been laid down by fibroblasts that have migrated into the wound. The wound has contracted near its surface and the re-epithelialized wound is slightly higher than the surrounding surface (Permission obtained by Nature Publishing Group) [19].

1.3 Wound characteristics, the chronic wound

Wounds can be classified in many different ways, but all classifications reflect somehow the required treatment and the expected time and prospects of healing. Differentiations are made considering the type of the injury (pressure, trauma, venous leg ulcer, and diabetic foot ulcer), the extent of tissue loss, presence of an infection, foreign bodies or exposure of vital parts. A general distinction considering the tissue loss allows the division of “wounds with no tissue loss” and “wounds with tissue loss”. The tissue loss can be caused by burning, trauma or abrasion, or be the result of chronic ailments like venous statis, diabetic ulcers or pressure sores. Tissue loss can also be included in the treatment of the wound as skin grafting or derma- abrasion. The extent of the wound can be described in terms of the involved layers. Superficial wounds involve only the epidermis; partial thickness wounds also involve the dermis. In a full thickness wound the subcutaneous fat and the deeper tissue are affected, too [20].

Wounds can also be denoted as acute or chronic. An acute wound is defined as a recent wound undergoing all stages of wound healing [21]. It is a result of an incision or a trauma and heals in a timely and orderly manner as described in the previous chapter. Chronic wounds fail to heal in an anticipated time frame and orderly fashion. In general, all wounds have the potential to become chronic wounds [22]. The factors that can be involved in the non-healing of a wound can be classified as intrinsic and extrinsic. The intrinsic factors, also called local factors include ischemia, infection, presence of necrotic tissue, and foreign bodies in the wound. Extrinsic or external factors are diabetes mellitus, cancer, chronic disease, steroid use, radiation injury and malnutrition that further contribute to poor wound healing [23]. Aging plays a crucial part in the non-healing of the wounds. Reduced skin elasticity and collagen replacement influences healing as well as the nutritional status and vitamin deficiency [24,25]. Common chronic wounds are venous ulcers, arterial ulcers, diabetic foot ulcers, pressure ulcers, vasculitis and pyderma gangrenosum [17,26]. In addition to the intrinsic and extrinsic factors, systemic factors play a crucial role in impaired wound healing. The events of early wound healing reflect a fine- balanced environment in which proteolytic activity and matrix synthesis occur under tight regulations; this balance is lost in chronic wounds [27]. A reduction in tissue growth factors, an imbalance between proteolytic enzymes and their inhibitors and an excessive infiltration of neutrophils is often seen in chronic wounds, the wound is locked into a state of chronic inflammation with associated reactive oxygen species and destructive enzymes [28]. Reduced levels of active growth factors in the wound environment may partly explain why certain wounds fail to heal. Chronic ulcers are known to have reduced levels of platelet derived growth factor, basic fibroblast growth factor, epidermal growth factor, and transforming growth factor compared to

acute wounds [29]. Growth factors may become trapped by extracellular matrix molecules [30] or may be degraded by proteases to an excessive degree, resulting in non-healing [31]. Imbalance between proteinases and their inhibitors, excessive proteinase activity in chronic wounds, probably from overexpression of matrix metalloproteinases, results in abnormal degradation of the extracellular matrix. [32]. In addition, the neutrophils release an enzyme called elastase that is capable of destroying important healing factors such as PDGF and TGF-ß [33]. Dermal fibroblasts have an age related decrease in proliferation potential, called senescence [34]. Fibroblasts in chronic wounds have impaired responsiveness to growth hormone, which may be due to an increased number of senescent cells [29].

1.4 Wound infection and infection detection

Infection is a common local cause for delayed wound healing, broken skin facilitates the colonisation of the wound. Bacteria prolong the inflammatory phase of wound healing and elaborate toxins and proteases, compete for oxygen and nutrients and further damage the cells [26]. Wound infection is a clinical diagnosis, the progression from colonization to infection is not directly correlated with microbiological density of bacteria within a wound [35]. Bacterial density and the virulence of the invading organism are clearly important factors, as well as the host's immune response [36]. Adverse effects on the patient can be direct, due to the bacterial pathogenicity and indirectly mediated by the immune response to eradicate the microorganisms. Direct measurements of the bacterial burden include the production of bacterial toxins, for example, endotoxins resulting in the elevation of pro-inflammatory cytokines, for example, interleukin-1β and tumour necrosis factor–α [37]. Exotoxins produced by common wound bacteria tend to have quite broad substrate specificity, attacking many types of cells with subsequent tissue necrosis [26]. This necrosis may be exacerbated by local hypoxemia from vessel occlusion. Wound infection results in many changes in the inflammatory process, including increased consumption of complement proteins resulting in decreased chemotaxis. Tissue damage is increased by the production of cytotoxic enzymes and free-oxygen radicals. Local thrombosis depletes platelets and results in localized tissue hypoxia. Increased levels of interleukins and TNF may contribute to the imbalance between levels of matrix metalloproteinases and their inhibitors. In infection, bacterial metabolites and proteases may not only inhibit re-epithelisation but also degrade dermal proteins and polysaccharides [38].

Undiagnosed and untreated soft tissue infection in chronic wounds increases the likelihood of complications such as a sepsis and extends the human suffering [39–41]. The identification of an infection in a chronic wound is problematic. The traditional signs of infection are redness (rubor), warmth (calor), swelling or induration (tumour), tenderness and pain (dolour) and purulent secretions [42]. These diagnostic features, however, may be partially masked in patients in whom the inflammatory processes and immune system are dysfunctional, for example, in cases of diabetes, ischemia, and steroid therapy [43]. New guidelines were developed by The International Working Group of Diabetic Foot (IWGDF) and the Infectious Diseases Society of America (IDSA) that describe both, the presence and the severity of an infection [44]. Despite the guidelines, clinical signs are still seen as controversial as criteria for diagnosing infection [45]. Assessment of microorganisms in wounds is the silver standard in infection detection, but the presence of microorganisms per se does not in itself define a clinical infection. All wounds are exposed; therefore their micro flora will represent the

surrounding environment [46]. Generally, the presence of microbes in wounds can be divided into 4 different stages. In a contaminated wound non- replicating microorganisms are present, without any host reactions in contrast to the colonisation state, where replicating microorganisms can be found. The release of toxins, in the critical colonisation state causes a delay in wound healing without manifest signs of inflammation. The wound can be diagnosed as infected when an host reaction is associated to the presence of replicating microorganisms [47]. As a guideline, it is proposed that the excess of the critical number of bacteria ($>1x10^5$ per gram of tissue) points out an infection. However, not only the relative number of microorganisms present in wounds, but also their pathogenicity and invasiveness contribute whether a wound becomes infected or not [48]. Biopsy is considered as the gold standard microbiological method so far, but it is hardly carried out in clinical practice due to concerns over harm to the patient [49,50]. Since the commonly used methods are either imprecise, time consuming or hardly feasible, it might be of special interest to gain insights into the local wound microenvironment by assessing biochemical markers in wound fluid, which might give a clue about the local processes reflecting the current status of wound healing [51].

1.5 Inside the wound fluid

Wound fluid, also known as “wound exudate”, or “wound drainage” is produced in response to a complicated interaction between wound aetiology, wound healing physiology, wound environment and compounding pathological processes. Wound fluid per se has not to be considered as “bad” as it assists the healing process by preventing the wound bed from drying out, aiding the migration of tissue repairing cells, providing essential nutrients for cell metabolism enabling the diffusion of immune and growth factors and assisting separation of dead or damaged tissue [52].

Depending on the wound and the used bandage materials, several wound fluid sampling techniques exist. One very common method is the usage of occlusive dressing materials and the harvesting of the wound fluid by aspiration using a micropipette, syringes or needles [53]. Another technique is the extraction of the wound fluid of diverse dressing materials like gauze, foams or cotton mesh [54–56]. The material can therefore be squeezed through a syringe or be centrifuged to get rid of the debris [56]. The use of microdialysis relies on the principle of passive diffusion across a concentration gradient and is used for monitoring small molecules as glucose and lactose in diabetic foot ulcers but is not very commonly used for wound fluid sampling [57]. A quite convenient method is the swabbing of the wound bed with polyester tipped applicators or micro-flocked nylon swabs. The micro-flocked nylon swabs work through capillary action without fluid entrapment enable a fast wound fluid sampling even in wounds with little exudate preserving the original fluid composition for further analysis [58,59]. The wound fluid provides an insight in the local microenvironment of the wound, as it contains a dynamic combination of factors that reflect the current status of the wound. Markers of the immune defence like neutrophils and their released enzymes could act as biomarkers for infection detection as their number rises dramatically in case of an infection in the wound bed.

1.6 Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes, are the main phagocytes circulating in blood on their way to connective tissues. They develop from the pluripotent stem cells in the bone narrow and represent 35-75% of the population of circulating leukocytes [60]. They comprise a multilobed nucleus and so called primary, secondary and tertiary granules [61]. As the granules do not stain with either the basic or acidic dyes used or blood smear, they are called “neutrophils” [62].

In the normal wound healing process, neutrophils are known to be the first cells to arrive at the site of tissue injury, moving out of the blood vessels, following a gradient of inflammatory stimuli [63]. Even if their half-life is only in the order of a few hours, they can reach a concentration of 1,5-5 x 10^9 cells/ litre [64]. They are the leading parts in the sterilization of microbes, the production of molecular signs to limit the amassing of additional neutrophils and in the initiating of an anti-inflammatory, tissue restorative process involving macrophages. Neutrophils engulf invading microorganisms into a phagosome, which fuses with intracellular granules to form a phagolysosome. After the phagocytosis there are two mechanisms known for microbe killing, one is oxygen dependent and one is oxygen independent. The oxygen dependent mechanism is also called “respiratory burst” as there is a 50-100 fold increase in oxygen consumption. Membrane bound NADPH oxidase produces a reactive oxygen species (ROS) known as superoxide which is converted to hydrogen peroxide. The myeloperoxidase, stored in the azurophilic granules uses the hydrogen peroxide and available halide ions like Cl- to produce hypochlorite (HOCl), whose action in the presence of hydrogen peroxide degrades the bacterial membranes [65,66].

The oxygen independent mechanism involves the degranulation of the other granules into the phagolysosome, where the combination of enzymes, antimicrobial peptides, and proteins act to physically degrade the enclosed bacteria or cell debris [67]. Azurophilic granules (or primary granules) are known to be the first to be synthesized but they are the last to undergo exocytosis. They contain the highest concentrations of antimicrobial substances and are the only peroxidase positive neutrophil granules and additionally contain lysozyme, defensins and serine proteases [68]. The specific or secondary granules contain antimicrobial molecules, lactoferrin and MMP-8 [69]. Like the secondary granules, the tertiary granules also contain MMP-8 but especially high levels of MMPs with gelatinase activity can be found.

In chronic wounds, signals from the inflammatory microenvironment may be aberrant, and the neutrophil invasion continues, causing harmful effects to the tissue as the activity of many enzymes and chemicals used by neutrophils to kill microbes is not specific to pathogens, so they can damage host tissues when released extracellularly [61]. Different

enzymes of the neutrophils will be elevated in an infection reaction and could therefore be suitable as infection markers.

1.6.1 Myeloperoxidase

The myeloperoxidase (MPO) was purified for the first time in the early 1940s, although a presence of a peroxidase in the azurophilic granules of the neutrophils was suggested since the early 1900s [70]. It is the most abundant protein in the neutrophils, representing 5% of the dry weight of the cells and was formally known as verdoperoxidase as it gives pus its green colour [71]. In much lower concentrations, MPO is also localized in monocytes and tissue macrophages. Macrophages do not have the ability to synthesise MPO, they receive the enzyme by the uptake of whole neutrophils or MPO alone [72]. The enzyme is synthesised during myeloid differentiation in the bone narrow (as the product of a single gene) as a precursor and finalized prior to PMN entering the circulation [73,74]. The end product is a 150- kDa haeme-containing homo-dimer, which each monomer consisting of a 467amino acid heavy (α) and a 112 amino acid light (β) subunit. The two heavy subunits are linked by a disulphide bond and contain covalently bound haeme [75]. The incorporation of the haeme (carrying Fe3+) is a part of the maturation process of the enzyme and implies the enzymatic activity of the precursor [76]. MPO belongs to the mammalian family of peroxidases with a reaction optimum at pH 5.5, but the activity remains over a wide range of pH. It is a strongly basic protein with an isoelectric point >10, thus binding avidly on negatively charged surfaces, coating engulfed microorganisms or biological membranes when released outside of the cell [70]. Myeloperoxidase can perform peroxidase reactions as well as the unique chlorination reaction. As the primary function of PMNs is the destruction of microorganisms, it was early taken into account that MPO contributes to the killing, but the mechanisms remained unclear until 1967, because the availability of hydrogen peroxide (H_2O_2), alone had no toxic effect. H_2O_2 is a product of the respiratory burst reaction where NADPH oxidase forms a superoxide anion, a reactive oxygen species (ROS), which then is converted to H_2O_2 [77,78]. Klebanoff reported in 1967 that MPO, H_2O_2, and chloride, bromide, or thiocyanate form a powerful antimicrobial system in neutrophils [79]. Chloride is abundant in physiological fluids (100-140 mM) and therefore thought to be the main substrate for MPO. MPO catalyses a 2-electron oxidation of chloride by H_2O_2, which results in formation of hypochlorous acid (HOCl), a powerful chlorinating oxidant that is critically important for the microbicidal properties of the neutrophils. Essentially any oxidizable group on the organism can be oxidized, consequentially leading to a loss of membrane transport, suppression of DNA synthesis or an interruption of the membrane electron transfer chain [80]. Besides the primary physiologic function, it is proposed that it plays a protective role in

infectious diseases by detoxification of several microbial toxins [81]. Despite the positive effects of the enzyme, there is evidence that the MPO has the potential to damage normal tissue if it is unnaturally released in the extracellular fluid [82]. It is associated with the induction of lung injury and other inflammatory diseases with a special recent interest in the involvement of MPO in the development of atherosclerosis [82]. Detection methods are therefore further developed, and new techniques appear every year [83]. MPO was also presented as valuable marker of infection in wound fluids that is taken as basis for new devices in wound infection determination [84].

1.6.2 Lysozyme

Lysozyme, also known as muramidase or peptidoglycan N- acetylmuramoyl hydrolase, is an enzyme capable of hydrolysing glycosidic bonds between the N- acetylmuramic acid and N-acetylglucosamine residues of the bacterial cell wall peptidoglycan of gram positive bacteria, while gram-negative species are resistant to enzymatic degradation by this enzyme [85,86]. The enzyme consist of a single chain of 129 amino acid residues with four disulphide bonds and has a pH optimum between 6 and 9 [87]. It is abundant in a number of mammalian secretory products like tears, salvia, human milk and mucus, as well as in tissues, and is well-known for its antibacterial properties but also exhibits antiviral, antitumor, and immune modulatory activities. It has been detected in hematopoietic cells, as well as in granulocytes, monocytes and macrophages where lysozyme is synthetized continuously, in various exocrine glands, cartilage and the kidney. In pathological conditions, such as inflammation and neoplasia, most lysozyme positive cells at the site of disease are either granulocytes or members of the mono-nuclear phagocytic system [85,86] . The assays for the determination of lysozyme activity are based on the hydrolysis of the *Micrococcus lysodeikticus* cell wall [88]. Besides the commonly used turbidimetric assay, *Micrococcus luteus* cells modified with Remazol brilliant blue R (RBB) were described by Ito et al. and Hardt *et al.* as a method more sensitive for the determination of the concentration of lysozyme in serum [89,90]. The measurement of lysozyme concentrations in serum or urine is a useful task for the diagnosis or screening of diseases and high lysozyme concentrations could furthermore act as a biomarker of active chronic inflammation [91]. Moreover, investigations of wound fluids from infected chronic wounds and non-infected chronic wounds showed significant elevated enzyme activity levels in terms of infection [92].

1.6.3 Proteinases

Proteinases are in general enzymes that cleave peptide bonds in the central regions of polypeptides. Depending on their biochemical mechanisms responsible for their catalytic activities, they can be divided in serine, metallo, cysteine and aspartic proteinases [93,94] [95].

1.6.3.1 Neutrophil matrix metalloproteinases

Matrix metalloproteinases (MMPs) are also termed “neutral proteinases” due to their optimal activity at neutral pH. For their full activity they are dependent on intrinsic Zn+ ions and extrinsic Ca+ [31]. There are 18 members known, 8 of them expressed by leucocytes. They can be subdivided in five groups depending on their substrate specificity. Concerning this work, the focus is set on the MMPs derived from the polymorphonuclear leucocytes (PMN). MMP-8, the neutrophil collagenase, belongs to the interstitial collagenases, whereas MMP-9 is known as gelatinase, or type IV collagenase. Collectively, they can degrade all components of the extracellular matrix (ECM). Substrates for MMP-8 are the collagens I-III, VII, X, gelatine and proteoglycans, meaning that it is the only mammalian enzyme capable of initiating the degradation of the triple helix of native fibrillar collagens [96]. MMP-9 is able to degrade the collagens IV, V, VII, X XI, as well as elastin and fibronectin [97]. These MMPs are stored in the PMN, MMP-8 mainly in the specific or secondary granules, MMP-9 within the tertiary granules [94]. The regulation of MMPs occurs ether at the level of transcription, the activation of proMMPs or the inhibition of active MMPs by tissue inhibitors of matrix metallo proteases (TIMPs) [98]. Normally, MMPs are rather synthesized on demand than stored, except MMP-8 and MMP-9 as they are rapidly released from PMNs following cellular activation [99]. The activation of proMMPs is performed by proteinases (plasmin, stromelysin, cathepsin G and bacterial proteases) and reactive oxygen species (ROS). MMPs are inhibited by the TIMPs, which are synthetized by connective tissue cells and leucocytes [100,101]. MMPs play an important role in the controlled degradation of the ECM in normal wound healing, the removal of damaged components and the laying down of the basement membrane. The excess production of MMPs over TIMPs may contribute to matrix destruction, including cartilage degradation in both osteoarthritis and rheumatoid arthritis and tissue damage in periodontal disease and pulmonary fibrosis [102–104]. Chronic wounds contain significantly higher levels of MMPs than is found in wound fluids of surgical wounds or healing open dermal wounds. The highly proteolytic environment is promoted due to the reduced levels of inhibitors [58]. Since they play a significant role in wound healing, a study

within this thesis was performed to investigate the MMP levels in infected and non-infected wound fluids and to figure out the usability of these enzymes as infection biomarkers.

1.6.3.2 Serine proteases

Serine proteases are the largest class of mammalian proteinases; they have a catalytically essential serine in residue at their active site and are active at neutral or slightly alkaline pH. Their activity depends on a catalytic triad consisting of residues Asp ^{110}, His ^{57} and Ser ^{195} [105]. Human leucocyte elastase, cathepsin G and proteinase 3 are stored in the in an active form within the leucocyte granules. They are secreted upon release of azurophilic granules during neutrophil phagocytosis, stimulation and cell lysis.

Human neutrophil elastase (HNE)

The term elastase describes an enzyme capable of releasing soluble peptides of insoluble elastin, but this does not necessarily imply that its activities are always physiologically related to the digestion of this target. The human neutrophil elastase (HNE) consists of a single polypeptide chain of 218 amino acid residues and contains two aspargin- linked carbohydrate side chains. It is a highly cationic enzyme, with a strongly basic isoelectric point (pH 10-11) [106]. It preferentially cleaves bonds that are carboxyterminal to small, hydrophobic residues.

The synthesis of HNE is on the one hand regulated at the transcriptional level during granulocyte development as well as at the post-translational level before they are stored within neutrophil azurophilic granules. As serine proteases are mainly stored in the neutrophil azurophilic granules, HNE is also localized in the nuclear envelope [107]. Upon the activation of the neutrophils, granular HNE is secreted extracellularly, whereas some molecules remain on the cell surface [108]. Due to stimulation of the neutrophils, the intracytoplasmic granules migrate to the phagosomes and the plasma membrane to release their content. These stimuli include cytokines (TNF-α), chemo attractants (platelet- activating factor, or IL-8) or bacterial lipopolysaccharides [109].

Upon release HNE is involved in inflammatory process regulation and pathogenic agent killing [110]. The direct intracellular killing of phagocytised bacteria is performed in combination with myeloperoxidase and reactive oxygen species [111]. The targets are outer membrane proteins like the outer membrane protein A of *Escherichia coli* and other virulence factors of *Salmonella enterica*, *Yersinia enterocolitica* and *Shigella flexerni*. Extracellular HNE could cleave the proinflammatory bacterial virulence factor flagellin and degrade leucotoxin, which lyses leucocytes and inhibits neutrophil functions [112–114].

The proteolytic activity of HNE is tightly regulated to avoid degradation of connective tissue proteins. The main inhibitors include chelonianins, macroglobulins and serpins, which represent the largest and most diverse family of protease inhibitors [115].

Excessive, inappropriate or prolonged proteinase activity can mediate tissue injury affecting most organ systems. Elevated HNE activity is involved in a number of inflammatory disorders as rheumatoid arthritis, chronic obstructive pulmonary disease, adult respiratory distress syndrome, ischemia/reperfusion injury, emphysema, cystic fibrosis and tumour progression as well as in chronic skin wounds. In chronic wounds, HNE could contribute to tissue injury by degrading fibronectin and other matrix proteins, damaging the endothelial cells and degrading growth factors which are important in the reparative phase of wound healing [116,117].

Elevated HNE levels are described at the very beginning of an infection. They are suited as target for the detection of wound infection in chronic wounds as there can be found a significant activity difference in wound fluids obtained of non-infected and infected chronic wounds [118].

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2 Aim

The detection of wound infections in chronic wounds is still a challenging topic in medical care. As described, the levels of elastase or myeloperoxidase could be elevated in chronic wounds compared to acute wounds. Nevertheless, a threshold could recently be determined to distinguish between infected and non-infected wound fluids addressing the enzyme levels of lysozyme, myeloperoxidase and elastase. Based on these results, new innovative, but easy to handle point of care devices should be developed. A comprehensive study with various wound fluids that are simultaneously tested for infection with silver standard methods in an external microbiological laboratory should state the applicability of these enzymes for infection determination. Extensive literature study in terms of a review focussing on the newest developments in point of care technology should demonstrate the advantages of enzyme responding devices in medical care. In comparison to the common used methods, approaches should be analysed, from new colour changing substrates, to immobilization of the enzyme substrates for innovative applications. A medical device has to point out the wound status, allowing the personal to decide on the further medication of the patient. Therefore, the aim is to invent easy to handle strategies for further hand hold devices that change their colour in case of infection, when incubated with the patient´s wound fluid or during a bandage change. No complicated or invasive steps for the patient should be involved to minimize the impact for the patient and the effort for the personnel.

3 Biomarkers for infection: Enzymes microbes and metabolites

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Wound infection is a severe complication causing delayed healing and risks for patients. Conventional methods of diagnosis for infection involve error-prone clinical description of the wound and time-consuming microbiological tests. More reliable alternatives are still rare, except for invasive and unaffordable gold standard methods. This review discusses the diversity of new approaches for wound infection determination. There has been progress in the detection methods of microorganisms, including the assessment of the diversity of the bacterial community present in a wound, as well as in the elaboration of specific markers. Another interesting strategy involves the quantification of enzyme activities in the wound fluid secreted by the immune system as response to infection. Colour changing substrates for these enzymes consequently have been shown to allow detection of an infection in wounds in a fast and easy way. Promising results were also delivered in measuring pH changes or detecting enhanced amounts of volatile molecules in case of infection. A simple and effective infection detection tool is not yet on the market, but innovative ideas pave the way for the investigation of fast and easy point-of-care devices.

3.1 Introduction

The prevention of wound infection is challenging and still a problem due to time consuming or uncertain wound diagnostics. In developed countries it is estimated that 1 to 2% of the population will experience a chronic wound during their lifetime, predominantly affecting patients older than 60 years - and the number is likely to increase as the population ages [1,2]. The classical clinical signs such as redness (rubor), heat (calor), swelling (tumour), pain (dolour) and impairment of function (functiolaesa) may be reduced due to neuropathy, vascular disease, venous disease, and impaired leukocyte function in diabetic patients. To this aim, experts permanently revise the assessment of clinical infection including signs specific to secondary wounds [3,4]. The International Working Group of Diabetic Foot (IWGDF) and the Infectious Diseases Society of America (IDSA) presented schemes that describe how to define both the presence and the severity of an infection [5–7]. Despite the guidelines, clinical signs are still seen as controversial as criteria for diagnosing infection [8,9]. Assessment of microorganisms in wounds is the silver standard in infection detection, although the presence of microbes per se may not be indicative for infection [3]. Several states such as colonisation, contamination, “critical contamination” and infection have to be considered [10]. The lack of standardised protocols for wound fluid collection (swabbing the wound bed) could mislead the diagnosis, requiring further research to improve its validity [11]. Biopsy is considered as the gold standard microbiological method but it is hardly carried out in clinical practice due to concerns over harm to the patient [12,13]. In this review, current methods and novel approaches for wound infection determination are compared and discussed. Three major chapters focus on bacteria, enzymes or proteins and metabolites as biomarkers. Within these chapters the presented strategies are compared concerning their specificity, the analysis time and subsequent actions linked to the diagnosis (diagnostic value). Highly specific methods often need time consuming preparations, whereas fast and easy applications sometimes only cover few detection parameters (Figure 4).

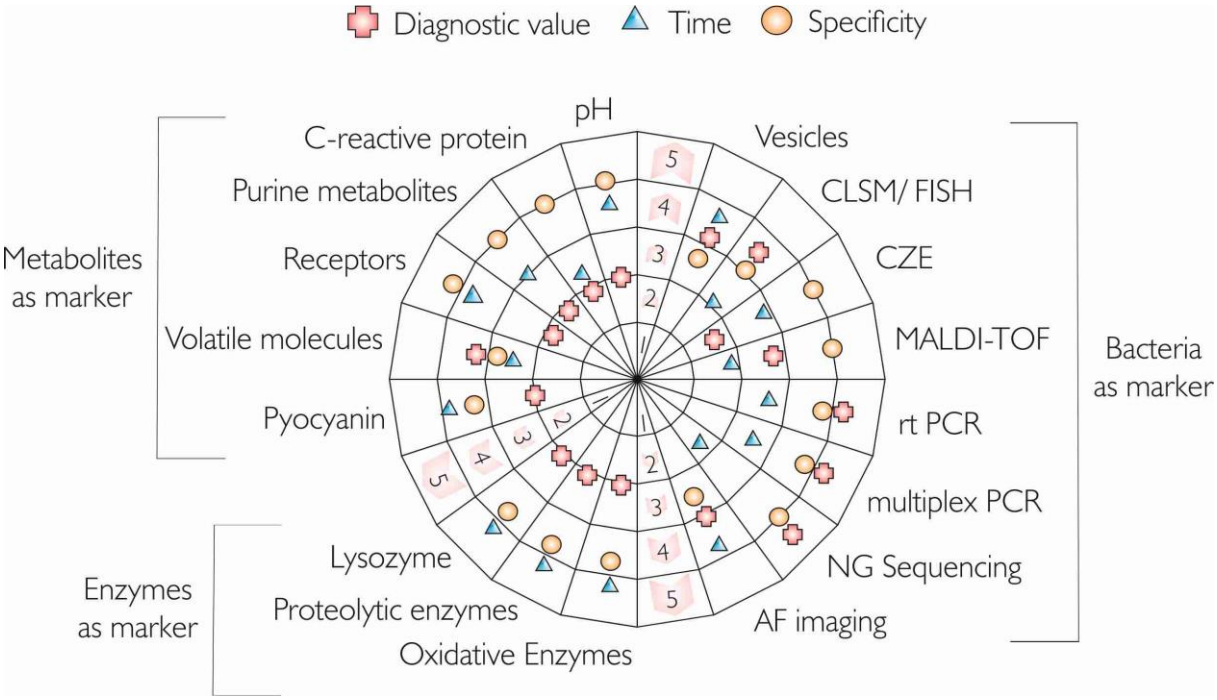

Figure 4: Overview of three main groups of biomarkers: Bacteria, enzymes and proteins and metabolites. Within these main groups, the specific targets or in case of bacteria as markers, the strategies are shown. A comparison is made concerning the specificity of the method, the total analysis time and possible actions based on diagnostic result (diagnostic value). The rating was performed from 1 to 5, whereas 5 is the highest rating.

The determination of the bacterial burden is challenging, but nowadays facilitated by a variety of modern techniques ranging from real-time PCR to pyrosequencing which, however, require special equipment and experienced staff [14,15]. In addition to microbiological analyses, assessment of further markers of infection including enzyme activities, volatile molecules or other metabolites in wounds as well as different methods for pH change are presented. Novel approaches also include sensors and devices that rely on bacterial biochemistry, odour or temperature [16–19].

The wound fluid per se has been an upcoming target for wound infection determination. Detailed analysis of the wound fluid provides an insight into the local extracellular microenvironment of the wound and reflects its current status [20–22]. The composition of wound fluid regarding biomolecules like enzymes is constantly changing due to their secretion into the wound. Enzymes of the neutrophils appear at a very early stage in the infection process and, consequently, analysis of neutrophil-derived enzymes could lead to a new perspective in wound status monitoring [23–25]

Infection detection is a race against time since there is a limited availability of fast diagnostic devices. Enzymes of the immune system could be the basis for fast and simple detection

methods with the help of the rapid conversion of labelled molecules resulting in colour changes. Furthermore, new sensors detecting volatile molecules as well as metabolites could deliver fast leading to a customized treatment. The addressed markers for infection and the respective methods for their assessment are listed in table one and two. Figure 5 depicts the variety of approaches for infection detection that are well established or have been reported recently.

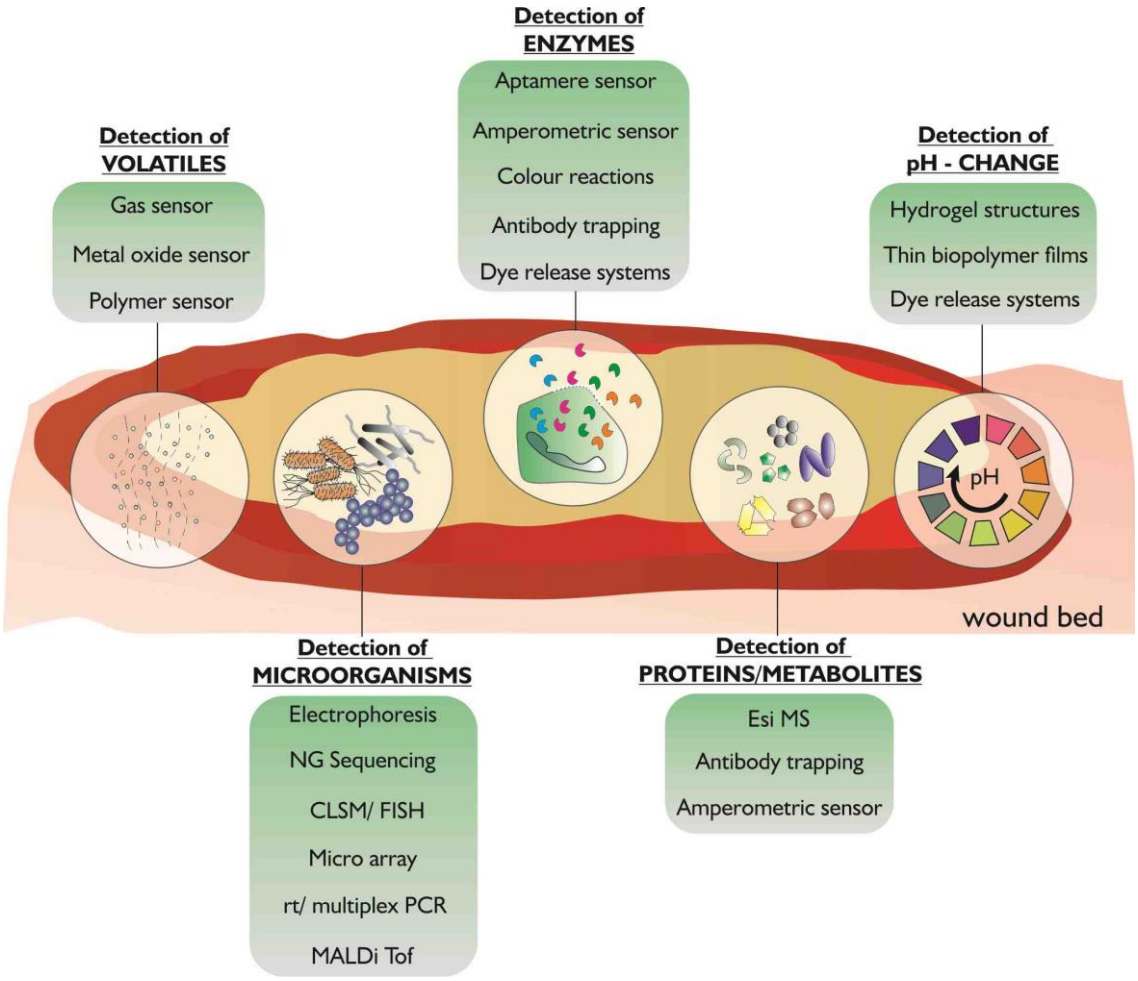

Figure 5: Overview of markers for wound infection and methods for their assessment.

Table 1: Enzyme based biomarkers for infection detection and methods for their assessment

Approach	Marker	Method	Source	References
Proteolytic Enzymes	Human neutrophil elastase	pNA-peptide sequences immobilized on various matrices	Wound fluid	[24,49][24]
		Quartz crystal microbalance (QCM) measurement - specific peptide recognition sequences	Enzyme solution	[50]
		Fluorescence aptamer sensors	Enzyme solution	[52]
	Cathepsin G	Visual detection via pNA-peptide sequences	Wound fluid	[24]
		Plasmon resonance imaging (SPRI)	White blood cells, saliva	[53–55]
	Gelatinases and Matrix metalloproteinase	Sensors based on porous silicon (PS) photonic films	Enzyme solutions	[56]
		Fluorescein-labeled gelatin beads	Wound fluid	[47]
		Woundcheck™ protease status	Wound fluid	
Oxidative Enzymes	Myeloperoxidase	Guajacol, Fast Blue	Wound fluid	[23,82]
		ELISA combined with peroxidase substrates	Human fluids	[113]
		SIEFED	Tissue extracts	[85]
		Lateral flow immunoassay (LFIA)	Human serum	[86]
		Strip-based amperometric biosensor	Human serum	[87]
		Glassy carbon electrodes	Human serum	[88,89]
		Electrochemical magneto immunosensors	Human serum	[90]
	Xanthin oxidase	Anti-XO polyclonal antibodies	Wound fluid	[92]
		HPLC	Wound fluid	[92]
Lysozyme		Stained agarose/peptidoglycan gels	Wound fluid	[25,62]
		Zymography	Termite gland secretion	[63]
		Resonance scattering	Enzyme solutions	[64]
		Aptamer sensors	Enzyme solutions	[66–68,70]
		Fluorescence nanosenor	Human serum	[51]
		Quartz crystal microbalance (QCM) nanosensor	Hen egg white	[72]

Table 2: Other markers for infection detection and methods for their assessment

Approach	Marker	Method	Test conditions	References
Bacteria	*E. coli*	Capillary zone electrophoresis (CZE)	Wound fluid	[27]
	P. aeruginosa	Multiplex PCR	Wound fluid	[34]
	Aerobic bacteria	Real-time PCR	Wound fluid	[31]
	Pathogenic bacteria	Auto fluorescent (AF) imaging	Wound bed	[40]
		Dye-containing lipid vesicles	Bacterial solutions	[41,42]
	Antibiotic resistant bacteria	Stained beta-lactam antibiotics	Theoretical consideration	[44]
	Bacteroides fragilis group	Quantitative real time PCR	Wound fluid	[33]
	Microbial populations	Next generation sequencing	Wound fluid	[38]
		MALDI-TOF	Bacterial solutions	[29]
		FISH combined with CLSM	Wound fluid	[39]
Proteins and Metabolites	Receptor 1, MMP-9, HSL	Electrochemical impedance immunosensing	Artificial wound fluid	[94]
	Purine metabolites	HPLC in combination with ESI-MS/MS	Wound fluid	[92]
		Screen-printed electrodes (SPE) for urate detection	Blood	[95]
		Carbon fiber based devices	Blood, serum, blister fluid	[96]
		Pad-imprinted carbon-uric acid electrodes	Artificial wound fluid	[97]
	C-reactive protein	Immunoturbidometry	Wound fluid	[99]
	Pyocyanin	Electrochemical sensor based on carbon fibers	Bacterial solution	[101]
	Volatile molecules	SnO_2 gas sensor arrays	Bacterial cultures	[103]
		Conducting organic polymer sensors	Wound beds	[104]
		e-nose (metal oxide sensors and gas sensors combined with feature extraction)	Animal model	[105–109]
pH		Immobilized indicator dyes on Ormosil	Artificial wound fluid	[111]
		2D luminescence based on FITC and $(Ru(dpp)_3)$	In vitro and in vivo	[112]

3.2 Bacteria as biomarker for infection

An increasing bacterial burden can lead to an infection in the wound bed. The early detection of an upcoming infection is challenging, due to the lack of rapid and easy applicable detection methods. Within this section we focus on strategies despite the silver standard diagnostics, pointing out the advantages and disadvantages and the feasibility of the methods. The use of bacteria as biomarkers is a challenging topic; Methods with a high specificity often require cost and time expensive sample preparations and long incubation times, whereas fast and easy applications cannot cover the whole bacterial community.

3.2.1 Cappillary zone electrophoresis

Capillary zone electrophoresis is a well-established method for the analysis of various analytes in pharmaceuticals and several other complex compounds [26] Within a clinical study, the suitability of capillary zone electrophoresis (CZE) for a fast detection of *E.coli* in wound samples was investigated [27]. Secretion samples of *E.coli* infected ulcerations were taken and directly applied to CZE. A distinct single peak for *E.coli* was identified to be characteristic for its occurrence and was chosen as the pathogenomic picture for an *E.coli* infection. By applying this method, 86.7% sensitivity and 85% specificity was obtained, and the pathogen could be identified within 30 min. CZE is a rapid diagnosis for *E.coli* and was already applied in a clinical study. Nevertheless, detecting only one pathogen of a complex bacterial flora might not be sufficient for an accurate medication, it could be used as additional and fast information on the bacterial burden.

3.2.2 MALDI-TOF

Mass Spectrometry is applied to various disciplines facilitating the interpretation of complex samples. Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry allows the analysis of large biomolecules and recently rendered a suitable method for bacterial identification [28].

De Bruyne et al developed a standard procedure for the detection of bacterial strains using the reference organisms *Leuconostoc, Fructobacillus* and *Lactococcus* [29]. The data were acquired in a standardized matrix and solvent and analysed by the cell smear method as well as the cell extract method. In combination with a machine learning approach, the mentioned bacterial species could be identified in the samples. The best identification was observed for different genera of *Lactococcus*. This method was not yet investigated in complex wound fluid samples.

The MALDI-TOF analyses stands out due to its speed and especially its accuracy and could furthermore give a more comprehensive information of wound colonization by multiple bacteria [30]. Biswas et al recapitulated recent bacteria identification methods using MALDI-TOF analysis [28].

3.2.3 Real- time PCR

Real-time PCR (rt-PCR) constitutes a powerful technique for the detection and quantification of DNA targeting the biome of a sample. It consequently facilitates the investigation of wound samples towards bacterial pathogens. The detection of the entire aerobic bacterial flora was investigated for assessment of the wound infection status [31]. A real-time PCR assay was applied to investigate chronic wound samples towards their biome by targeting 14 clinically relevant aerobic pathogens. Methicillin-resistant *S. aureus* (MRSA) was found to be the most abundant organism in the investigated chronic wounds followed by *P. aeruginosa* and Group B *Streptococcus*. In comparison to previously reported real-time-PCR assays, the presented approach indicated significantly higher specificity in complex environments.

The identification of anaerobic bacteria like *B. fragilis* is an advantage compared to the common methods [32]. The only possibility for the detection of anaerobic bacteria so far relies on the preparation of an anaerobic culture, which involves time-consuming processes. Furthermore, a quantitative real-time PCR assay was established for the detection of 10 prominent members of the *B. fragilis* group [33]. A variety of surgical wound infection samples was investigated using this method and higher counts were determined using the PCR method, compared to cultivation. Controversially, *B. uniformis* was detected to be the most frequent species within the sample pool whereas *B. fragilis* was detected to be the major representative according to common cultivation methods. This approach delivers new insights in the detection of the anaerobic genus *Bacteroides* but cannot be seen as tool for wound infection detection as it targets only one bacterial species, which cannot constitute an ultimate proof of an existing infection.

Methods for the detection of the entire bacterial community within the wounds environment are a promising approach to enable an appropriate infection therapy. The increased availability of PCR machines in medical institutions makes these assays suitable for the application in routine diagnostics. However, experienced staff is required in contrast to ready-to-use diagnostics.

3.2.4 Multiplex PCR

Multiplex PCR represents a powerful method for the detection of high genotypic diversity. Several pathogenic bacteria show this diversity which hampers their specific detection. For

the detection of *Pseudomonas aeruginosa* in wound samples, a multiplex PCR method was established that resulted in enhanced detection specificity in comparison to similar PCR procedures and biochemical methods [34]. Briefly, after bacteria isolation and DNA extraction, specific genes in *P. aeruginosa* were targeted by multiplex PCR. An enhanced detection was obtained in comparison to biochemical methods investigating pus samples. Unfortunately, despite the general potential of this method it does not seem suitable for fast and simple infection detection.

3.2.5 Next generation sequencing

Next generation sequencing (NGS) summarizes higher- throughput sequencing strategies when compared to the Sanger method. Pyrosequencing as one application, is an alternative for *de novo* sequencing and is used in many applications such as Single Nucleotide Polymorphism (SNP) genotyping, fungal and viral typing as well as the identification of bacteria [35] [36]. The 16S rRNA gene-based pyrosequencing has the advantages of accuracy, flexibility, parallel processing and can be easily automated. It enables a high resolution analysis of the entire wound microbiome, which is of high significance targeting appropriate therapeutic actions [37]. Compared to culture based methods, a wide range of bacterial taxa including anaerobic pathogens can be observed. Important information about the dynamics of bacterial communities in wounds can be delivered and influences on the composition of the bacterial taxa due to diabetes or clinical treatments can be detected. [15]. A microarray together with next-generation sequencing was developed to detect the microbial population in wounds [38]. The developed microarray was able to monitor all sequenced pathogens and detected *Acinetobacter* to be associated with wound failure. Interestingly, the presence of enteric bacteria was correlated with successful wound healing. No connection could be ascertained between the quantity of bacteria within the wound and its infection status. Due to the short analysis time, the approach could function as a fast infection indicator once a critical microbial pattern can be assigned to the infection level. The assessment of the microbiome within the wound could give an advice for appropriate therapeutic actions due to the knowledge of the contamination constituents.

3.2.6 Fluorescent in situ hybridization / Confocal laser scanning microscopy

Biofilms are known to protect the microbiome towards antibacterial molecules like certain enzymes or antibiotics. A method that combines fluorescent in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM) was used exploring the microbial flora of biofilms in infected wounds [39]. In vitro biofilms and in vivo biofilms were investigated by the application of peptide nucleic acid (PNA) probes. *P. aeroginosa* was detected as a major

bacterial species in the investigated biofilms of chronic skin wounds. CLSM investigations determined the bacterial composition of clusters within these biofilms whereas FISH analyses explored the bacterial load in wound biofilms. The combination of these identification methods renders a powerful tool for the investigation of bacterial clusters within infected wounds, although long analysis intervals are critical for timely infection detection. Nevertheless, comprehensive information about bacterial constituent within the wound is obtained representing a support for subsequent therapeutic actions.

3.2.7 Auto fluorescence imaging

Auto fluorescence (AF) imaging enables the distinction between connective tissue within a wound and bacterial contaminations. While endogenous connective tissue shows green fluorescence, bacteria like *S. aureus* induce red fluorescence caused by endogenous porphyrins. Wu et al (2014) developed a device for auto fluorescent (AF) imaging of pathogenic bacteria in wounds, called PRODIGI (Portable Real-time Optical Detection, Identification and Guidance for Intervention). This diagnostic contains a charge-coupled device (CCD) sensor-based camera that acquires AF images as well as white light images. Within a preclinical study on a mouse skin wound model, the bacterial flora was simultaneously visualized and quantified over time. This implies the ability to monitor wound healing upon antibiotic treatment. Although the method is suitable for infection detection, no conclusion can be drawn to distinct therapies since no information about specific bacteria is obtained.

3.2.8 Responsive vehicles

Responsive materials undertake physicochemical changes upon interaction with a distinct matter, resulting in conformational changes or degradation of the respective material. Bio responsive systems are already applied to various disciplines in science including medicine.
For the detection of infection, responsive vesicles were prepared (Zhou et al 2010; 2011) responding to bacteria-derived virulence factors. Upon degradation of the vesicles, antimicrobials and dye indicators were released. The functionalization of fabrics like wound dressings with vesicles that are responsive to pathogenic bacteria could become a powerful tool for directed drug delivery into the wound. Giant unilamellar vesicles (GUVs) were synthesized, attached to plasma deposited maleic anhydride, and investigated towards their lysis and release properties. Furthermore fabrics with stabilized vesicles containing carboxyfluorescein were treated with suspensions of *P. aeruginosa* and *S. aureus*; the release of carboxyfluorecein was detected photographically under UV light after 15min. This

vesicle approach could act simultaneously as wound infection indicator and drug delivery system (Fig.6). As the respond of the different vesicles is related to specific bacteria, the use of this approach for infection detection is more likely than for specific therapeutic actions.

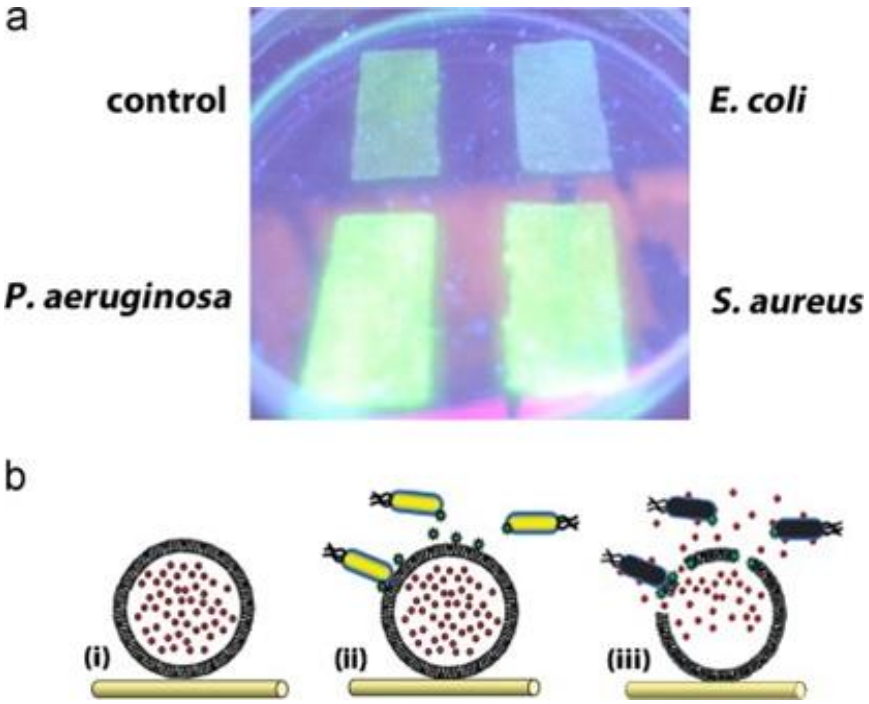

Figure 6: (a) Polypropylene fabrics impregnated with vesicles release carboxy- fluoroescein as seen under UV light in the presence of pathogenic bacteria in comparison to non-pathogenic controls. (b) The mechanism for bursting of the vesicles by bacteria releasing the fluorescent dye or an antimicrobial agent. (i) vesicle prior to rupture, (ii) toxins from bacteria lyse vesicle wall and (iii) contents of vesicle released. Adapted from [17,41,42] Permission obtained by Elsevier

3.2.9 Cephalosporin derivatives

A major reason for the need of an early stage detection of infection is the dramatic increase of bacterial resistance towards antibiotics. It is very time consuming to investigate whether a resistant group of bacteria is on site or not, although this information is crucial for therapy.

As example, extended spectrum β-lactamase (ESBL)-producing organisms pose unique challenges, as the laboratory detection of ESBLs can be complex and, at times, misleading. ESBLs are an example of the increasing number and diversity of enzymes that inactivate β-lactam- type antibacterials mostly expressed by *Escherichia coli* and *Klebsiella pneumonia* [43].

A promising strategy for the visual detection was recently proposed [44]. The approach takes advantage of the cleavage of the beta-lactam antibiotics. A dye is attached a beta-lactam functional group and, when capable bacteria are present, it is cleaved off the drug residue upon degradation by beta-lactamase (Fig. 7). Thus, a dye-drug conjugate tethered on a

wound dressing would constitute an efficient biomarker for the visual detection of antibiotic-resistant bacteria. Additionally a possibility is discussed to convert this detection system into a drug delivery system whereby the dye is replaced by antibiotics to which bacteria are sensitive. However, an experimental proof of this concept was not published to date.

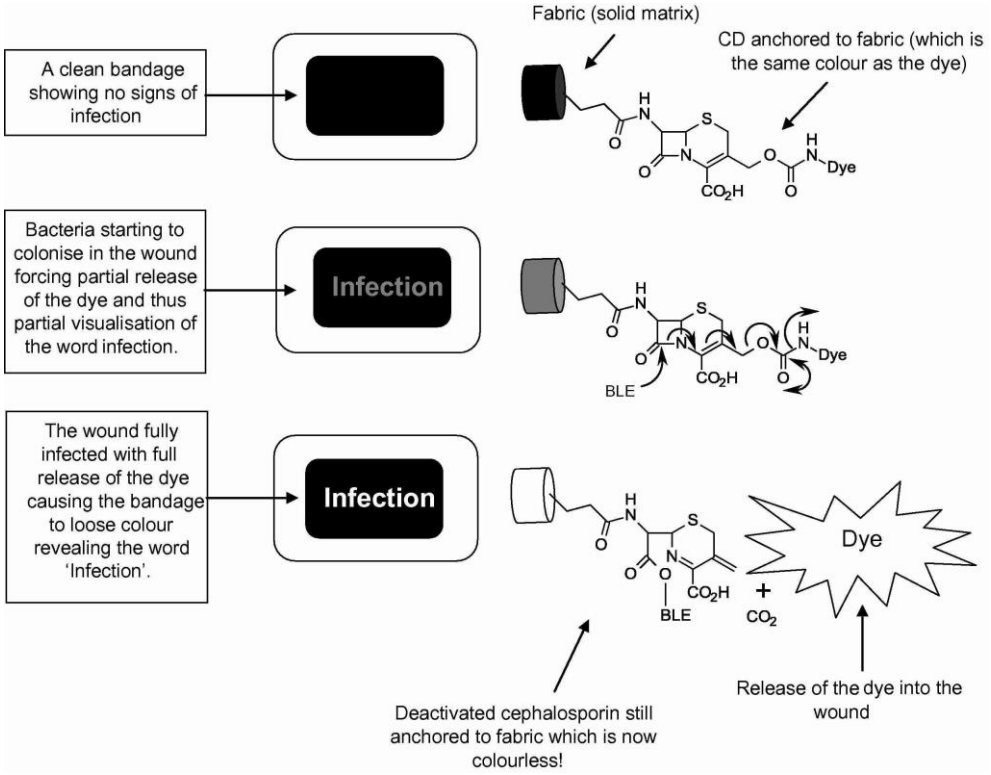

Figure 7: The mechanism of action for early stage detection of an infection, using a cephalosporin-dye bandage [44]. Permission obtained by Elsevier

3.3 Enzymes as biomarker for infection

3.3.1 Proteolytic enzymes

Protease activity is essential for an efficient wound healing process [45]. These enzymes hydrolyse different proteins like the extracellular matrix (ECM) and connective tissues, degrading damaged matrix and foreign material. Thus, the formation of new tissue is enabled facilitating wound closure. Elevated protease activity in healing wounds is observed only for some days. In non-healing wounds protease activity remains elevated for longer time intervals resulting in a damage of reconstructed tissue [46]. Moreover, increased proteolytic activity in the wound bed often results in wound infection. Heinzle et al. (2013b) detected significantly elevated activities of several proteases in infected wounds when compared to non-infected wounds indicating their potential as biomarkers for the detection of wound infection.

3.3.1.1 Detection of human neutrophil elastase (HNE)

Human neutrophil elastase (HNE) is a serine protease that belongs to the chymotrypsin superfamily. Including proteinase 3 (PR3) and cathepsin G (CatG), these enzymes are the major bioactive constituents of the neutrophil azurophilic granules. Elastase is released from neutrophils and macrophages during acute neutrophil-mediated inflammation and it takes part in the degradation of components of the ECM and in the cleavage of inflammatory mediators, due to its broad substrate specificity [48].

Significantly altered activities of this enzyme were measured in infected wound fluids by applying N-methoxysuccinyl-AAPV-p-nitroanilide (MeOSuc-AAPV-pNA) as chromogenic substrate. Sensor materials were synthesized for wound status monitoring that enable the detection of infection at an early stage. In these systems, the chromophore para-nitroanilide is released from a carrier material resulting in colour formation. Different materials were used as carrier for this enzyme substrate. Sililca gel was modified with APTS and subsequently MeOSuc-AAPV-pNA was immobilized using EDAC. Furthermore, a cysteinamide derivative of the HNE substrate, Cys-Suc-AAPV-pNA, was covalently attached to several surfaces, namely polyamides, polyethylene terephthalate, collagen films and matrices of hyaluronic acid. The immobilized substrates were incubated with either infected or non-infected wound fluids. For all substrates treated with infected wound fluid, a colour formation was observed [24].

Another immobilization strategy [49] of elastase substrates was based on attachment onto a cross-linked ethoxylate acrylate (CLEAR) resin. Suc-AAPV-pNA and suc-AAPA-pNA was coupled via a glycine spacer on CLEAR resins. The release of para-nitroanilide upon incubation with elastase solution was detected photometrically. Higher hydrolysis rates were observed for substrates in solution when compared to their resin-bound counterparts, whereby a slightly higher reaction rate was monitored for the alanine analogue. Swelling of the CLEAR resin prior to incubation with the enzyme improved the hydrolytic cleavage of the chromophore. In order to circumvent light scattering at higher amounts of resin, substrate analogues were prepared by saponification of the substrate containing resins. Solubilized resin conjugates led to an improved dose response. The suitability of these substrates for a visible detection of released para-nitroanilide was also investigated. To do this, the adsorption of the chromophore on a polypropylene surface was monitored, whereby only a minor percentage of available substrate was attached to the surface [49] .

Stair J.L. et al. (2009) detected elastase activity by the use of dextran hydrogel films coated on a quartz crystal. Oxidized dextran was cross-linked with the peptide AAPVAAK, which is a substrate for HNE. Upon degradation of the hydrogel film, a change in resonance frequency was detected at the quartz crystal microbalance (QCM) measurements. The hydrogel degradation rate correlated with elastase activity and could also be controlled by its cross-link density.

Aptamers can be used for a highly selective and sensitive method for detection of HNE. These single strand DNA/RNA oligonucleotides can be designed to specifically bind to selected molecules. Aptamer strategies combine several advantages in comparison to traditional antibodies since they are obtained by a chemical approach. Song et al (2014) reported recent advances in the field of aptamer-based biosensors.

For the detection of HNE, a fluorescence based DNA aptasensor was constructed. In combination with a S19 scrambled sequence (SS) and a fluorescent molecular beacon (MB), a selective signal could be obtained. A low detection limit 47 pM HNE was determined. Due to a decreased binding affinity of SS to the aptamer, 10°C incubation temperature was necessary. [52].

3.3.1.2 Detection of cathepsin G

Like HNE, Cathepsin G (CatG) is a component of the neutrophil azurophilic granules and constitutes an important factor in the early immune response. Its proposed role is to hydrolyse proteins, including the ECM and hormonal factors, similar to HNE. The activity of CatG was determined using the same procedure as for HNE, however, using N-Suc-AAPF-pNA as specific substrate [24]. Similarly to HNE, the activity of CatG was found to be

significantly elevated in infected wounds. Substrates for CatG were immobilized on APTS-modified silica gel. A colour change was only visible upon incubation with infected wound fluid. Further investigations revealed that Suc-AAPF-pNA is only hydrolyzed by CatG but not by HNE. The inverted phenomenon was observed for MeOSuc-AAPV-pNA that is only degradable by elastase.

One powerful tool for the detection of substrate-ligand interaction is surface plasmon resonance imaging (SPRI). The applications of SPRI biosensors enable a highly sensitive quantification of bound ligands to their immobilized substrates and inhibitors without the need for labeling. In order to monitor CatG activity in solution, a SPRI sensor was developed using the MARS-115 peptidyl inhibitor [53,54] that was immobilized on a gold chip via cysteamine. A low detection limit of 0.23 ng/ml was determined. Selectivity of the inhibitor to CatG was investigated whereby no influence of other proteases on CatG determination was monitored. The sensor was further applied to white blood cell samples of leukemia patients, saliva samples and endometrial tissue.

Another CatG inhibitor, MARS-116, was applied in a colourimetric active-site-specific immunoassay and could detect CatG in few micrograms of cell lysate [55].

3.3.1.3 Detection of gelatinases/matrix metalloproteinases

Gelatinases are proteolytic enzymes that promote the hydrolysis of gelatin. Matrix metalloproteinases (MMP) are prominent members of this enzyme class, in particular MMP-2 and MMP-9, and play an important role in the human immune defense. These Zn^{2+}-dependent endopeptidases take part in the regulation of ECM and in other physiological processes. Elevated activities of MMPs in combination with other proteases like HNE are not only sensitive indicators for an upcoming wound infection, but also play an important role in the wound healing process [46].

An approach to detect gelatinolytic activity in general was implemented, covering MMPs as well as serine proteases like HNE that are likewise able to digest gelatin [47]. Enzyme activities were monitored using fluorescein-labeled gelatin as well as gelatin beads with a covalently attached dye. A 23-fold higher activity was monitored in infected wounds in comparison to non-infected wounds. An up to 32-fold increase was measured due to blue dye release of the gelatin beads in infected wound fluids (Fig.8) Studies with specific inhibitors for either MMPs or serine proteases (HNE) demonstrated the considerable contribution of HNE to gelatinolytic activity found in wound fluids [47].

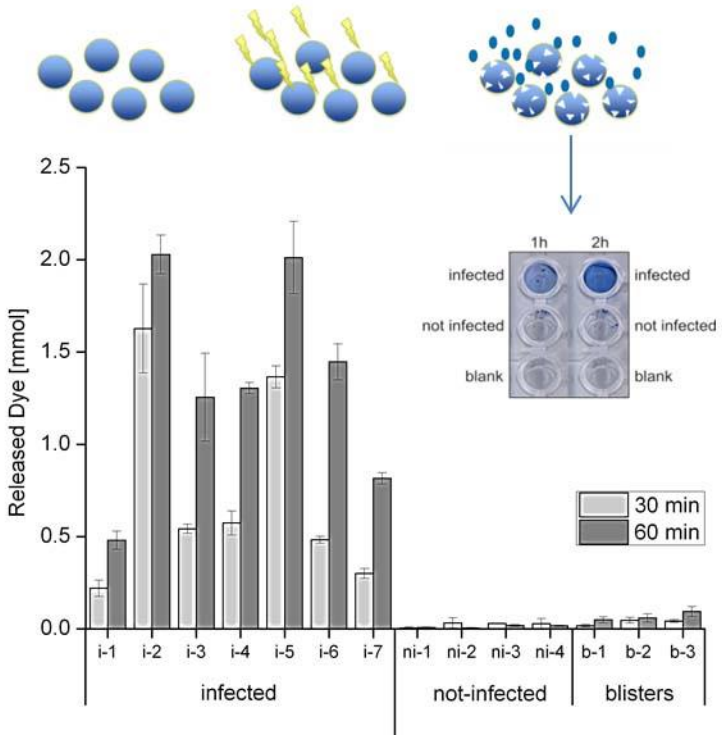

Figure 8: Schematic representation and measurements of blue colour release upon wound infection due to gelatinolytic enzyme activity. Formatted figure based on [47]. Permission obtained by John Wiley and Sons

A method of detecting gelatinases like MMP-2 was developed using sensors based on porous silicon photonic films [56]. Gelatin was spin-coated onto porous silicon (PS) by applying a drop MMP solution. A subsequent digestion of the gelatin film enabled the entrance of hydrolysis products into the pores. As soon as air in the pores is replaced by a liquid medium a colour change can be observed. Since water diffusing into the pores would also result in a colour change, drying of the senor and the employment of glycerol was necessary after the reaction. The PS photonic sensor could detect MMP-2 in concentrations down to 1 ng/ml.

Martin et al used porous silicon microcavity (PSiMc) structures for detection of MMP-8 [57]. For the binding of a streptavidin conjugated anti MMP-8 human antibody, biotinylated BSA was used for the attachment to PSiMc.

An already commercialized diagnostic from Systagenix, WOUNDCHECK™ Protease status, is based on the detection of elevated protease activity to assess whether neutrophil-associated proteases are present in the wound or not.

3.3.2 Detection of lysozyme

Lysozyme is a well-known enzyme, which was already mentioned in 1922 [58]. Antimicrobial activity of the enzyme was even published in 1909 by Laschtschenko who discovered antimicrobial properties of hen egg white [59]. It is categorized as a glycosidase capable of hydrolyzing the glycosidic linkages of peptidoglycan (PG) in bacterial cell walls. Elevated enzyme activities in serum often relate to diseases [60].

Elevated lysozyme activity was demonstrated in infected wounds fluids thus indicating the potential of this enzyme for wound infection detection [25]. In a correlative study, the increasing bacterial burden in infected wounds was in line with a significant increase in lysozyme activity [61]. Based on the natural lysozyme substrate, PG-based devices for detection of elevated lysozyme activities in wound fluids were developed [25]. Agarose/PG gel layers were prepared, in which PG is degraded and a resulting change in turbidity can be related to different hydrolysis activities. In order to improve the sensitivity of such devices, PG stained with Remazol Brilliant Blue (RBB) was incorporated into the layer system to enable a photometrical detection. No loss in turnover rate was detected using the stained PG derivative. A system for visual detection was implemented, where the hydrolysis of an upper PG layer led to the appearance of the stained hydrogel that was placed underneath [25].

Schneider at al developed a multi-layer system by using an agarose/PG hydrogel with incorporated laccases that operate as so-called enhanzymes [62]. These enzymes were covalently attached to PG and incorporated into the gel by increasing their size via PEGylation. The digestion of PG by lysozyme led to the release of laccase, catalysed a colour forming reaction, and thereby dramatically enhanced the detection signal.

Another elegant approach for detecting and quantification of lysozyme in complex samples is based on the use of a special electrophoresis method called zymography [63]. After an electrophoretic separation, the enzymes are allowed to renature, which enables them to convert a substrate that is incorporated into the electrophoresis gel. As substrate, *Micrococcus lysodeikticus* cells were incorporated into polyacrylamide gels. The cells were stained with Remazol Brilliant Blue to allow a detection of the enzyme activity. After 1 h of reaction time, clearing zones were observed in the gel due to release of soluble dye-cell fragments. A detection limit of 1.4 ng of lysozyme was determined after incubation for 12 h.

Based on the specific resonance scattering peaks of *M. lysodeikticus* (ML), Jiang et al detected lysozyme activity by the evaluation of resonance scattering spectra [64]. ML cells were incubated with a lysozyme solution for 10 min, before resonance scattering spectra were acquired. The hydrolysis of ML led to dissolution of the bacteria and hence a decrease in interference between solid and liquid, which results in a loss of signal intensity. Optimal

parameters for the assay were investigated and competitor molecules were applied in order to control the selectivity of this method. A detection limit of 0.014 U/mL was determined.

3.3.2.1 Aptamer sensors

The use of aptasensors for the detection of enzymes was previously described for the monitoring of HNE activity. Likewise, several aptamer-based systems were developed for the detection of lysozyme. These sensor systems generally reveal good selectivity towards the target molecule [65]. Nevertheless, the selectivity of aptasensors in complex environments like wound fluids towards a distinct target has not been investigated so far.

The detection of lysozyme by electrochemical impedance spectroscopy (EIS) was successfully implemented [66]. To do this, a chitosan-graphene oxide conjugate was immobilized on a pencil graphite electrode (PGE), and the required DNA-aptamer was covalently attached to graphene oxide. After incubation of the aptasensor with lysozyme, EIS experiments were performed. A decrease of electron-transfer resistance (R_{ct}) was observed with increasing lysozyme concentration. The detection limit of lysozyme was observed to be 0.38 µg/ml. Mixing lysozyme with thrombin or bovine serum albumin validated the selectivity of the system.

In order to enable a spectrophotometrical detection of lysozyme, a fluorescent aptasensor was developed [67]. A hairpin probe (HP) was used that contains the aptamer sequence to lysozyme, as well as a signal probe (SP) that was labeled with carboxyfluorescin. Signal amplification could be achieved utilizing exonuclease III for fluorophore liberation. Finally, still intact SPs were adsorbed on a graphene oxide surface, which is why its fluorescence was quenched. A fluorescence signal was obtained that could detect lysozyme down to a concentration of 0.08 µg/ml. The selectivity of this aptamer system was evaluated by the addition of several competitors.

A label-free alternative to fluorescence-based aptamer sensors constitutes the determination of aptamer oxidation upon the binding to lysozyme [68]. The group therefore grafted the respective aptamer onto a carbon paste electrode (CPE). The attachment of the enzyme to the aptamer sequence accompanies a decrease of the oxidation signal of guanine and adenine, monitored by the use of square wave voltammetry (SWV). The detection limit was determined to be 36nM for guanine and 18nM for adenine. In a common approach, the selectivity of the designed sensor was investigated whereby no interferences could be detected. However, this device was not tested for complex samples like wound fluids.

A piezoelectric aptamer sensor was used for the quantification of lysozyme in saliva samples [69]. A thiolated aptamer was immobilized on the SPQC-IDE (piezoelectric quartz crystal-interdigital electrode) gold surface. The piezoelectric device could detect a frequency shift

because of molecular changes of SPQC-anchored compounds. High selectivity and a low detection limit of 0.5nM were reported for this sensor system.

Amongst electrochemical aptamer sensors, a highly sensitive aptasensor was developed based on gold nanoparticles that were immobilized with the aptamer DNA [70]. In the presence of lysozyme, a Fc tagged DNA is liberated from the aptamer, by which the increasing distance between the Fc tag and the electrode resulted in a signal decrease of faradaic current. The specificity of the gold aptamer system was investigated by the addition of several proteins like albumin and haemoglobin. No interference and signal change were observed, which makes this aptasensor a sensitive (detection limit 0.1pM) and selective detection system (Fig. 9).

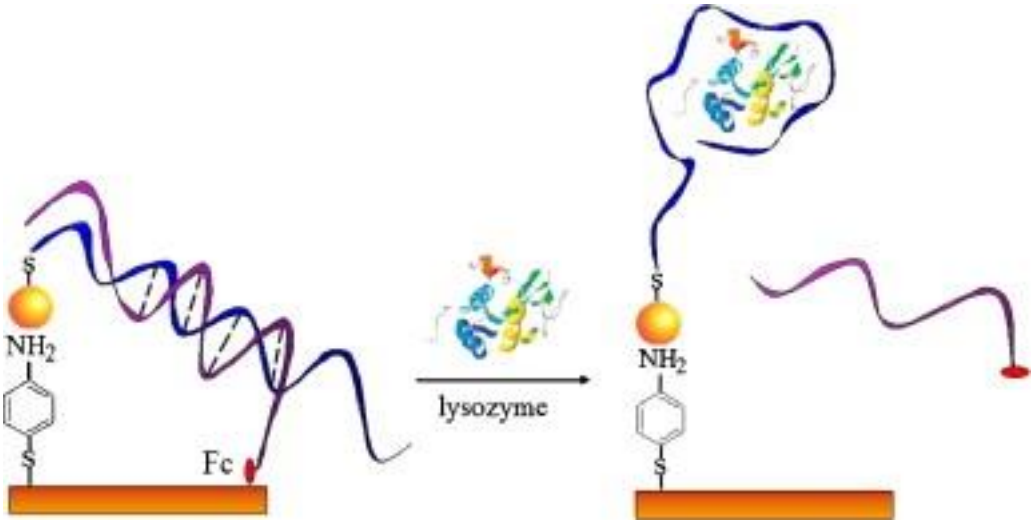

Figure 9: Proposed mechanism of a signal-off electronic aptamer-based sensor for the detection of lysozyme [70]. The release of the Fc tagged aptamer leads to signal decrease. Permission obtained by Elsevier.

3.3.2.2 Nanosensors

In the past decade, nanosensors gained great interest, not at least in the field of medicine. Their ability to acquire data in nanoscale is especially interesting for monitoring small changes in biological processes. The development of nanosensors comprises various fields like optometry, chemistry and electrochemistry, amongst others. The transducing compound mainly comprises biological recognition elements that induce physicochemical changes after interaction with the analyte. These changes are converted into measurable parameters like electrical or optical signals [71].

For the detection of lysozyme a fluorescence nanosensor was developed based on modified CdTe quantum dots (QDs) [51]. Carboxymethyl chitosan (CMCS) was attached onto the dots via electrostatic interactions. Based on the strong binding ability between Zn^{2+} and CMCS

onto the quantum dot surface, an enhanced photoluminescence signal was obtained after addition of Zn^{2+}. Due to the ability of lysozyme to degrade chitosan as well as CMCS, hydrolysis of the latter results in the release of Zn^{2+} and in the adsorption of lysozyme onto quantum dots. This process leads to the quenching of photoluminescence. The linear range of this behavior was determined to be within 0.1-1.2 ng/mL lysozyme with a detection limit 0.03 ng/mL. The applicability of this nanosensor for medical diagnosis was investigated in interference studies as well as in the detection of lysozyme in human serum. In both cases no significant interferences were detected.

A nanoscale device for lysozyme detection based on the change in weight was achieved by the preparation of a quartz crystal microbalance (QCM) nanosensor [72]. Lysozyme-imprinted (MIP) PEDMAH nanoparticles were synthesized that were subsequently attached onto the gold surface of the QCM sensor. Nanocavities in the nanoparticles specifically led to lysozyme adsorption from the surrounding medium driven by a concentration gradient. A resulting mass shift on the QCM sensor was detected and found to be linear between a range of 0.2-100µg/mL with a detection limit of 1.2ng/mL. The specificity of the nanosensor was investigated by the use of albumin as a challenger molecule. The result of this competitive assay as well as the application for lysozyme detection on hen egg white indicated high selectivity.

3.3.3 Oxidative enzymes

Oxidative enzymes like oxidases and peroxidases are commonly used for the determination of hydrogen peroxide (H_2O_2) and organic hydroperoxide. Biosensors for glucose, alcohols, glutamate and choline are based on a two-enzyme system, where one highly specific oxidase delivers H_2O_2, which is then detected by a co-immobilized peroxidase via a colour reaction. Probably the most important application of peroxidases in bioanalytics is the enzyme-linked immunosorbent assay (ELISA), in which peroxidase-coupled antibodies are used for detection of toxins, pathogens, cancer risk in bladder and prostate, and many other analytes via simple peroxidase catalysed colour reactions [73].

3.3.3.1 Detection of myeloperoxidase

Myeloperoxidase is stored in the azurophilic granules of the neutrophils and is released until activation and degranulation of neutrophils [74]. MPO is a haeme-containing enzyme, consisting of two dimers connected by a disulphide bond. Each dimer comprises a beta-heavy subunit (59 kDa) and a light alpha-subunit (14 kDa) [75]. MPO catalyses the production of hypochlorous acid from chloride ions in the presence hydrogen peroxide

(H_2O_2). Hypochlorous acid is the most powerful bactericidal oxidant produced by neutrophils [76]. MPO furthermore oxidizes many phenolic compounds like guajacol. Since the recruitment of neutrophils increases with bacterial invasion, MPO-activity can indicate infection at a very early stage. Elevated MPO activity has been shown to be linked to a multitude of diseases, including atherosclerosis [77], myocardial infarction, atrial fibrillation [78], multiple sclerosis [79], Alzheimer's disease [80], and transplant rejection [81]

Hasmann et al (2013b) reported significantly higher MPO levels in infected wound fluids using guajacol as a substrate (92.2+45.0 versus 1.9+1.8 U/mL) . Moreover, elevated MPO activities were in accordance with the elevated bacterial burden in wound fluids [82]. Apart from natural phenolic compounds, a number of synthetic substrates including the synthetic dye Fast Blue RR were shown to be suitable markers for infection detection. This dye was coupled to siloxanes allowing printing to a wide range of surfaces. Colour formation of the immobilized dye was observed only upon incubation with infected wound fluids. Apart from the MPO oxidation activity, the chlorination activity – i.e. formation of hypochlorid acid (HOCl) - was tested in different wound fluids as an alternative method towards development of sensors. A correlation with MPO oxidation and chlorination activity in wound fluids was found and highly significant activity differences were likewise obtained (P = 0.01) in infected wound fluids compared to non-infected wound fluids [82]. Based on this, an electrochemical sensor for the detection of MPO in wound fluids was developed based on the consumption of H_2O_2 by MPO. The required H_2O_2 was supplied by *in situ* generation by immobilized glucose oxidase directly on the sensor from glucose present in wound fluids to avoid instable H_2O_2-containing system reagents [82]. Indeed, this system could clearly distinguish infected and non-infected wound fluids (Fig. 10).

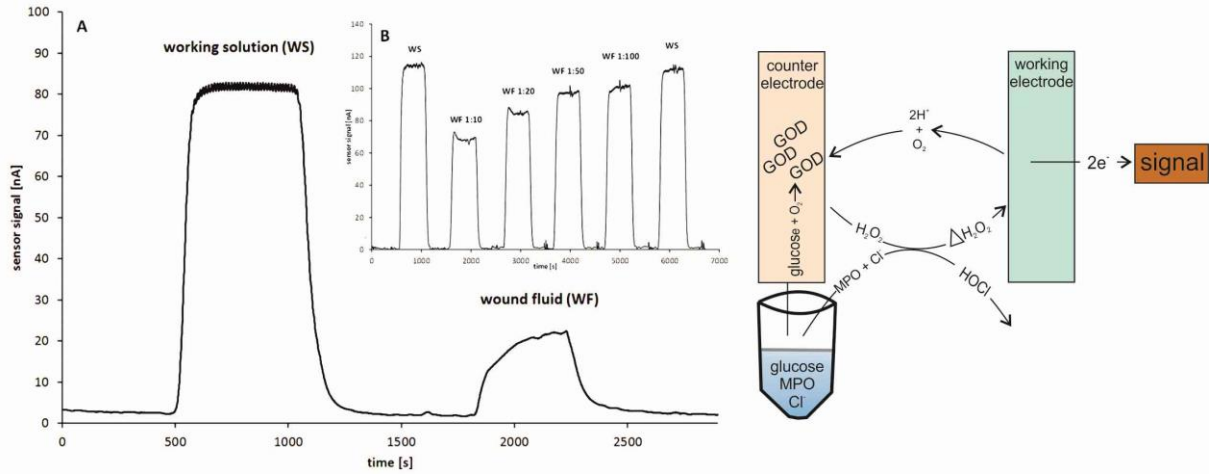

Figure 10: Detection of wound infection based on amperometric detection of H_2O_2 consumption by MPO. A constant supply of H_2O_2 was achieved by glucose oxidase directly immobilized on the sensor

from glucose present in wound fluid. Inlet: Different dilutions of infected wound fluid lead compared to a control (WS working solution). Permission obtained by Elsevier.

Regarding to the importance of the enzyme in other diseases, a huge variety of enzyme assays has been reported. Pulli et al. (2013) compared different assays for MPO quantification in human fluids and tissue samples. General peroxidase substrates (e.g. TMB, o-dianisidine, guaiacol) and 10-acetyl-3,7- dihydroxyphenoxazine (ADHP) were used as well as chlorination activity substrates (39-(p- aminophenyl) fluorescein ((APF) and 39-(p-hydroxyphenyl) fluorescein (HPF)) paired with an antibody capturing assay (ELISA). Bromide-dependent chemiluminescence with luminol was determined as described by Haqqani et al. (1999). As an ELISA guarantees high specificity, the best results were obtained by combining this method with MPO activity determination using ADHP as substrate.

Another approach to measure MPO activity is the “Specific Immunological Extraction Followed by Enzymatic Detection” (SIEFED) performed in plasma, bronchoalveolar fluids (BALs) and tissue extracts [85]. The difference to ELISA tests is the measurement of active MPO activity in comparison to total MPO content. This study highlighted the main advantages of this method as follows: the easy immuno-capture of MPO out of a biological sample without any preparation, the elimination of interference compounds, the specificity since the specific antibodies only capture human MPO, the high sensitivity due to the combined use of Amplex Red as fluorogenic substrate in combination with nitrite as an enhancer for the reaction and the measurement of the activity by referring to a calibration curve made with pure human MPO. SIEFED and ELISA can be used as complementary immunological techniques to determine the active/total MPO ratio in biological samples.

Cardiovascular diseases are, as wound infection, a tremendous problem in the western world. Common ELISA tests (as mentioned above) are commercially available but faster tests with less machinery are desired. The development of a high-affinity avian-MPO-specific recombinant antibody enables a detection of MPO in one step and avoids sandwich signal enhancement steps. The incorporation into a rapid, simple, and sensitive one-step lateral flow immunoassay (LFIA) facilitated the sensitive detection of MPO in MPO-depleted serum within clinically relevant reference ranges [86]. Electrochemical sensors are also considered suitable for MPO detection as well as in wound fluids for infection determination as in serum for CVD prevention. As substrate 3,3-5,5-tetramethylbenzidine (TMB) is often used. Windmiller et al. (2010) used TMB as a redox mediator to enable highly sensitive quantification of physiological levels of MPO. They investigated a screen-printed strip-based amperometric biosensor. Under the optimal conditions, physiological levels of MPO were

detected in human serum using flexible screen-printed electrodes (SPE) within 30 minutes. Another approach is the coating of modified glassy carbon electrodes with anti-MPO antibodies (Lu et al 2010; 2011) This method greatly improves the electrochemical behaviour and enhances the sensitivity of the immunosensor. The sensor was tested in human serum and illustrated several advantages such as high sensitivity, a wide linear detection range for the detection of MPO and no need for multiple labelling procedures. Carbon nanotube wiring for signal amplification of electrochemical magneto immuno sensors was presented by Herrasti et al. (2014). The chronoamperometric MPO detection was accomplished using immunofunctionalized magnetic microparticles (MPs), disposable carbon screen-printed electrodes (C-SPEs), and a ready-to-use commercially available TMB-based substrate. The MPO was captured and concentrated due to the MPs and monitored using TMB. The immuno capturing can be done within 15 minutes, also in complex fluids like serum.

3.3.3.2 Detection of xanthin oxidase

Xanthine oxidase (XO) is a complex oxidoreductase containing a molybdoflavo group. It is described as a key enzyme in the purine catabolism, whereby hypoxanthine is converted to urate in a two-step biosynthesis [91]. Elevated levels of XO and urate were detected in chronic venous leg ulcers, which lead to the assumption that this enzyme plays a crucial role in inflammatory processes of chronic wounds. Due to these recent observations, XO is a potential biomarker for wound infections. The enzyme itself was monitored in wound fluid by western blotting using anti-XO polyclonal antibodies [92]. The activity of XO was also indirectly determined by the analysis of uric acid in wounds fluids of chronic wounds via HPLC. Significantly higher concentrations were detected in comparison to human serum.

3.4 Detection of proteins and metabolites

The environment of an infected wound is a complex setting involving a lot of organisms and compounds. A variety of substances are secreted into the wound, either from the invading microflora or triggered by the host immune response [93]. Their concentration strongly depends on the wound status, which makes them important biomarkers for wound infection. Several determination methods for suitable targets were recently published and are discussed within this subchapter.

3.4.1 Detection of receptor 1, MMP-9 and HSL

Ciani et al (2012) implemented electrochemical impedance immune sensing for the detection of wound infection. The sensor was designed for the detection of 3 previously chosen analytes, namely receptor-1 expressed on myeloid cells (TREM), matrix metallopeptidase 9 (MMP-9) and N-3-oxo-dodecanoyl-l-homoserine lactone (HSL). These compounds are known to play a crucial role in the host immune response and as quorum sensing molecules. Specific antibodies were immobilized on gold screen-printed electrodes and investigated for their suitability by testing in mock wound fluid. An increase in electron transfer resistance was observed for all analytes, and the highest limit of detection was obtained for HSL (1.4nM). Due to the high dynamic range and sensitivity of the sensor, a promising detection system for early infection detection was developed. Since the biomarker derives from the induced immune system, no conclusion on the composition of contaminating bacteria within a wound bed can be drawn. This implies no hints for the optimal therapy strategies and the method can consequently only prove whether an infection is present or not.

3.4.2 Detection of purine metabolites

In recent studies, elevated concentrations of purine metabolites were observed in wound fluids of chronic venous leg ulcers [92]. In particular, accumulation of uric acid was detected resulting from high activity of xanthine oxidase, which converts hypoxanthine into xanthine and finally leads to the production of uric acid. Consequently the level of purine metabolites indicates the infection status of a wound and therefore acts as an infection biomarker similar to xanthine oxidase discussed above. Similary to enzymes as biomarkers, purine detection only targets whether a wound is infected or not and does not give detailed information that could support therapists in their decisions.

In parallel to the detection of xanthine oxidase, Fernandez et al detected purine metabolites using HPLC in combination with ESI-MS/MS. Purine standards were used for the calibration

of mass spectrometry and the respective metabolites could subsequently be quantified. In combination with multiple reaction monitoring, an accurate evaluation of the metabolite levels was achieved. Unfortunately, the time consuming analytical effort disables timely monitoring of the wound infection status [92].

A prominent purine catabolite is uric acid. Its accumulation affects pH changes in wounds. The development of a simple and disposable pH sensor based on screen-printed electrodes (SPE) utilizes urate for infection detection [95]. The sensor was constructed as layer system by laminate sheeting including a cellulose filter paper to enable a satisfactory distribution of sample liquid. The prepared sensor was applied to buffer solutions and blood samples. The plasma-treated and anodised SPE led to enhanced responses to urate in solution in comparison to the non-anodised counterparts. Urate in blood samples was successfully detected, and a decrease of the signal was observed with increasing scan number**.**

Sensors based on carbon fiber mesh for the detection of uric acid were developed [96] and tested for their applicability in blood, serum, blister fluid and microbial culture. The laminated carbon fiber devices were prepared in a multi-layer approach using the fibers and resin-polyester. Further anodisation was necessary to circumvent disturbing factors like tryptophan. In all tested fluids a short term monitoring was possible without alteration of sensor parameter.

In subsequent studies, pad-imprinted carbon-uric acid composite electrodes were prepared with incorporated uric acid acting a pH probe [97]. Fouling of the electrode could only be prevented by the use of 1,2-diaminobenzene. The pH could be monitored in a range of pH 4 to 10 in buffered solutions as well as in simulated wound fluid. The developed devices indicate great potential for a clinical application, however, the monitoring time may need to be prolonged to assess a long lasting wound healing process.

3.4.3 Detection of C-reactive protein levels

An already well-known indicator for occurring infections is the C-reactive protein (CRP). It plays a crucial role in the humoral immune response by activating the complement system to defend the host against bacterial colonization [98].

Neumaier and Scherer (2008) investigated the CRP levels in a variety of postoperative wound using an immunoturbidometric approach. According to the obtained results, a CRP concentration above 96 mg/L was defined as the cut off for a deep wound infection 4 days after surgery. The assessment whether a wound is infected or not in a point-of-care manner is hardly possible by only monitoring the CRP level since its concentrations change with the clinical situation of the patient.

3.4.4 Detection pyocyanin

P. aeruginosa is a major constituent of the microbial flora of infected wounds. For the growth within the emerging biofilm quorum sensing was found to play an important role. The regulation of gene expression is triggered by the secretion of small molecules, which affects the bacterial virulence and accelerates the infection process [100]. The quorum sensing system of *P. aeruginosa* results in the production of pyocyanin, a redox-active phenazine dye. An electrochemical sensor based on carbon fiber tow electrodes was constructed monitoring pyocyanin concentrations by square wave voltammetry [101]. The sensor was successfully applied for quantification of pyocyanin. Further studies focused on the detection of *P. aeruginosa* in mixed populations to assess interferences. No interferences were observed and a detection limit of 0.03 µM was reported. Furthermore the sensor was also proven to work without oxygen, which renders this device suitable for its application in wound environment. Despite information that is obtained about a certain pathogen by targeting pyocyanin, no conclusion about the severity of infection is possible.

3.4.5 Detection volatiles

Wound-invading bacteria play a crucial role in the alteration of a variety of complex processes taking place. During bacterial colonization different metabolites are generated whose composition and concentration depends on the species and the growth phase of the bacteria [102]. Many of these substances are volatile, often causing typical foul-odour of wounds upon infection. The composition of the headspace metabolites is specific for occurring bacteria within the wound and for their growth phase status. Volatile molecules do not necessarily involve a sample taking, since monitoring can be conducted directly upon release from the wound. A detailed analysis of the concentrations of volatile organic compounds (VOCs) above the wound can result in an accurate monitoring of the wound infection status. Since a conclusion of bacterial constituents within the wound can be drawn, the detection of volatile molecules can support the choice of an appropriate therapeutic agent.

A strategy to give a conclusion of the bacterial flora in the wound environment relies on the use of a SnO_2 gas sensor array. The dynamic response of the sensor array towards volatile compounds was detected [103]. Briefly, after wound swabbing, cultures were prepared where *E. coli*, *P. aeroginosa* and *S. aureus* were selected as infection biomarkers. Subsequently the analysis by the sensor array was conducted in a flow control system. Additionally standard microbial investigations were performed with the remaining cultures. The impact of surrounding influences like humidity and temperature were monitored and

found to have only minor effects on the measurement outcome. However the obtained data varied with the stage of the culture growth. Due to the fact that a culture growth is necessary for further investigations with the prepared sensor array, a timely detection of wound infection is not achievable. A direct application of this sensor on wounds would save preparative effort and detection time.

In the work of Bailey et al. (2008) several headspace molecules were chosen according to results of a previous GC/MS analysis of bacterial species that are commonly found in infected wounds. This selection included ethanol, acetic acid, ammonia, acetone, acetaldehyde, 2-butanone and butyric acid. For detection of these metabolites, conducting organic polymer sensors were developed as well as hybrids of these polymer sensors with metal oxide sensors. The obtained data was processed online by neural network techniques. The investigated array could detect the targeted metabolites, however, properties like resistance and conductivity were not satisfactory. Further development was necessary for its application on complex biological samples.

Subsequent investigations led to the implementation of an electronic nose (enose) system by utilizing metal oxide gas sensors and an electrochemical gas sensor to a sensor array [105]. The system used a feature extraction method and a neural network classifier that enabled the sensor to detect seven bacteria species including the wound colonizing pathogens *P. aeruginosa* and *S. aureus*. Enabling an accurate detection, the enose sensor array consists of several units namely a headspace-sampling unit, the sensor array together with a signal conditioning circuit followed by a data acquisition and processing unit. First experiments were conducted by analyzing the headspace of inoculated agar slants of single populations as well as of pathogen mixtures.

The presented enose approach was improved [106] using a multi fold amount of gas sensors as well as a method utilizing background elimination, namely direct spatial correlation of wavelet transform coefficients. Mice with infected wounds by *P. aeruginosa*, *E .coli* and *S. aureus* were found to be a good model because of the mouse smell resulting in high background that requires a good specificity of the chosen approach to detect the analyte. The recovered signal from background resulted in a significantly improved signal. In recent studies [107–109] several feature selection and feature extraction methods were investigated towards the improvement of obtained signals and the elimination of occurring background.

3.5 pH as infection indicator

During the healing process of a wound different parameter alter in concentration and activity. The change of the pH in the course of healing progression constitutes a long known phenomenon, which accompanies with alteration of enzyme activities and the oxygen update rate of the wound, amongst others. The common range of the pH of an intact epidermis encompasses a pH of 4 to 6. Upon tissue damage and induction of the immune defence, the pH is shifted towards basic conditions. In previous studies, designated pH values could be assigned to distinct phases of the wound healing process (Schneider et al., 2007). These discoveries render the pH value to a powerful biomarker for timely infection detection.

Based on the well-known pH indicators bromocresol green and bromocresol purple, a silicate pH sensor was developed for a continuous monitoring of the wound pH [111]. Therefore the respective indicator was immobilized on tetraethoxysilane (TEOS) films to form an organically modified hybrid sol-gel (Ormosil), whereby a chip-LED enabled illumination of the respective pH indicator. Due to a shift of the indicating pH range after immobilization, only bromocresol green exhibited the required pH section and was consequently used for further investigations. A fast response time was observed for pH measurements in selected buffer solutions whereas a prolonged response was obtained for detections in artificial wounds, which complicates potential application in wound environments.

Another interesting approach for a visual detection of the pH pattern within wounds constitutes the application of 2D luminescence based on time-domain luminescence imaging [112]. The dyes fluorescein isothiocyanate (FITC) and ruthenium (II)tris-(4,7-diphenyl-1,10-phenanthroline) ($Ru(dpp)_3$) were therefore incorporated into micro particles which were subsequently immobilized on polyurethane hydrogels. A time-gated CCD camera was chosen to detect the luminescence. The acquisition period was divided into two time gates, namely the excitation part and the emission part. Whereas the total luminescence was detected in the excitation phase, only the pH-independent signal of ($Ru(dpp)_3$) could be observed in the emission phase because of a much longer luminescence life time compared to FITC. This fact rendered the ($Ru(dpp)_3$)-signal the reference of the pH-dependent FITC. The internal reference system remediated confounding factors and irregularities in particle distribution. The pH sensor was successfully applied in vitro as well as in vivo and is thus a promising approach to pattern a wound pH map.

3.6 Conclusion

Fast and appropriate diagnosis of a wound infection constitutes an indispensable task in wound management. The development of diagnostic tools for online monitoring of the wound status would facilitate trained personnel to choose an appropriate therapy early enough. Recent statistics demonstrate the urgent need of rapid infection detection and demographic developments towards extended age of patients furthermore clarify its importance. In the recent years, a number of infection biomarkers were identified and a variety of methods was established for their detection. These diagnostic methods situate in different stages of development whereby to date most of them are not implemented in clinical applications and daily use. In the near future the number of publications referred to detection of wound infection is going to increase tremendously due to the high demand on point of care devices. Enzymes seem to be interesting biomarkers since their activity in wounds directly pictures the intensity and type of the immune response. They will play a relevant role in fast and easy wound monitoring towards a “yes/no answer” concerning infection in the wounds. Several other infection biomarkers, like bacteria and their metabolites, represent promising indicators; however wound infection cannot be identified only by a distinct concentration and pathogenicity of wound contaminants, but is much more complex. Recent developments of sophisticated PCR and sequencing methods created a bunch of data that deepened the understanding of wounds and infection processes. The impact of various bacteria in wounds of different origin could be elucidated by the help of enhanced sequencing methods. Combined with enzyme based approaches for infection detection systematic application of antibiotics and wound care could be possible. However in respect to efficient therapies, the clinical evaluation of novel diagnostic devices is crucial and constitutes a major challenge in the near future.

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4 Assessment of infection in chronic wounds based on the monitoring of elastase, lysozyme and myeloperoxidase activities

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4.1 Introduction

Infection in wounds affects about 2% of the population in developed countries at least once in their lifetime, and the lack of tools for its rapid diagnosis is still a problem [1]. Standard procedures of infection detection include the judgement of the classical clinical signs, the detection of signals specific to secondary wounds, or the quantification of the microbial load [2–5]. The determination of the microbial load is a time-consuming standard procedure, although the presence of microbes per se is not indicative of infection [2]. Biopsy is still considered as the gold-standard method but is not often carried out in clinical practice to avoid unnecessary pain [6-7]. The wound bed, including the wound fluid, harbours numerous biomarkers including enzymes that provide an insight into the current infection status [8–10]. Detection of enzyme activity, especially myeloperoxidase (MPO), human neutrophil elastase (HNE) and lysozyme (LYS), is a new approach in wound status monitoring [11–13]. Fast enzyme assays, including visible colour changes due to increased enzyme activities indicate a change in the wound bed. A combined measurement of three immune-system-derived enzymes enhances the sensitivity of an infection detecting system, considering the individual enzyme variations of each patient.

4.2 Material and methods

Over a period of four months, chronic or acute wound samples from 95 patients were examined, obtained from ulcers, diabetic feet, post-operative wounds, decubitus wounds and blisters (negative control). The wound bed was swabbed to obtain the wound fluid for microbiological and biochemical (enzyme) analysis. In this blind study, the enzyme analysis was performed in a research laboratory as index test, whereas the microbiological determination of the wounds (as reference) was performed by a clinical microbiologist. The biochemical analysis included measurements of total protein content (BCA kit from Novagen) and enzyme activities. MPO was measured based on oxidation of guaiacol [11], HNE based on hydrolysis of N-methoxysuccinyl-ala-ala-proval-p-nitroanilide leading to yellow colour, and LYS based on loss of turbidity due to the hydrolysis of peptidoglycan [14].

To compare the enzyme activities in infected, possibly infected and non-infected wound fluids, two-sample t-tests assuming equal variances were performed. A P value of less than 0.05 was considered as statistically significant.

Microbiological investigations involved MALDI-TOF analysis and microscopy after Gram staining, given as + (<1) to ++++ (>1000) counts per ocular field. Results were categorized based on the presence of potentially pathogenic microorganisms (ppmos) relative to the general microbiological flora.

"Infected": one or more ppmos were present without general microbiological flora, or more than 3 sorts of ppmos were present;

"possibly infected": ppmos present as well as general microbiological flora in the same amount;

"good healing/ non-infected": no ppmos or ppmos in lower amount than general microbiological flora.

The “clinical look” evaluations (infected/ non-infected) were performed according to hospital guidelines accompanied by a questionnaire.

Exclusion criteria for this prospective study were the use of antibiotics in the last seven days and no measurable protein content in the sample. Permission to collect wound fluid was obtained from the Ethics Committee of the Medisch Spectrum Twente, Enschede.

4.3 Results

Of the 95 patients considered, 20 were excluded to avoid false negative results due to lacking protein content or the use of antibiotics in the last seven days; the 75 included are described in table 3. Nine out of the 75 wounds were described as clinically infected by the attending doctors and by microbiological analysis, while 5 wounds were labelled as non-infected by both. All wound status interpretations (infected/ possibly infected/ non-infected) are summed up in table 4. In 65% of the samples, the results of the superficial wound swabs were not in accordance with the visual clinical reports, excluding the possibly infected results (only yes/no; no/yes). The enzyme activities were measured based on colour changes or a decrease in turbidity (leading to the appearance of the colour on the base of the well, figure 11). Augmented enzyme levels were observed due to increasing bacterial load with potential pathogenic microorganisms (ppmos) (fig. 11).

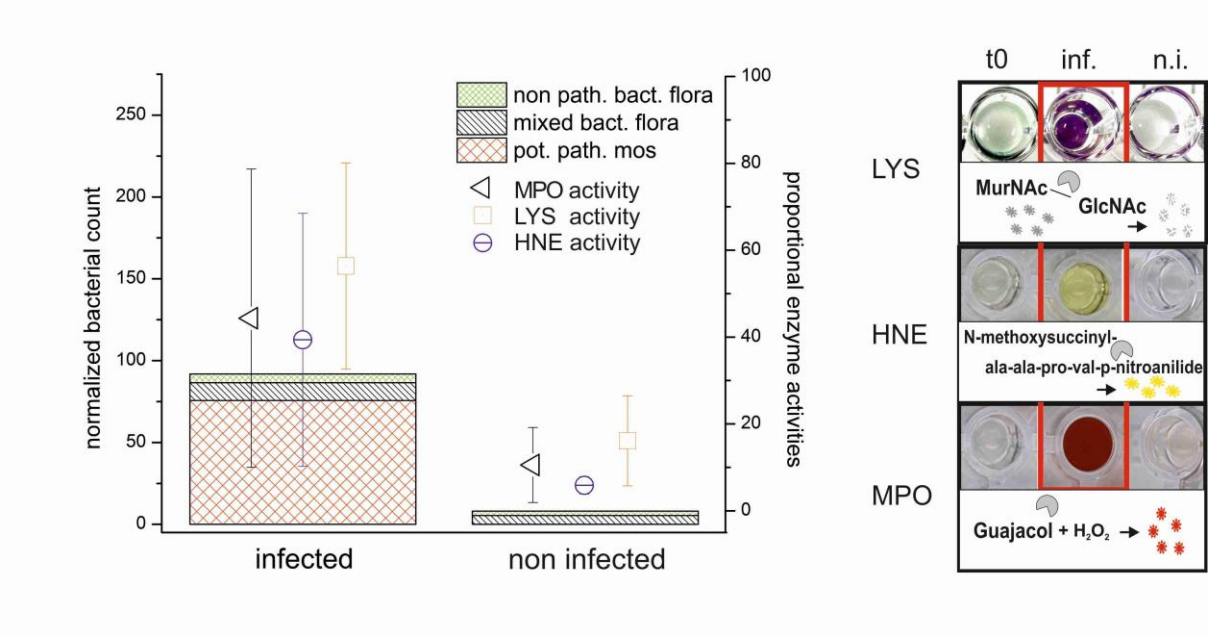

Figure 11: Left: Comparison of lysozyme, elastase and myeloperoxidase activities (absorbance) and bacterial burden (ppmos) of wound fluids. Right: Distinction of infected (inf.) and non-infected (n.i.) wound fluid after incubation compared to the substrates at t=0 based on colour change or loss of turbidity leading to appearance of the colour at the base of the well (indirect colour change).

Table 3: Characteristics of the 75 patients and ulcers included (n=75)

		Frequency (%) / mean (sd)
Patient characteristics	Female gender	33 (44)
	Male gender	42 (56)
	Age in years (mean (sd))	66 (16)
	Diabetes	30 (40)
	Cardiac disease	12 (16)
	Pulmonary disease	7 (9)
	Arterial disease	26 (35)
	Venous disease	9 (12)
	Hypertension	18 (24)
	Rheumatism	3 (4)
	Gout	4 (5)
	Renal disease	4 (5)
	Antibiotics last month	13 (17)
Wound characteristics	Arterial ulcer	6 (8)
	Venous ulcer	2 (3)
	Diabetic foot ulcer	22 (29)
	Traumatic ulcer	14 (19)
	Pressure ulcer	9 (12)
	Amputation wound	6 (8)
	Oncological ulcer	2 (3)
	Mixed arterial / venous ulcer	2 (3)
	Other	12 (16)
	Wound duration in days (mean (sd))	167 (557)
	Wound length in cm (mean (sd))	4 (5)
	Wound width in cm (mean (sd))	2 (3)
Wound appearance	Partial wound necrosis	23 (31)
	Serous exudate	51 (69)
	Ensanguined exudate	10 (13)
	Sanious exudate	5 (7)
	Ensanguined/ sanious exudate	1 (1)
	Foul wound smell	12 (16)
	Dry wound bed	8 (11)
	Moist wound bed	49 (65)
	Wet wound bed	13 (17)

Table 4: Summary of the wound status interpretation. An assumed infection is indicated as "+", no infection as "-". Uncertain microbiological results are indicated as "p" for possible infection.

	+/ - / p					
Superficial swab analyses (microbiology)	+	-	+	-	p	p
Clinical look investigation	+	-	-	+	+	-
Count of wound fluids	9	5	20	6	9	26

Moreover, all three measured enzyme activities were in accordance with the microbiological results (fig. 12) with significant differences between infected and non-infected wounds with a P value (two-sample t-test assuming equal variances) of 0.01 concerning HNE and LYS and a P value of 0.08 for the MPO measurements. Seven out of the 9 infected samples were diagnosed as infected according to all three enzyme levels. In the remaining two samples, the activities of two enzymes clearly confirmed infection which demonstrates the importance of assessing three different enzymes as markers.

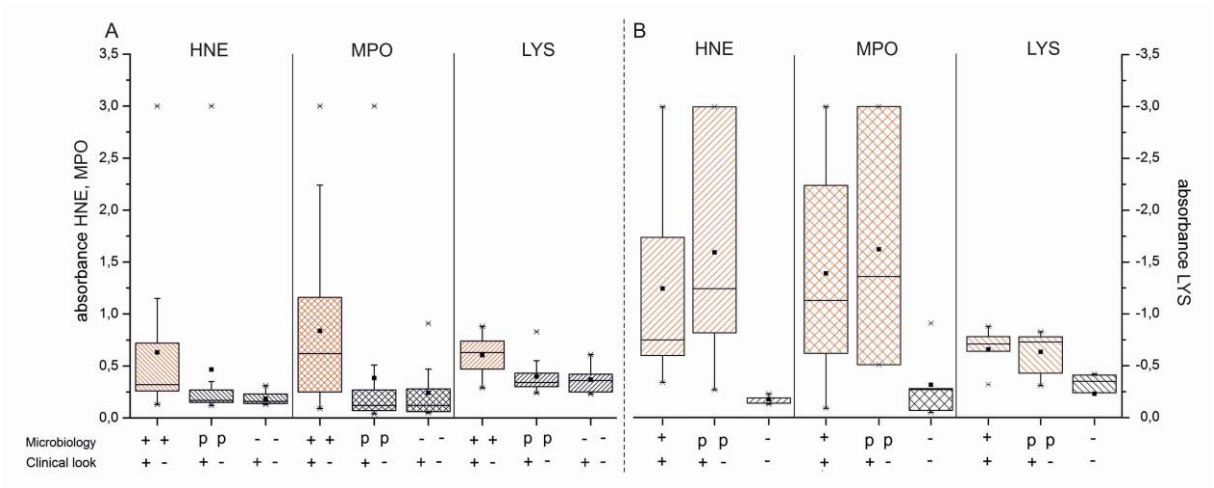

Figure 12: Measurement of enzyme activity values of 75 patients. A: Enzyme activities of infected (+), non-infected (-) and possibly infected (p) superficial wound swabs according to microbiological analysis. The visual clinical inspections (both positive and negative) were assessed in the same data set. Lysozyme activity (right y-axis) is based on loss of turbidity (reverse absorbance values). Box plot results show significant differences in enzyme activities in wounds seen as infected and non-infected as well as infected and possibly infected. B: Comparison of enzyme activities in six of the possibly infected wound fluids according to microbiological assessment with high absorbance values. The middle box of each enzyme refers to samples indicated as possible infection, "p", by the superficial wound swab and + or – infection according to the visual clinical inspections. These six samples show

significant differences to wounds indicated as non-infected by both investigations and, in return, the significant difference cannot be observed concerning infected wound fluids.

Interestingly, six of the possibly infected samples (microbiological evaluation) showed high enzyme activities comparable to the levels of wounds predicted as infected (microbiological evaluation). Figure 2B highlights these six samples from the possibly infected sample pool, showing a significant difference to non-infected fluids whereas no significant difference to infected wound fluids was found. The inclusion of all three enzyme activities could facilitate the identification of infected wounds if the microbiological assessment does not provide clear results.

4.4 Discussion

The combined activity levels of three enzymes (MPO, HNE, LYS) indicate infection based on simple colour changes and on spectrophotometric analysis when compared with silver standard microbiological analyses. Not all wounds tested positive for the enzyme activity of all three enzymes, with some wounds only testing positive for two enzymes, demonstrating that at least two of three positive enzyme reactions are sufficient for the diagnosis of infected wound fluid. In 85% of the wounds only described as infected by the microbiologist but not by the doctors, a precise detection of infection based on the three enzymes was possible. The combined activities enable an optical and measurable differentiation of infected wounds where the microbiology and clinical judgment provide uncertain results. This enzyme-triggered and rapid diagnostic tool is based on a simple “readout” system for wound status monitoring. No invasive biopsies are needed, as the wound fluid collection is equivalent to the superficial wound swab for microbiology. The information of this silver standard method concerning the wound status can be complemented with wound activity measurements of three different wound enzymes as shown here. The results could be confirmed with an improved gold standard for infection, such as biopsy.

4.5 References

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5 An electrochemical sensor for fast detection of wound infection based on myeloperoxidase activity

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Infection can lead to severe complications during wound healing. We have developed an electrochemical sensor for fast and simple detection of wound infection based on the quantification of myeloperoxidase activity as a marker for infection.

Applicability of the enzyme was confirmed with a correlation study with silver standard wound diagnostics. Significant higher enzyme activities comparing non infected and infected wound fluids were determined (P = 0.01). To eliminate supplemental substrate addition, the chlorination activity of the enzyme - the formation of hypochlorous acid (HOCl) from chloride and hydrogen peroxide - was investigated in different wound fluids and correlated with the peroxidation activity measurements. Significant activity differences were likewise obtained (P = 0.01). Based on this we constructed an electrochemical hydrogen peroxide sensor system for the quantification of chlorination activity in wound fluids. Furthermore, immobilized glucose oxidase was integrated into the system to provide hydrogen peroxide required by myeloperoxidase.

Infected wound fluids were indeed identified by using the sensor system quantifying the consumption of hydrogen peroxide consumed by myeloperoxidase. Thereby, immobilized glucose oxidase was shown to produce enough hydrogen peroxide for the myeloperoxidase reaction from glucose present in wound fluids. There is a strong need for a simple but effective sensor system to determine infections in wounds. This sensor measuring hydrogen peroxide consumption could effectively identify infected wound fluids based on the myeloperoxidase activity.

5.1 Introduction

Infection is a common problem in chronic wounds resulting in prolonged hospital stays, non-healing wounds and increasing mortality of the patients. For example about 1% to 2% of individuals are estimated to suffer from leg ulceration during their lifetime requiring a substantial portion of the health care budget and the number is likely to increase as the population ages [1-4]. The correct identification of infection is a complex issue as not all clinical signs such as redness (rubor), heat (calor), swelling (tumour), pain (dolour) and impairment of function (functiolaesa) can be consistently observed. The concept of critical colonization is also controversial and not universally accepted [4-6]. Diverse guidelines have been developed to prevent upcoming infections [7,8]. Clinical examination by itself is unreliable for the diagnosis of wound infection. Quantitative biopsies of ulcers showed that 28% of participants had bacterial counts greater than 105 but were lacking any clinical signs of infection [9]. Despite on-going research with a focus on markers for wound healing, most of the available sensors still rely on bacterial biochemistry, odour, temperature or pH changes which are either time-consuming or unspecific [10-14]. Wound fluid per se has an enormous potential for fast detection of infection and for monitoring wound healing [15-18]. As the recruitment of neutrophil granulocytes is one of the earliest events in wound repair, the analysis of neutrophil-derived enzymes has a new perspective in wound status monitoring [19, 20]. We present a new sensor supported strategy for fast diagnosis of wound infection based on the detection of neutrophil derived myeloperoxidase (MPO). MPO is a haeme containing protein that uses hydrogen peroxide (H_2O_2) to oxidize not only chloride ions yielding antimicrobial HOCl, but also phenolic compounds like guaiacol [21-23]. Since the recruitment of neutrophils increases with bacterial invasion, the enzyme MPO-activity indicates the infection status at a very early stage [24, 25]. We used amperometric detection of H_2O_2 to determine the MPO chlorination activity in wound fluids. MPO requires H_2O_2 which was supplied by in situ generation by immobilized glucose oxidase to avoid instable H_2O_2 containing system reagents. Devices for fast diagnosis of infections are applicable in medical centres focussed on dermatological illnesses as well as in home care and retirement homes. They address not only long term ulcer wounds observed frequently but also wounds after amputations or decubitus wounds as well as post-operative wounds. A fast and easy applicable device should facilitate the recognition of an infection in a wound and therefore allow adequate wound treatment to avoid sepsis.

5.2 Material and methods

If not stated otherwise all chemicals were purchased from Sigma Aldrich, MO, USA.

5.2.1 Sample collection and preparation

Wound fluids from post-operative wounds, ulcer and decubitus wounds were collected by swabbing the wound bed with a nylon swab (Microrhelogics, Brescia, Italy) followed by dilution in 0.9% sodium chloride (NaCl) for further analysis. Uninfected wound fluids from blisters served as negative controls. Permission to collect wound fluid was obtained from the Ethics Committee of the Medical University of Graz, Austria.

5.2.2 Microbiological analyses

Before sample collection, wounds were cleaned with 0.9% NaCl (Sigma Aldrich, MO, USA) to remove superficial bacteria. Swabs were taken of the most contaminated and/or deep site of the wound bed and/or wound edges. The samples were analysed with MALDI TOF techniques, addressing the analyses of the occurring species, as well as gram staining dependent microscopy evaluations were performed to determine the bacterial load [26]. After gram staining, the slides were screened (magnification 1000x) and the bacterial count was reported. The semi quantitative reporting system was subdivided from + (<1) to +++ (>100) counts per ocular field [27, 28]. The microbiological test results were categorized as "infected", "critical", or "good healing", by looking for the presence of potential pathogenic microorganisms (ppmos) relative to the general microbiological flora. The following definitions were used:

Table 5: wound status definitions

wound status	definition
infected	one or more ppmos were present without general microbiological flora, or more than 3 different ppmos were present
critical	ppmos and general microbiological flora were present at the same amount
good healing	ppmos amount is lower than general microbiological flora

The results were evaluated by a microbiologist. Additionally an experienced medical doctor also examined the wounds following standard diagnostic procedures. E.g. a good healing wound was characterized due to size reduction of the wound bed as well as a good granulation and epithelisation.

5.2.3 Myeloperoxidase activity

For the detection of MPO-peroxidation activity 10 µL of wound fluid or MPO solution was mixed with 290 µL substrate solution containing 99 mM guaiacol and 0.017% (w/w) H_2O_2 in 50 mM potassium phosphate buffer pH 7.0 [25]. Enzyme standards containing 1.0 to 5.0 U/mL MPO (Human Myeloperoxidase, Planta, Vienna, Austria) were used. The formation of tetra-guaiacol was measured every 1s for 100 sec at 470 nm in 96 well plates using a Tecan infinite M200 platereader (Tecan, Maennedorf, Switzerland).
Detection of MPO-chlorination activity was based on trapping of HOCl formed from chloride and H_2O_2 by using taurin to produce stable taurinechloramine (MPO activity assay, Northwest, Vancouver, Canada) [29]. Taurinechloramine then reacted with 5-thio-2-nitrobenzoic acid forming colourless end products, resulting in a reduction of measurable absorbance with increased production of taurinechloramine, measured at 412 nm in 96 well plates.

5.2.3.1 Myeloperoxidase activity in the presence of glucose oxidase

Glucose Oxidase (GOD, from *Aspergillus niger*) was used to generate H_2O_2 which is required by MPO to produce HOCl. A 300 mM β-D-glucose solution (Glucose, Roth, Karlsruhe, Germany) was prepared in 100 mM potassium phosphate buffer at pH 7.0, and stirred for at least 3 hours to gain oxygen saturation. GOD was diluted in 100 mM potassium phosphate buffer to concentrations of 0 U/mL, 10 U/mL 20 U/mL and 30 U/mL. MPO (Sigma Aldrich, MO, USA) was diluted to concentrations of 2 U/mL. For the measurements, 100 µL of the GOD containing buffers were incubated with 100 µL of MPO buffers (2 U/mL; containing 61.5 mM guaiacol and 300 mM β-D-glucose) and peroxidation activity was measured in triplets in 96 well plates. The reaction was monitored over 45 minutes at 470 nm.

5.2.4 Glucose measurement

Glucose levels of 10 wound fluids, classified as infected, critical and good healing by a microbiologist and additionally by an experienced medical doctor, were determined according

to the protocol provided by ABCAM (Cambridge, UK; Glucose detection kit, ab102517). In this assay, glucose is specifically oxidized to a coloured product which was quantified spectrophotometrically at λ = 450 nm. The kit can detect glucose concentrations in the range of 20µM-10mM. The measurements were performed in 96 well plates using a Tecan infinite M200 platereader (Tecan, Maennedorf, Switzerland).

5.2.5 Sensor

A screen-printed amperometric H2O2 sensor was used to assess MPO activity in wound fluid samples. The sensor was used in a three electrode setting comprising a silver/silver chloride (Ag/AgCl) reference electrode, a carbon counter electrode and a modified carbon working electrode. The reference electrode and the conductive tracks of each electrode were screen printed with a polymer based Ag/AgCl ink which is cured for 1 hour at 85°C. The second production step is screen printing the counter- and working electrodes with carbon ink and a further curing step for 1 hour at 85°C. The carbon track of the working electrode is covered by an additional screen printing step using the carbon paste modified with 20% w/w manganese dioxide. For the amperometric detection of hydrogen peroxide a potential of 350 mV against Ag/AgCl was applied on the working electrode. The sensor was housed in a 2 µL measuring cell and was used in flow-through mode at a flow rate of 25 µL/min (Fig.13).

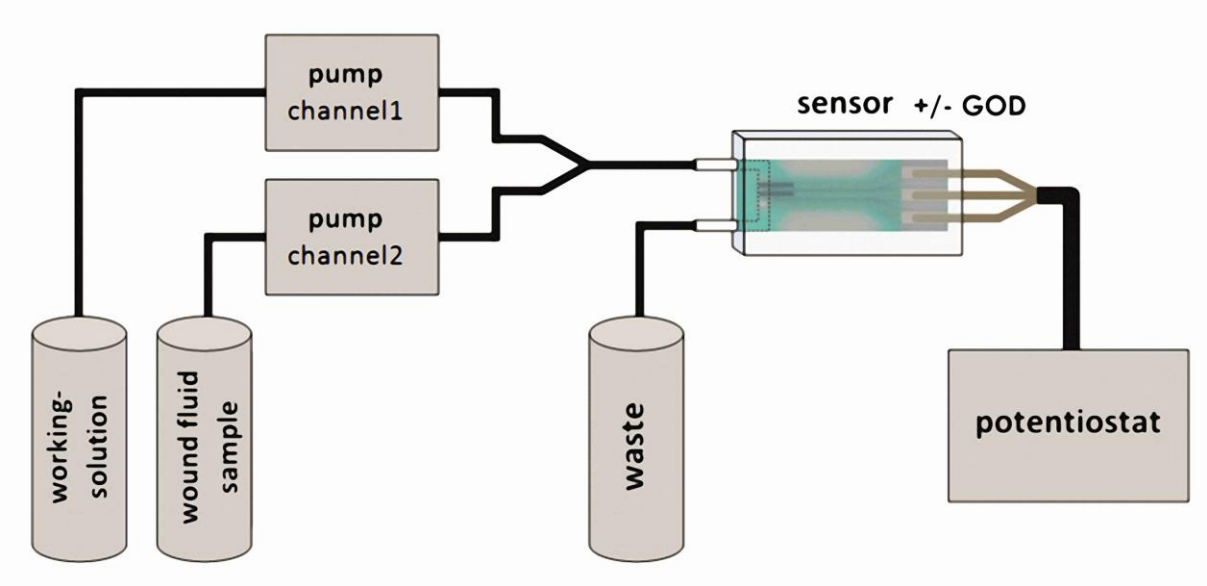

Figure 13: Sensor system: Schematic presentation of the MPO-sensor based on quantification of H_2O_2 consumption by MPO which is produced in-situ by GOD

10 µL of wound fluid were sampled using a capillary which was immersed in 90 µL of saline solution. This sample was then mixed with a solution of H2O2 (200 µM) containing 0.33 mM

KH2PO4, 66 mM Na2HPO4 and 0.9% NaCl (working solution) via a Y-connector which was placed before the inlet of the measuring cell. Alternatively, H2O2 was supplied by immobilized glucose oxidase as described below (Fig.14).

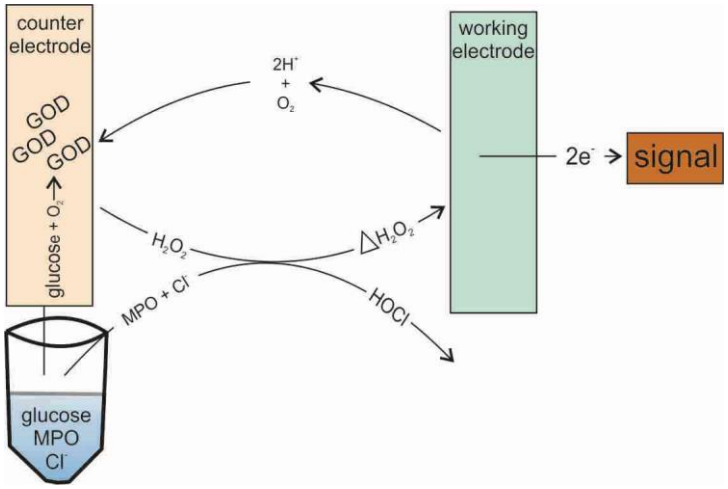

Figure 14: Scheme of the detection principle of the sensor: The sensor detects the H2O2 concentration present at the working electrode, which decreases with rising amounts of MPO. H2O2 can be delivered directly to the system or formed by immobilized glucose oxidase from glucose.

Prior to the addition of wound fluid, the sensor baseline was recorded by measuring the H2O2 solution or the H2O2 produced in situ by GOD, until a stable sensor current was reached. Upon addition of MPO solution or wound fluid the sensor current dropped, leading to a new equilibrium (Fig. 15). The relative difference of the sensor signal between the two equilibria was used to calculate MPO-activity which makes the system virtually calibration-free. As the system was used in-vitro where a working solution was mixed with the samples, we could completely control the availability of the necessary components for the reaction path. In one system arrangement a working solution containing Cl- at a concentration of 154 mM and H2O2 at a concentration of 200 µM was used. In a second system arrangement we used a working solution containing 154 mM Cl- and 1mM glucose that was converted by immobilized glucose oxidase to a maximum H2O2 concentration of 200 µM. Using a concentration ratio of 1/750 between H2O2 and Cl- ensured a sufficient excess of Cl- in the system as MPO converts equimolar amounts of H2O2 and Cl-. This native wound fluid samples naturally contained 0.3 mM glucose.

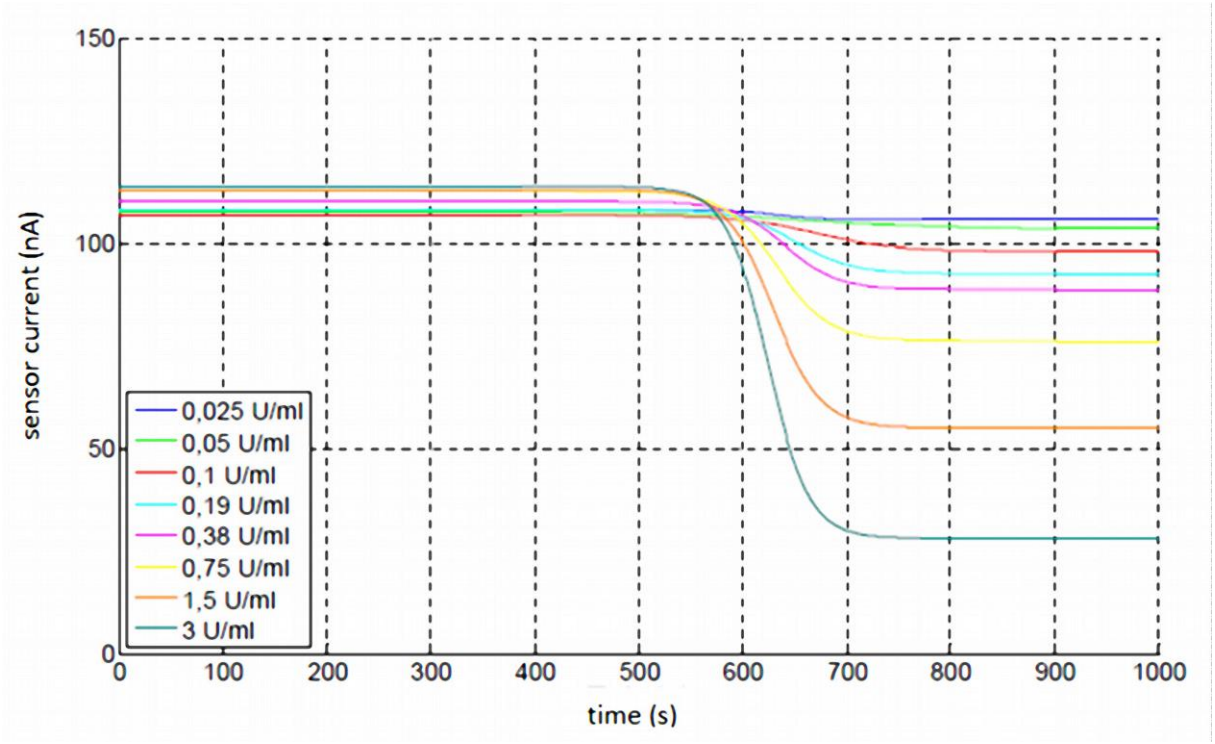

Figure 15: H2O2 measurement under different MPO concentrations: Amperometric detection of 100 µM H2O2 in presence of MPO ranging from 0.025 to 3.00 U/ml

5.2.6 Immobilization of GOD into the sensor material

10 mg GOD (200 U/mg) and 10 mg bovine serum albumin (BSA) were dissolved in 200 µL distilled water. Then 100 µL of glutardialdehyde solution (1.25 % in water) were added to the mixture to start the crosslinking reaction. The solution was then applied onto the working electrode or the counter electrode of the sensors by a microdispenser (Performus IV, Nordson EFD) and the sensor was dried at room temperature for 2 hours.

5.2.7 Statistical Analysis

To compare the different enzyme activities, two-sample t-tests assuming equal variances were performed. As described in the results section we assumed significance at P values less than 0.05. In figure 4 and figure 5 one star indicates a P value equal or less than 0.05, two stars indicate a P value equal or less than 0.01.

5.3 Results

5.3.1 Activity of myeloperoxidase correlates with bacterial burden

The determination of the bacterial load is a popular and often used standard procedure for wound analyses. To use the sensor as measurement tool it is important to compare the MPO activities in the wounds used for this study to the bacterial burden and wound status. Table 6 lists the microorganisms that were found in the different wound fluids. Apart from the non-pathogenic and mixed bacterial flora all belong to the class of potential pathogenic microorganisms (ppmos). The "+" indicate the severity of the bacterial burden whereas the number indicates the occurrence of the microorganisms, some of the microorganisms occurring more frequent than others throughout all samples. The evaluation was performed according to table 5, infection was characterized as appearance of different pathogenic microorganisms.

Table 6: Bacterial burden of the investigated wound fluids and occurrence throughout all samples; + 1 microorganism per ocular field; ++ 2-10 microorganisms per ocular field; +++ 11-100 microorganisms per ocular field

	Infected wounds		Critical wounds		Good healing	
Staphylococcus aureus	+/++	6	+	3		
Escherichia coli	++	1				
Sreptococcus equisimilis (group C)	+/++	3	+			
Pseudomonas aeruginosa	+	1				
Grampositive rods	+++	1				
Streptococcus constellatus ss constellatus	++	1				
Stenotrophomonas (Xant)maltophilia			++	1		
Serratia marcescens			+	1		
Acinetobacter bamanii complex			+	1		
Haemolytic Streptokocci group G			+	2		
Non-pathogenic flora	+	1	+/++	6	+	3
Mixed bacteria flora	+/++	3	+	1		
no flora						3

The results of the microbiological analyses correlated with MPO activity. A significant statistical difference in the enzyme activity levels of infected (P = 0.01) or critical (P = 0.02) wounds compared to good healing wounds were observed respectively (Fig 16). This correlation demonstrates the potential of MPO in a sensor for detection of infection.

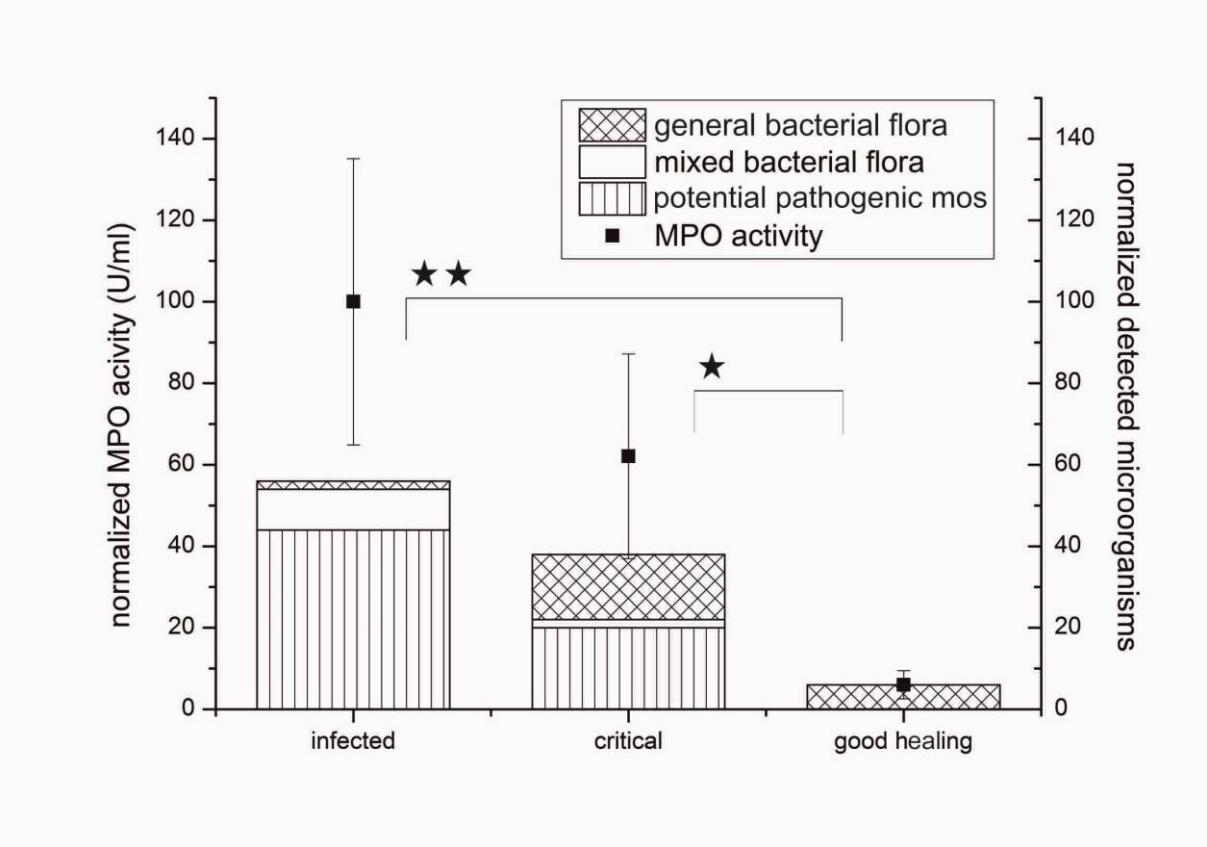

Figure 16: MPO activity correlated with the increase in bacterial burden: The outcome of the semi-quantitative microbiological study of the swabbed wounds was summarized and divided in 3 subgroups (non-pathogen bacterial- mixed bacterial flora and potential pathogen microorganisms). Additionally the average MPO activity was determined and normalized. As the appearance of ppmos is increasing in infected wounds, the MPO levels of infected and critical wounds are also significantly higher than MPO levels of good healing wounds. One star indicates a P value equal or less than 0.05, two stars indicate a P value equal or less than 0.01.

5.3.2 Chlorination activity in wound fluids

Recently we have shown that MPO-peroxidation activity on guaiacol is a suitable marker for the detection of infection in wound fluids [25]. However, guaiacol is not compatible with the MPO sensor system, causing interference signals and elevated baselines. In this study, in a first step MPO-chlorination activity – the formation of HOCl from chloride and H2O2 as a parameter for measuring wound infection was evaluated. Indeed, significantly higher MPO-

chlorination activity was detected in infected wound fluids when compared to non-infected "good-healing" (P = 0.01) wounds or blisters (P = 0.003). These results were compared to MPO-peroxidase activity of the identical wound fluid samples. Similarly, these significant differences were also found when comparing infected/ critical wound fluids and good healing wounds, which was in agreement with the clinical description resulting in P values of 0.01 for infected/ good healing wounds and P values of 0.02 for critical/ good healing wounds respectively. When plotting the MPO-chlorination and MPO-peroxidation activities of different wound fluids, the average result of each method was calculated and no significant differences regarding the two different methods were observed (Fig 17). Both MPO-activities actually fitted well to the clinical description. Consequently, for the first time we demonstrate that in systems (where guaiacol is not a suitable substrate for MPO) MPO-chlorination activity can likewise indicate the status of a wound.

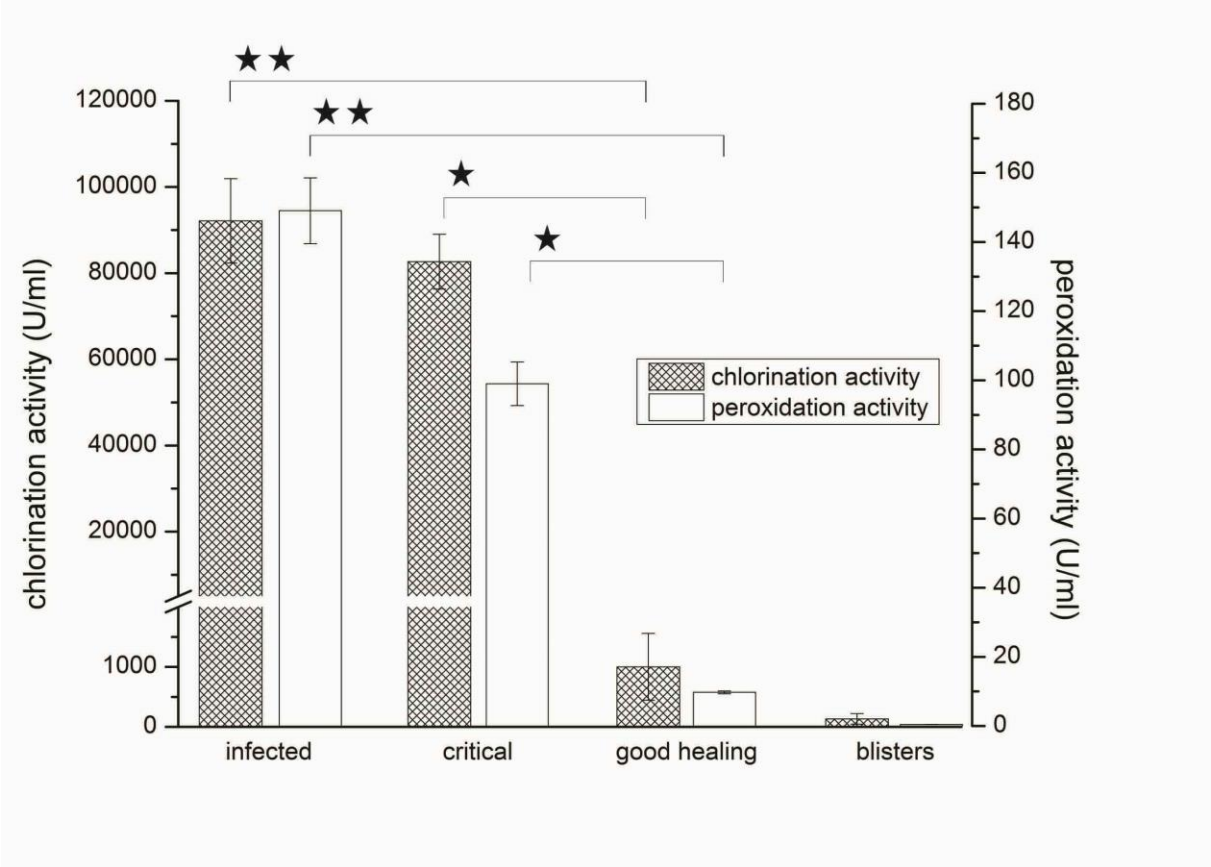

Figure 17: The chlorination activity of MPO is comparable to its peroxidation activity in wound fluids: Chlorination activity measurement was based on trapping of HOCl formed from chloride and H2O2 by using taurine. The differences in absorbance were measured in several wound fluids whereas guaiacol was used as a substrate to measure the peroxidase activities of the MPO. As a standard, commercial MPO was used to calculate the activities. The figure shows the means of the measured activities in each group. Significant differences between infected/critical and good healing/blister

wounds can be detected independent of the measurement method resulting in P values of 0.01 for infected/ good healing wounds and a P value of 0.02 for critical/ good healing wounds indicated with one or 2 asterisks in the figure.

5.3.3 In situ formation of H2O2 by glucose oxidase as co-substrate for MPO

The glucose levels of 10 wound fluids with different characterisations were tested. Glucose levels ranging from 0.1 to 4.2 mM with an average concentration of 0.9 mM and a standard deviation of 1.45 mM were measured. No correlation of the glucose concentration with the classification or the type of the wound was observed.

*O*xidation of guaiacol by MPO as well as the formation of HOCl require H_2O_2 as a co-substrate [23]. For test kits and/or sensor systems for the detection of wound infection, H_2O_2 as reagent should be avoided due to limited storage stability. Therefore, different concentrations of GOD in the presence of glucose, for *in situ* generation of H_2O_2, were tested over a long period of time. A stable signal regarding guaiacol oxidation rate was obtained when H_2O_2 was produced *in situ* as compared to *ex situ* addition of H_2O_2 (Fig.18). The best results were obtained with 10 U/ml GOD resulting in a constant guaiacol oxidation rate. Additionally we could show that 0.1 mM glucose is sufficient to provide enough H_2O_2 for visible substrate conversion. A supply of 0.5 mM glucose resulted in a saturation effect, no dependency of the guaiacol conversion (changes in the OD values) on the glucose concentration up to 200mM was observed (data not shown).

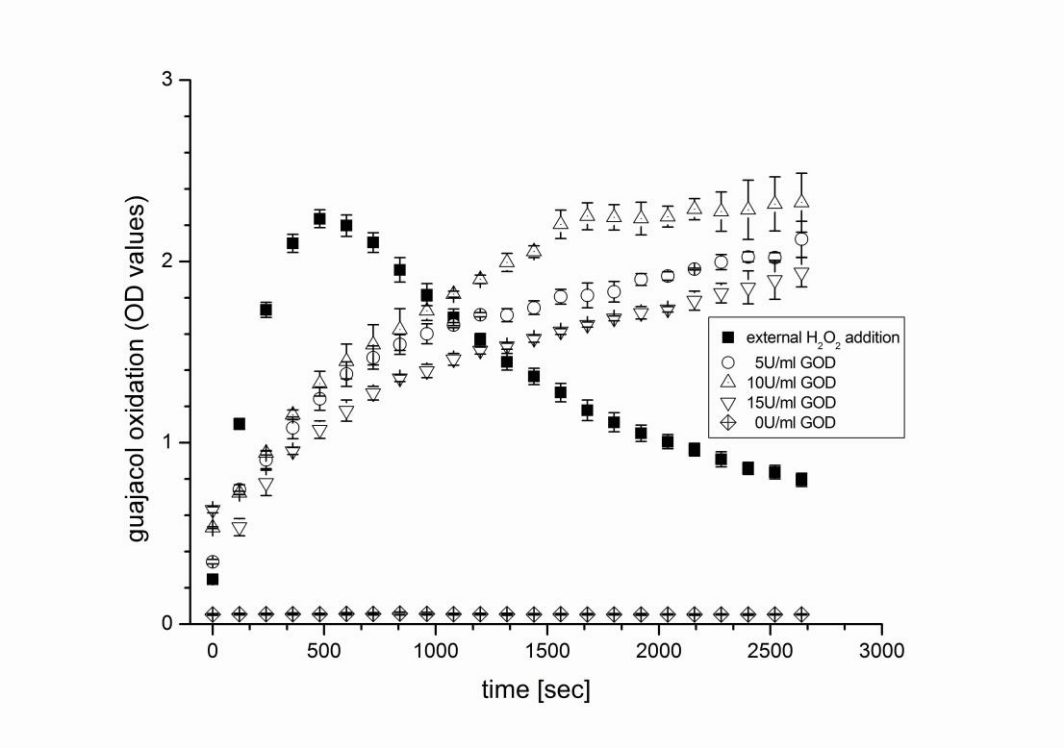

Figure 18: GOD provides stable H2O2 supply: Comparison between external addition of H2O2 and H2O2 generation by GOD required for guaiacol oxidation by MPO over a long time period. Reactions with added GOD for H2O2 supply stabilize the reaction system.

Interestingly, this enzymatic H_2O_2 production system was also adaptive to wound fluid environments. MPO-activity in blisters, good healing and critical wound fluids was determined in the presence of GOD instead of added H_2O_2. The increasing OD values (measured at 470 nm) were monitored for 45 minutes and the values regarding infection can again be related to the microbiological analyses. The statistical analyses were performed with a single factor variance analysis (ANOVA) of different time points and showed statistically significant differences (Fig. 19).

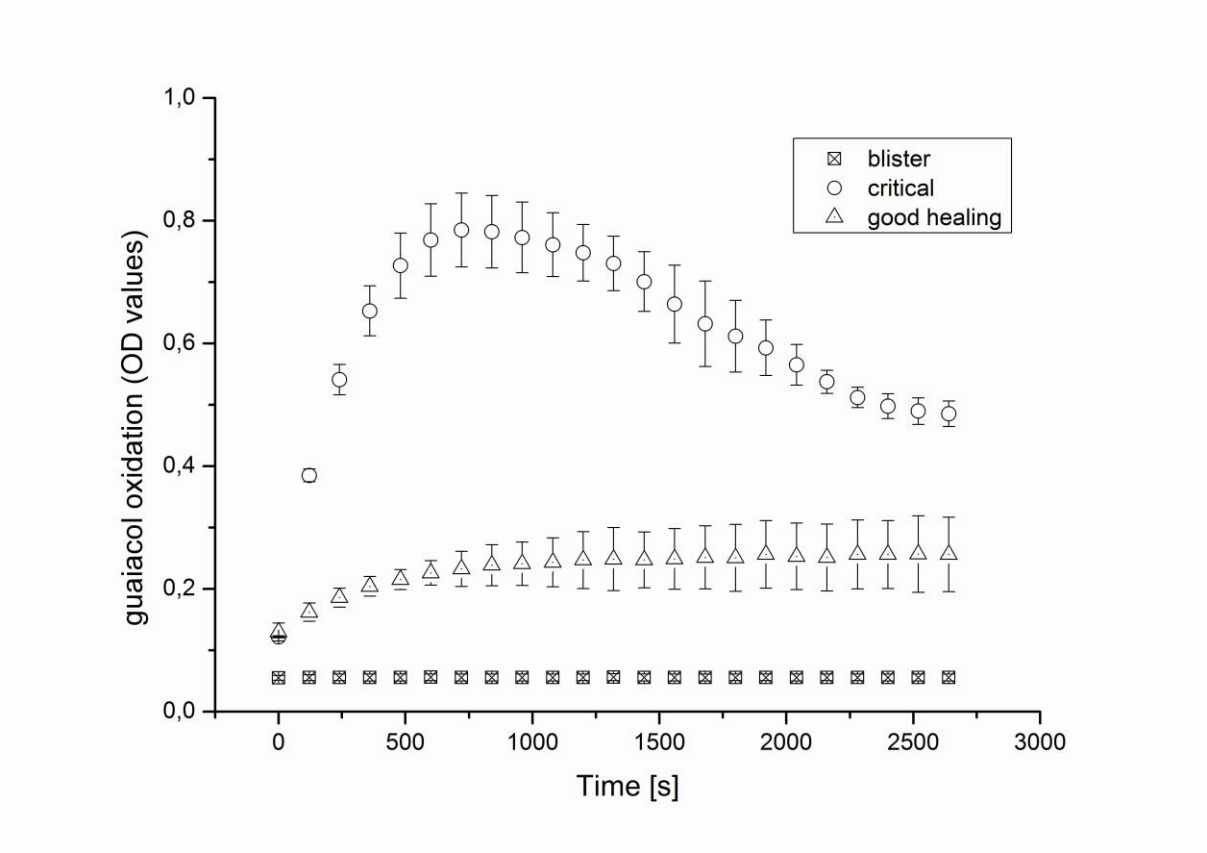

Figure 19: GOD can provide H2O2 supply in wound environments: Measurement of MPO activity over 45 minutes in three different groups of wound fluids in the presence of GOD for in-situ H2O2 production. The blister fluid (negative control) did not show a response to the GOD/MPO system. Critical wounds differ statistically significant in their guaiacol oxidation showing high absorption values over a long time period.

5.3.4 Measurement precision of the electrochemical H2O2 sensor

The sensors used to measure H_2O_2 concentrations exhibited excellent linearity in the range from 1 to 125 µM H_2O_2 (Fig. 20B). Sensor precision between measurements of a single sensor showed a coefficient of variation of 1.2%, as assessed by 10 consecutive measurements of a test solution containing 100 µM H_2O_2. The sensor to sensor variation was 17.4%, determined by 10 measurements with 10 sensors. The precision of the resulting signal decrease induced by an MPO activity of 0.75 U/ml was 2.4% (average ± SD: 16.2 ± 0.4%, n = 6). The sensor to sensor variability of the signal decrease was 11.3% (average ± SD: 14.2 ± 1.6%, n = 6). As uric acid can be present in wound fluids in a concentration of up to 751 µM [17], uric acid was tested as a potential interferent for the amperometric H_2O_2 sensor. 750 µM uric acid caused a maximum bias of 5% of the sensor current in 5 tested sensors which is assumed no significant interference according current guidelines [30].

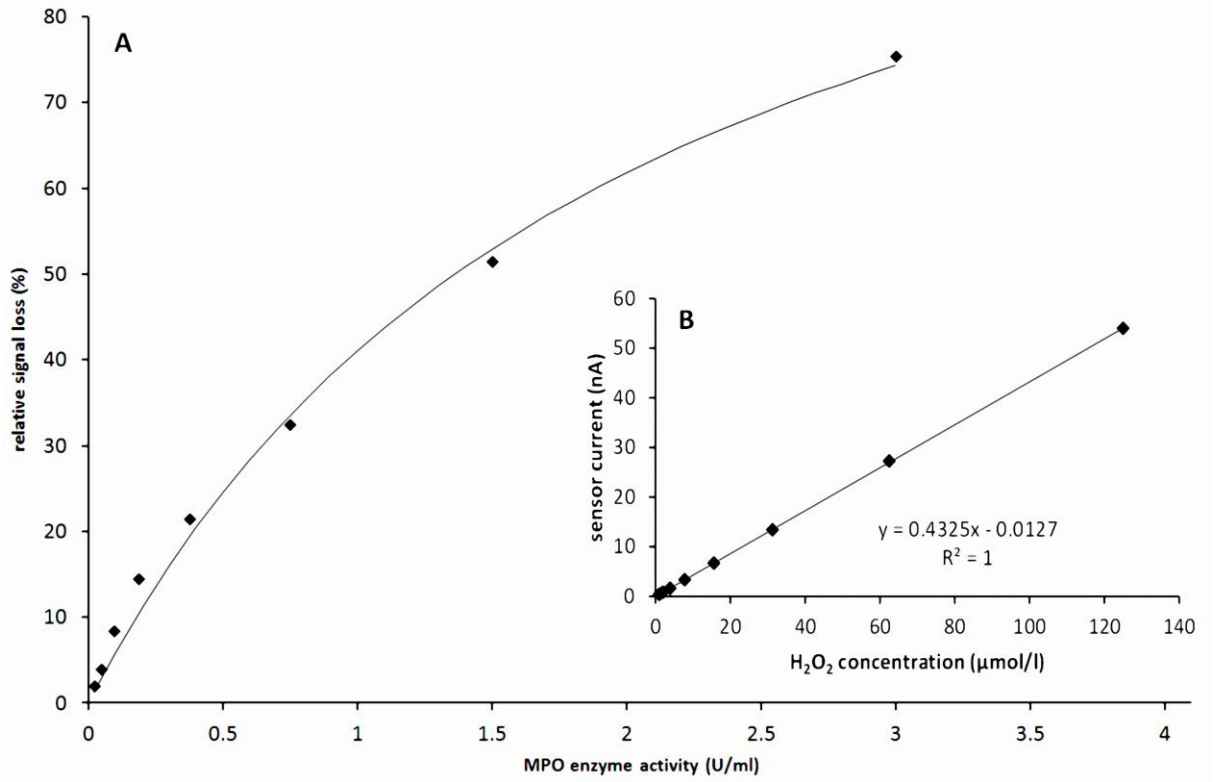

Figure 20: Calibration of the sensor: A: Characteristic calibration curve showing the correlation of MPO-activity and relative signal loss measured by the hydrogen H2O2 sensor (diamonds), which can be approximated by a Michaelis Menten like function (dark line).B: Dose response characteristics of the sensor covering the relevant H2O2 concentration range.

In general, the sensor signal decreases with increasing enzyme activity, although the relationship is not linear (Fig.20A) except at low enzyme activities, where the enzyme is present almost solely as enzyme-substrate complex over the entire reaction time of 30 seconds. The non-linear characteristics can be described by Michaelis Menten kinetics (see equation), except that, instead of reaction rate, the relative signal drop (SD) is used, and substrate concentration is replaced by enzyme activity (a),

$$SD = \frac{SD_{max} \cdot a}{K_a + a}$$

where SD_{max} is the maximum relative signal loss and K_a is the enzyme activity (in U/ml) at half SD_{max}. The model parameters were calculated as SD_{max} = 124.57% and K_a = 2.03 U/ml. Because too few data points were recorded in the saturation range (K_a > 3 U/ml), the calculated SD_{max} value exceeded the theoretical maximum value of 100%.

The linear range of the measuring system can be tuned by varying the H_2O_2 concentration in the working solution. The measuring range could be extended to higher enzyme activities by increasing the H_2O_2 concentrations in the working solution or by reducing the reaction time. Since for infection detection high sensitivity in the low activity range is important the parameters were set to reach balance between highly sensitive measurements in the low activity region and also sufficient sensitivity for higher enzyme activities.

5.3.5 Sensor measurement of clinical wound samples

15 different wound samples classified as infected, critical or non-infected were measured with the H_2O_2-sensor. The difference between infected, critical and non-infected samples was statistically significant for all measuring techniques (Fig.21).

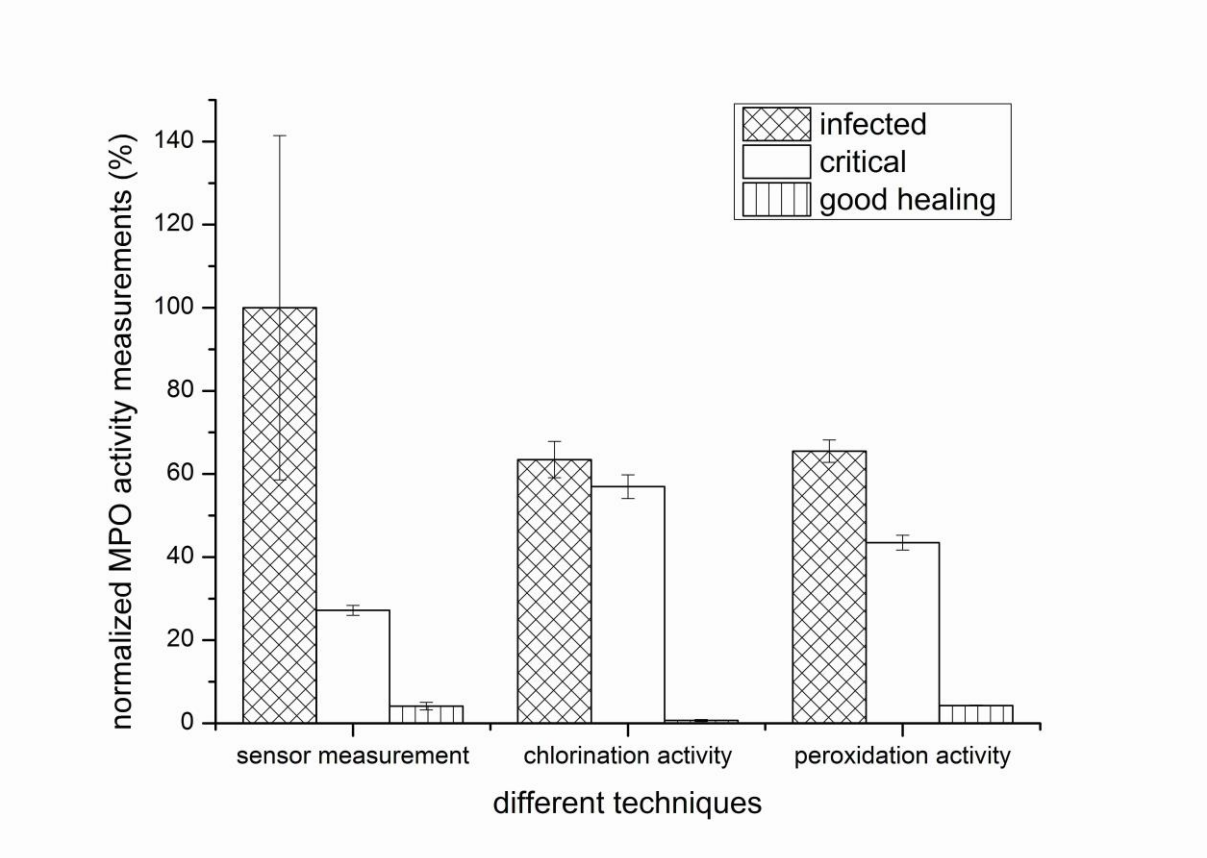

Figure 21: Comparison of different analyses of wound fluid: H_2O_2 -sensor based analysis of wound samples in comparison to MPO-chlorination/ peroxidation activity measurements. In all three experimental setups the same infected/ critical and good healing wound fluids were used. To compare the different techniques, the highest activity was scaled to 100% and the other activities normalized to this value. The figure shows the means of the normalized activities in % of each group. Significant differences between infected /critical and good healing / blister wounds were detected independent of the measurement method.

However, despite successful differentiation of infected and non-infected wounds the actual MPO activity in infected wounds showed a larger variation than in non-infected wounds. This may be due to the fact that the response of the human immune system to infection in terms of recruitment of neutrophil granulocytes and consequent enzyme activities depend on many parameters like the patient, appearance of the wound (dry/ moist wound bed) and on the origin of the wound (ulceration, amputation).

5.3.6 Implementing the GOD / H2O2 system in the electrochemical sensor

In a first layout GOD was immobilized on the working electrode of the sensor. The enzyme produced H_2O_2 in close vicinity to the working electrode where H_2O_2 was immediately detected. For this layout a fast sensor response was expected, but it took some time to reach equilibrium in terms of H_2O_2 consumption by MPO present in the wound fluid. In a second layout GOD was immobilized onto the counter electrode of the sensor which was located in front of the working electrode in flow direction. The response time to H_2O_2 was increased in this layout but as H_2O_2 was produced on the counter electrode there was more time to reach the equilibrium in terms of H_2O_2 consumption by MPO. For this reason a higher sensitivity of the sensor to MPO activity was expected when the GOD was immobilized onto the counter electrode. The sensor currents measured with this layout and test solutions containing 1mM glucose were comparable to 100 µM hydrogen peroxide. The hydrogen peroxide concentrations generated by GOD was sufficient for the detection of wound infection via the proposed MPO route (Fig.22).

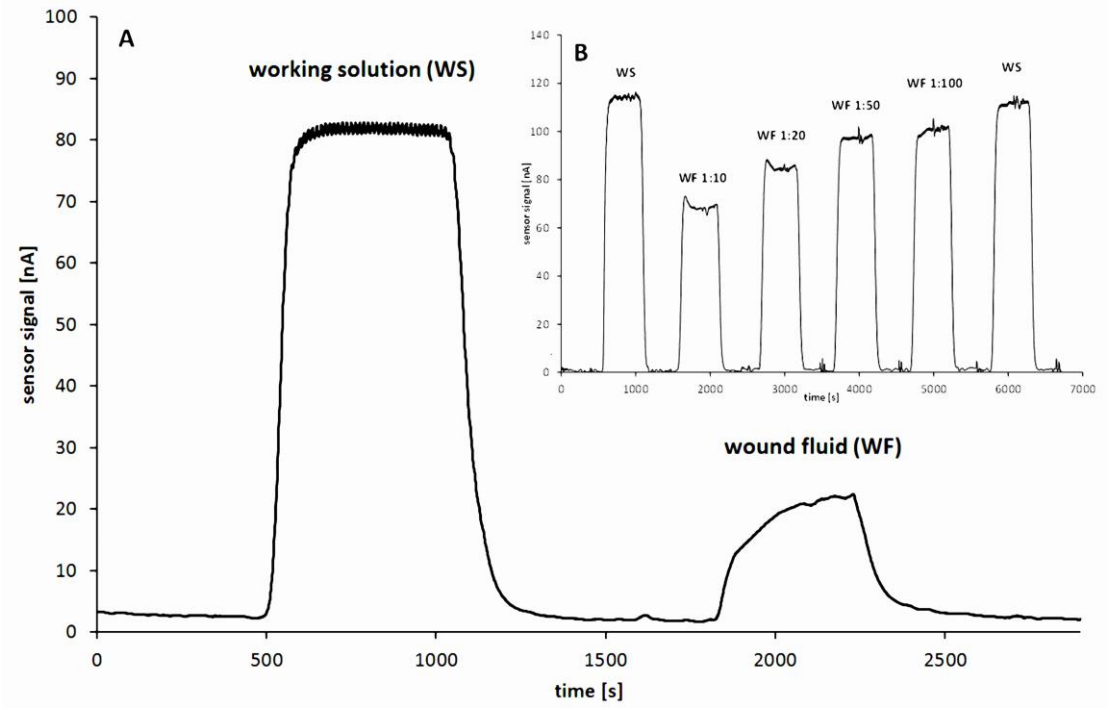

Figure 22: The sensor system uses glucose in wound fluids as GOD substrate: A: Analysis of wound fluid samples with an H2O2 sensor containing immobilized GOD; working solution containing 1 mM glucose and native wound fluid sample naturally containing 0.3 mM glucose; B: Working solutions containing 1 mM glucose with wound fluid in various dilutions.

5.4 Discussion

Fast detection of infection in chronic wounds and surgical sites followed by an adequate treatment is essential to support the natural healing process and can be a life - saving issue [31-33]. Although a variety of analysis methods ranging from optical to chemical (i.e. pH) have already been assessed for wound characterizations, there is still no simple and fast device for the detection of wound infection available [34-38].

We have developed a new electrochemical sensor system based on recent reports on neutrophil dependent enzymes as warning markers for wound diagnostics via the detection of MPO-activity in wound fluids [19, 25]. Compared to standard techniques, the MPO activity correlated with the increase in bacterial burden and appearance of pathogen microorganisms. A significant difference in enzyme activity between wound fluids diagnosed as infected and good healing wounds ($P = 0.01$) as well as critical wounds and good healing wounds ($P = 0.02$) could be determined. MPO activity has been quantified in wound fluid samples based on oxidation of guaiacol due to haemoglobin interference with other substrates [25, 39]. However, substrates based on phenolic compounds are not suitable when consumption of H2O2 is to be followed with electrochemical sensors [40]. Therefore,

MPO-chlorination activity was assessed as an alternative despite little knowledge on occurrence of this reaction in the wound matrix. Nevertheless, the results of this study indicate a very good correlation of MPO-chlorination and MPO-peroxidation activity in wound fluids with both showing significant differences in infected and non-infected samples ($P = 0.01$)..

A second improvement of the sensor system was the addition of a H_2O_2 providing enzyme in the system, in situ H_2O_2 production. As glucose is known to be present in wounds, we demonstrated that immobilized GOD can supply enough H_2O_2 as required by MPO simply from glucose present in wound fluids [17, 18]. GOD is a very robust enzyme which is widely used in commercial glucose sensors and previously employed for H_2O_2 supply in many applications [41-45]. The major advantage of in situ production of hydrogen peroxide production by GOD is to avoid limited storage possibilities of working solutions potentially containing hydrogen peroxide [46]. Former guaiacol based measurements showed that a concentration of 0.1mM glucose was sufficient for adequate in situ H_2O_2 production for guaiacol conversion. Concentrations exceeding 0.5 mM glucose resulted in substrate saturation (data not shown). The glucose concentration measurements of 10 wound fluids resulted in levels between 0.1 to 4.2 mM with an average concentration of 0.9 mM and a standard deviation of 1.45 mM glucose. This was in accordance to measurements of Iizaka et al [47] as well as Trengove et al [17], presenting medians of 2.2 mM and 1.8 mM respectively. These studies also included 25% - 30% patients suffering from diabetes. Both studies and our measurements did not show elevated glucose levels, indicating that glucose levels in wound fluids from diabetes patients do not differ from those of patients without diabetes. The double enzyme system was suitable to detect infection in wound fluid environments based on MPO activity. Sensors measuring glucose at physiological concentrations from 3 to 30 mM need membranes which limit the glucose diffusion to the working electrode to achieve a dynamic sensor response over the full physiological concentration range. In our case, the enzyme glucose oxidase is freely accessible for glucose which allows unrestricted glucose diffusion. Glucose oxidase is therefore saturated with glucose even at low glucose concentrations of 1 mM. The corresponding hydrogen peroxide concentration produced by the enzyme is stable when the threshold glucose concentration is exceeded and only little influence was observed in the range from 0.1 to 1mM glucose. So the hydrogen peroxide concentration is not limiting the measurement of MPO activity.

We could demonstrate that the glucose level does not influence the response of the sensor since even at low glucose concentrations the hydrogen peroxide concentration is not limiting the measurement of MPO activity. Moreover high concentrations of glucose do not affect

MPO detection as glucose oxidase is saturated with glucose at a concentration of 1 mM. As we show in figure 10a, the sensor delivers reliable results using the glucose concentration present in this wound fluid sample. In other applications for infection detection where samples might not contain glucose, simple addition of 1mM glucose to the working solution as in figure 10b could be an alternative.

Summarizing the results of this study, we have demonstrated the potential of a new sensor system for the detection of wound infection based on the electrochemical quantification of H2O2 consumed by MPO-chlorination activity. Future investigations including a larger clinical study could pave the way towards a new and fast diagnostic system for infection.

5.5 References

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6 Enzyme responsive polymers for microbial infection detection

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There is a pressing need for point-of-care diagnostics indicating early stages of infection. Polymers can respond to enzymes secreted by microorganisms or released by the human immune system. This provokes either a direct colour reaction or a release of dyes, allowing early stage detection of wound infections and contamination of medical devices. Conventional methods for the detection of infection indicators are based on slow, laboratory-based procedures and consequently do not allow a timely assessment. In contrast polymer-based materials offer real-time responses in point-of-care devices that in turn allow therapists to amend treatment before infection has become firmly established. The use of protein, polysaccharide and mixed polymer systems provides a sensitive means to detect the low levels of proteases and glycosyl-hydrolases produced on infection initiation in the clinical setting. These polymers can be easily fabricated into various forms that can be directly applied in diagnostic devices.

6.1 Enzyme responsive systems

A central issue in the diagnosis and therapy of infection is to detect or predict a proto-infection before there are large numbers of microorganisms present. The logic is simple: where there are many microorganisms present, pathology is worsened, biofilm is already present and selection base for tolerance and resistance is dramatically increased. One approach to this issue is the implementation of infection detection systems at the point-of-care or point-of-use.

Vital tools in this approach are materials that respond to the infected or proto-infected state. Like other aspects of diagnostics where enzyme elevation is the main indicator of pathology, infection also leads to release of enzymes that are early biomarkers of the presence of pathogenic microorganisms.

Enzyme-responsive systems change their physical properties as a result of an enzymatic conversion of the material [1]. They can be used for rapid detection of contamination or infection in biomedical materials, body fluids and wounds, as well as in technical applications, food packaging or household devices. This report focuses on smart polymers that change their physicochemical properties in response to enzymes and distinguishes them from bio-responsive polymers that can generally react to a variety of stimuli like pH, temperature, light or electrical fields [2]. Various enzyme-responsive biopolymers are described in the literature including polysaccharides and proteins together with synthetic aliphatic polyesters and polyphosphoester (PPE) [3]. The selected systems react to the presence of enzymes in their environment producing a colour reaction allowing simple visual detection of contaminating microorganisms or of infection. Triggering enzymes secreted directly from pathogenic/contaminating microorganisms include proteases, cellulases and pectinases. Enzymes indicating infection in wounds include human neutrophil elastase (HNE), matrix metalloproteinases (MMP), cathepsin G, myeloperoxidase (MPO) and lysozyme, and are derived from the human immune system. Each can act on correspondingly derivatised polymers to generate indicator reactions. Figure 23 gives an overview on enzyme-responsive polymers together with their physical forms primarily used.

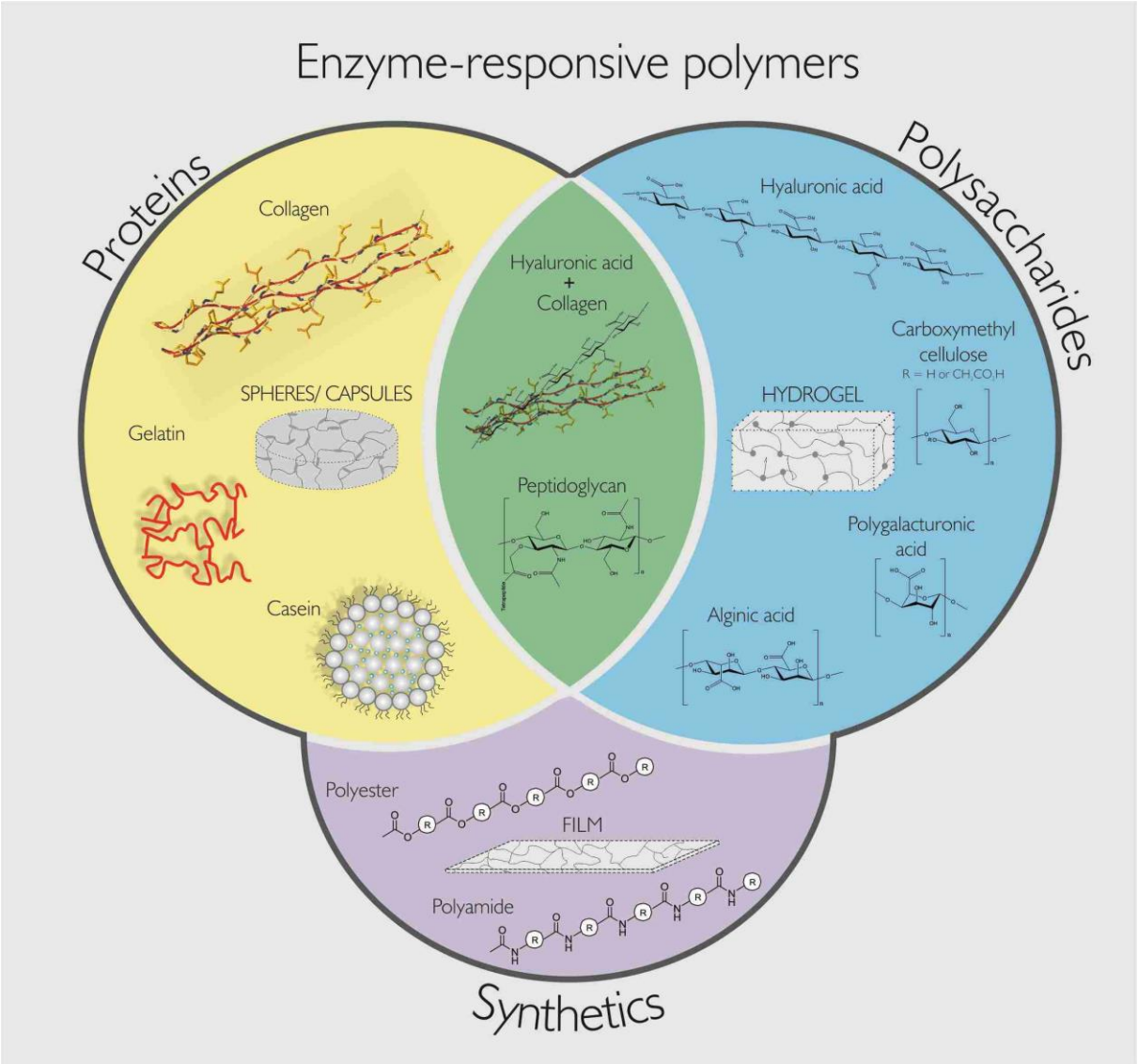

Figure 23: Overview of three distinct groups of enzyme-responsive biopolymers (proteins/polysaccharides) and synthetic polymers according to their main components, presented in this special report. Protein derived spheres or capsules mainly consist of casein, gelatine or collagen. Hydrogels are composed of different polysaccharides like hyaluronic acid, or peptidoglycan. The synthetic polymers composed of polyesters and polyamides are used and described as films. Overlapping of main groups was found for collagen and hyaluronic acid.

6.2 Enzyme responsive proteins

Infections in wounds or contamination of medical devices constitute a burden and risk for patients. In recent studies, activities of enzymes secreted by the human immune system were demonstrated to be elevated in infected wounds [4,5]. Due to an excessive stimulation of neutrophils and monocytes, and their subsequent lysis, the proteolytic enzymes human neutrophil elastase (HNE), cathepsin G, gelatinase and matrix metalloproteinases (MMPs) are released in the wound bed and can be detected by analysing the wound fluid per se [6,7]. HNE was detected using an amino acid sequence specific for HNE cleavage (MeOSuc-AAPV-pNA) and hydrolysis of the peptide leads to the release of the bright yellow p-nitroaniline indicating elevated enzyme activity. A variant of this specific HNE substrate was immobilized on enzymatically modified polyamide (PA), polyester (PES), and various protein-based materials such as collagen, collagen conjugates and modified hyaluronic acid [6][8]. Collagen, a structural protein, is involved in various steps of wound healing and due to its properties represents a very important biomaterial for chronic wound dressings [9]. The release of p-nitroaniline from these polymers differed between infected and non-infected wound fluids when using collagen or collagen/hyaluronic acid conjugates. Similarly, PA and PES derivatives could be used as a carrier for the HNE peptide substrate for infection detection. Enzymatic modification of the latter materials introduced novel amino and hydroxyl groups on the polymer surface allowing an increased substrate load that further enhanced colour formation. For clinical applications that directly contact the wound bed, a barrier has to be added to avoid leaking of the feasible substrate components into the wound bed.

A different strategy for infection detection involved enzyme-responsive protein particles with covalently bound dyes. In this approach the colour formation was not achieved by substrate conversion per se, but by the enzymatic degradation of the polymer particle subsequently releasing dyed fragments (Figure 24).

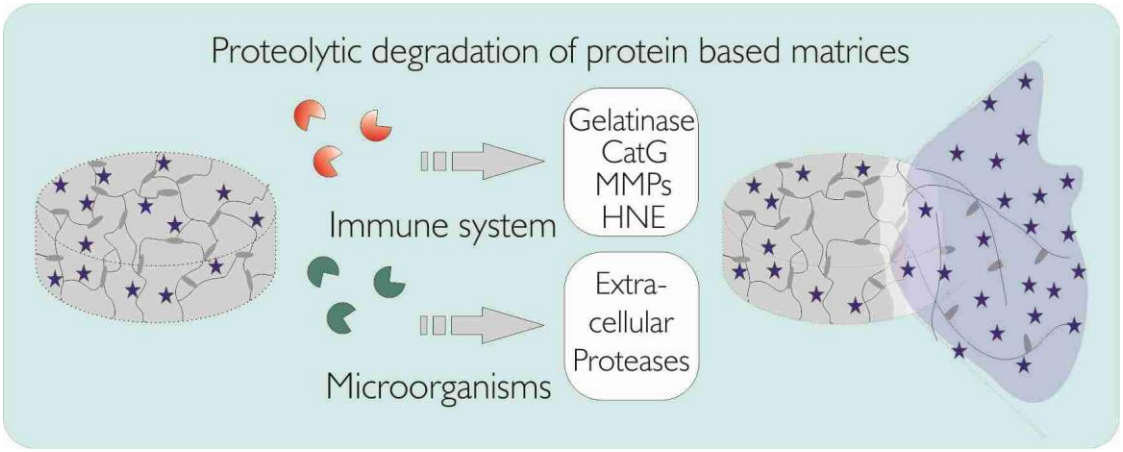

Figure 24: Schematic representation of proteolytic degradation of protein based particles. The particles were loaded with a respective dye. Immune system derived enzymes (Gelatine, CatG, MMps, HNE) or enzymes secreted by microorganisms (Extra cellular proteases) degrade the particle wall and enable a visible dye release.

For this purpose, gelatine was heated, dried and pressed through a sieve to obtain equal sized beads. Those beads were then loaded with Reactive Blue. High levels of MMPs, or gelatinolytic enzymes in general, led to a visible colour release within 30 minutes of incubation with the beads. The incubation of the beads with infected vs. non-infected wound fluids, caused a 34-fold increase of dye release (Figure 25) [7]. Beads are a good example for controlled release systems, used outside the wound bed, in e.g. test stripes, providing a high release output of dye (or dye fragments) due to their big surface.

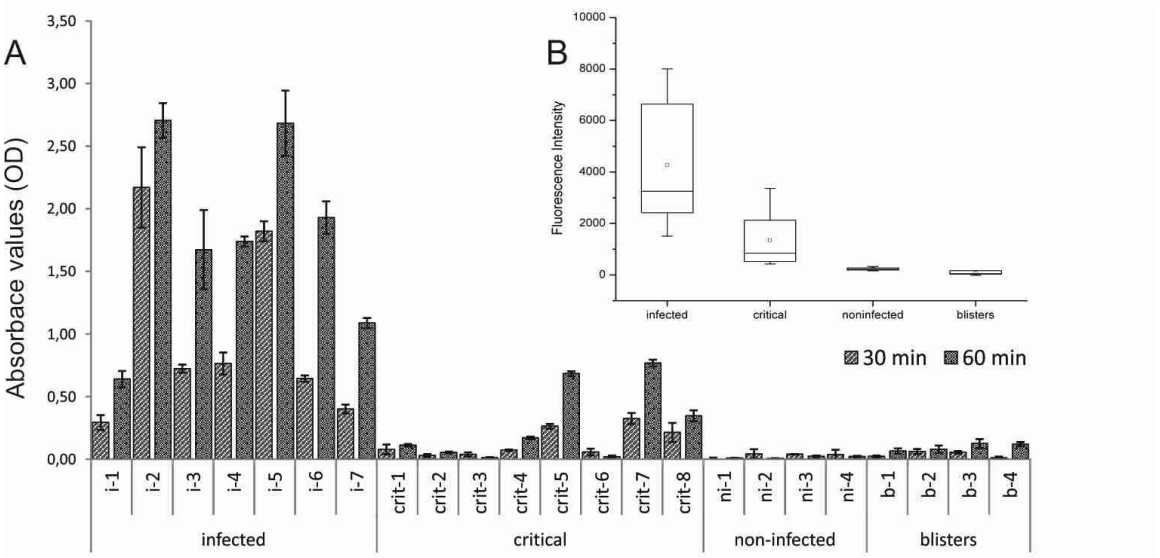

Figure 25: Box plot diagram displaying the gelatinolytic activities in wound fluid samples. The fluorescence activity of the samples was measured to calculate the gelatinolytic activity. Eight infected wounds, eight critical wounds, eight non-infected wounds, and five blisters were investigated. A

significant difference between enzyme activities in infected and non-infected wound fluids was observed ($p < 0.001$).

B: Incubation of gelatine beads with different wound fluids. The absorbance change was caused by the dye release of the beads. The measurements were performed upon incubation with wound fluids of infected, critical, and non-infected wounds for 30 and 60 minutes. Additionally, blister fluid was used as a negative control. Modified figure, permission obtained by Wiley [7].

Many surfaces suffer from contamination or infection, but also medical products and therapeutics can be minimally contaminated but not visibly changed. Medical devices and therapeutics like serum, platelet concentrates (PC) or other injectables would be improved if they had an integrated sensor device that indicated a non-sterile batch. [10].

Most saprophytic bacteria associated with human skin such as *Staphylococcus epidermis, S. aureus, Propionibacterium acnes* release extracellular proteases that can serve as contamination biomarkers [11]. An enzyme-responsive system consisting of methacrylated casein was established. Casein was labelled with Reactive Black and lyophilized. To enhance stability, the labelled protein was cross-linked with glycidyl methacrylate (GMA) using TEMED as polymerization starter (Figure 26).

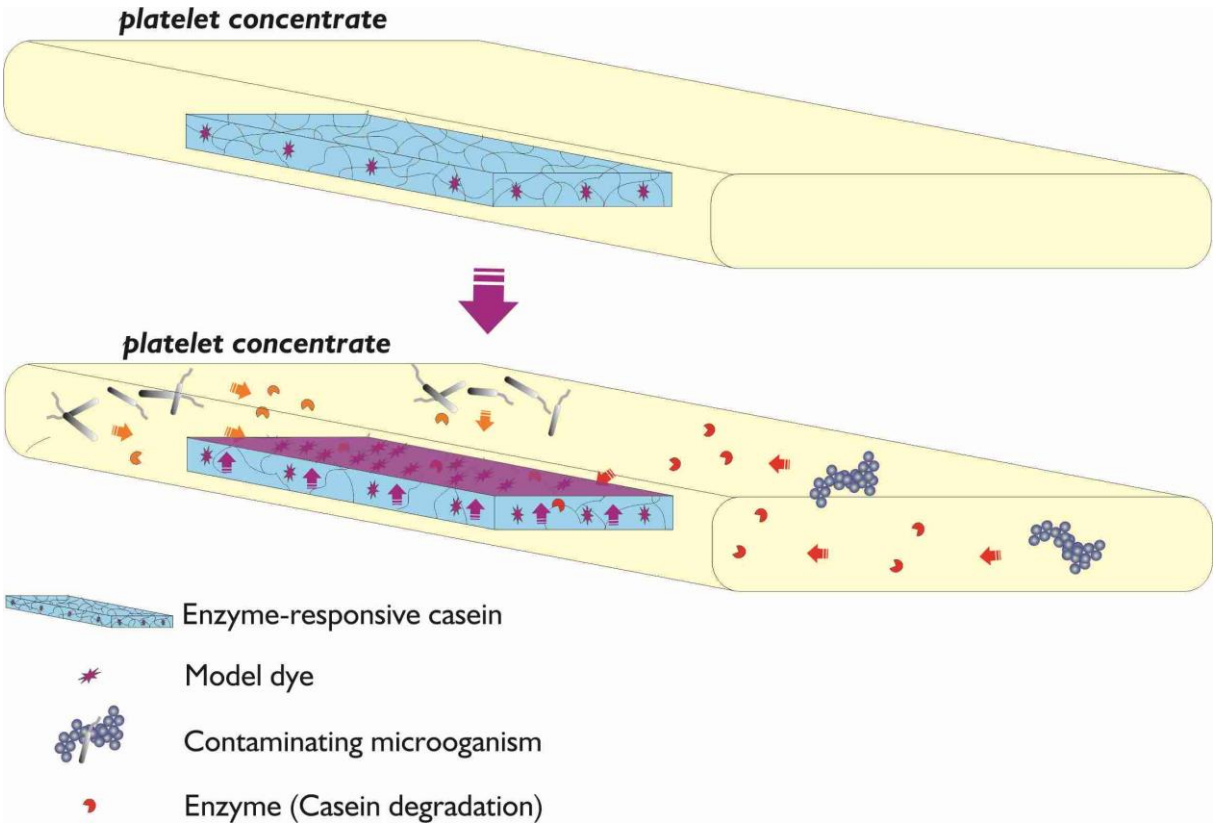

Figure 26: Schematic representation of the enzyme-responsive system integrated into a plasma concentrate bag. The system consists of a hydrogel of methacrylated and cross linked casein

functionalized with a dye (Reactive Black). Hydrolysis of the system by proteases secreted by contaminating microorganisms leads to a visible colour change.

The hydrogels were incubated with platelet concentrates containing either protease solutions or *S. aureus* suspensions. The colour change in the supernatant as well as the colour change in the hydrogel itself was recorded. A controlled colour release was observed after 20 minutes upon addition of proteases and after 24 hours incubation with 1 x 10^3 CFU/mL S. aureus at room temperature [11]. The rapid, enzyme dependent colour reaction of the enzyme-responsive polymer demonstrated the potential of enzyme-triggered systems to indicate sub-visible contamination [12]. Since the sensitivity was within the magnitude of the commercially available systems for contamination monitoring, the likelihood of transfusion-transmitted infections could potentially be lowered by the use of this indicator because it can be incorporated in all vessels and thus indicate breaches of hygiene not only in a batch, but also in specific containers which may be damaged in handling or storage. Conventional microbial analyses can, and is, applied to batch monitoring but cannot be feasibly applied to single containers. The model substances used in this study would have to be changed in future experiments according to legal regulations of medical products and a trapping of the soluble, released dye within the system has to be guaranteed.

6.3 Enzyme responsive polysaccharides

Polysaccharides are an essential component of microbial cell walls and membranes. Most organisms possess wall-degrading enzymes as a form of innate defence, the best known example being egg white lysozyme. Figure 27 schematically describes the enzymatically degradation of polysaccharide based matrices.

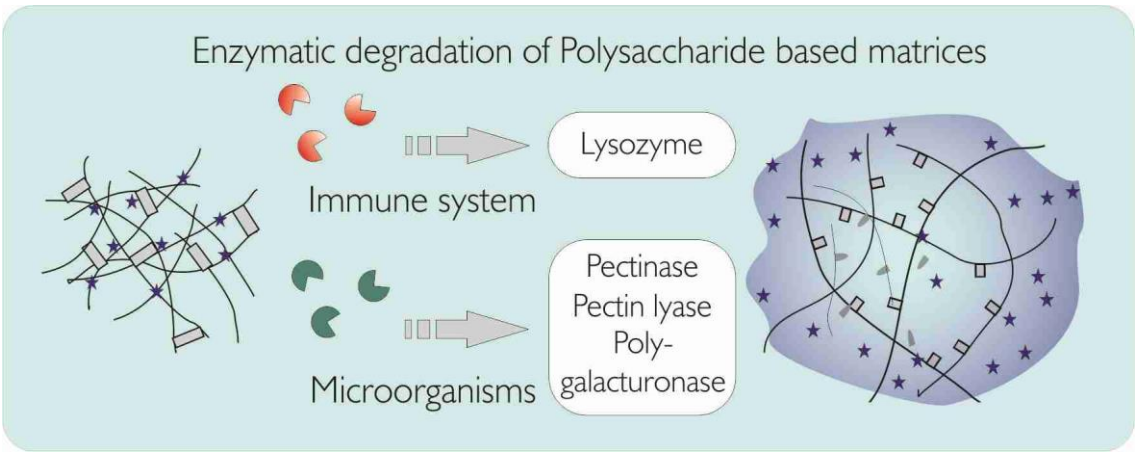

Figure 27: Schematic representation of the enzymatic degradation of polysaccharide based materials. Enzymes of the immune system (Lysozyme) or secreted enzymes of microorganisms (pectinase, pectin lyase, polygalacturonase) are capable of degrading the matrix and allow the release of covalently-bound dyes.

Amylase, pectinase and cellulase are all variants of this general glycosyl-hydrolase family. Peptidoglycan is a generic cell wall polysaccharide class found in human saprophytes and pathogens. Thus, changes in peptidoglycan integrity are an indicator of hydrolases like lysozyme which is also an integral enzyme of the mammalian innate immunity [13]. Enzyme-responsive systems with incorporated dyes were constructed using peptidoglycan [14,15], carboxymethylcellulose [16,17], methacrylated polygalacturonic acid and alginic acid [18]. Elevated bacterial burden in wound fluids is correlated to increased lysozyme concentrations in response to wound infection (Figure 28) [15].

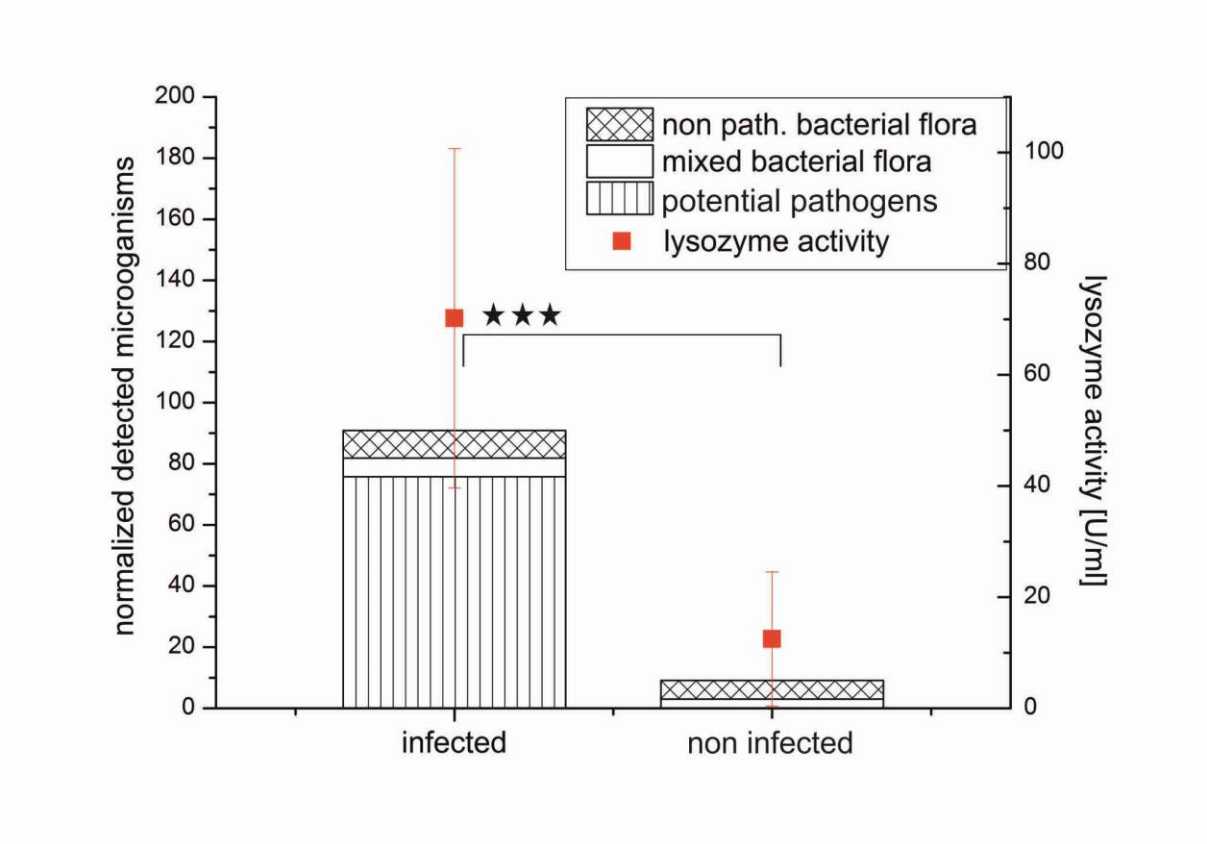

Figure 28: Increasing lysozyme activity is associated with increasing bacterial burden in infected wounds. 14 wound fluids from ulcer and post-operative wounds were swabbed and analysed. The bacterial load was determined by Gram staining and counting. The percentage per group was calculated related to the infected and non-infected wound fluids. The lysozyme activity was given in percentage of the average results. As bacterial count of potentially pathogenic microorganisms increased, there was an increase in lysozyme activity detected. Statistical analyses resulted in P values of 0.001 (indicated by the asterisks in the figure) using a two-sample t-test assuming equal variances when comparing the enzyme activities from infected and non-infected (good healing) wound fluids [15]. Permission obtained by Elsevier.

Lysozyme hydrolyses glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine residues of peptidoglycan (PG) [13]. When cast as a film, PG is opaque or translucent which means that its hydrolysis is associated with increasing transparency. Thus PG/ agarose monolayers, upon incubation with lysozyme preparations or wound fluids, become transparent. The blend layers were prepared with pre heated agarose mixed with peptidoglycan. The suspension was polymerized in 96 well plates overnight. The sensitivity and ease of detection can be augmented when covalently bound Remazol Brilliant Blue (RBB) coloured oligosaccharides are released by lysozyme and leads to a significant absorbance difference ($P < 0.005$) between infected and non-infected wound fluids. The labeling of bacterial cells was based on a standard all-in dyeing process [14]. A double layer

system was investigated for visual inspection of the wound status. It was composed of stained and unstained agarose/ peptidoglycan. The bottom layer contained RBB and agarose, whereas the upper layer contained unstained PG and agarose. Hydrolysis of the unstained upper layer led to the appearance of the stained layer beneath, thereby indicating infection based on high lysozyme activities [14].

Pre-dyed PG was further implemented in a lateral flow system displaying the infection status (Figure 29). An enzyme-responsive matrix composed of RBB-labelled PG delivered coloured oligosaccharides upon hydrolysis by lysozyme. A size exclusion membrane retained the stained full length PG polymer from diffusing into a subsequent capturing layer. Only oligosaccharides originating from lysozyme hydrolysis could pass the size exclusion membrane and were finally trapped in the ion exchange membrane. Both methods would serve as an external infection indicating system in a stand-alone tool or incorporated in a lateral flow device allowing simple and fast visual inspection of the wound status [15].

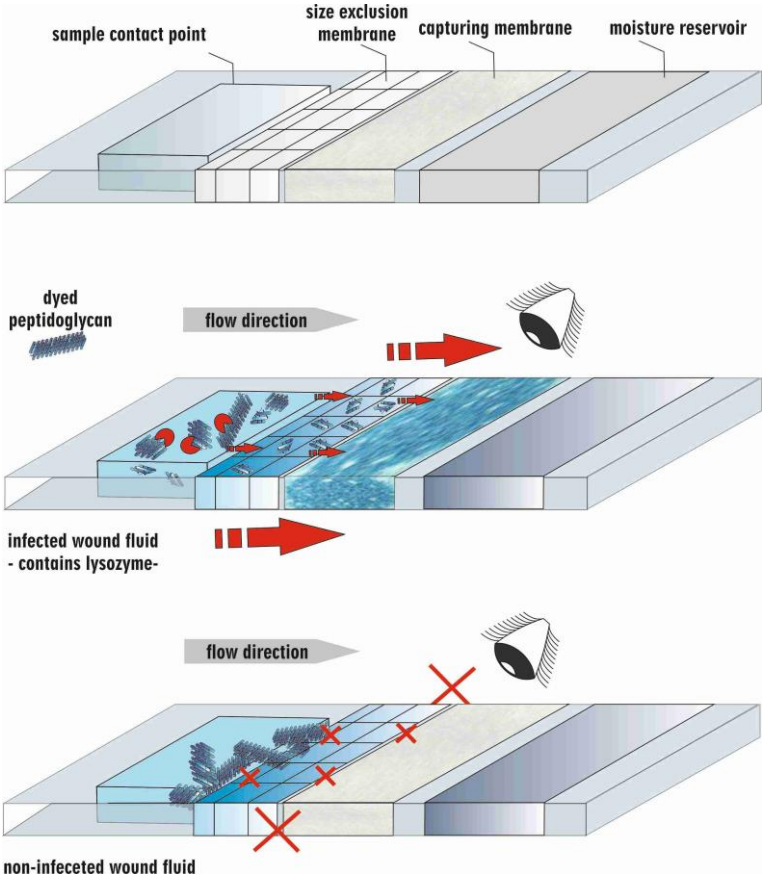

Figure 29: Schematic representation of the double-membrane system applied in a lateral flow device. The system responds to hydrolysis of Remazol Brilliant Blue labelled peptidoglycan by lysozyme from infected wound fluids. This results in coloured oligomeric fragments, which can pass through a size exclusion membrane to be trapped on an anion exchange membrane. The increasing enzyme activity is displayed due to augmented trapping of hydrolysed blue dyed peptidoglycan oligomers in the

capturing membrane. A moisture reservoir guarantees the continuous flow through to the capturing membrane [15]. Permission obtained by Elsevier.

There is a need for infection detection tools in wounds, the discovery of contaminations of medical or food products or installations such as air conditioning filters [19] [20]. Hydrogels consisting of polygalacturonic acid (PGA) and alginic acid were developed, comprising Alizarin as indicator dye [18]. Polygalacturonic acid, as well as methacrylated and cross-linked derivatives can be cleaved by enzymes including pectinases, polygalacturonases and pectin lyases released by potentially contaminating microorganisms. The triggered release of Alizarin due to pectin-degrading enzymes of microorganisms or pure enzyme solutions was investigated [18]. A simple hydrogel made by gelation of PSA in a calcium chloride solution and a more complex chemically modified PSA radically polymerized were constructed. The calcium chloride based polygalacturonic hydrogel (PSA–CaCl2) showed a lower stability in water when compared to the cross-linked system. The stability and sensitivity of the system was tuned by crosslinking of the polysaccharides using glycidylmethacrylate (GMA) as a covalently bound organic linker. Figure 30 shows the incubation of the PGA based hydrogel with a *Bacillus subtilis* suspension. GMA-crosslinked hydrogels showed a higher sensitivity and enhanced dye release stability vs. non-cross-linked material. Release of Alizarin after 24 hours and 48 hours of incubation was proportional to the concentration of purified galacturonase and pectate lyase as well as for various microorganisms [18].

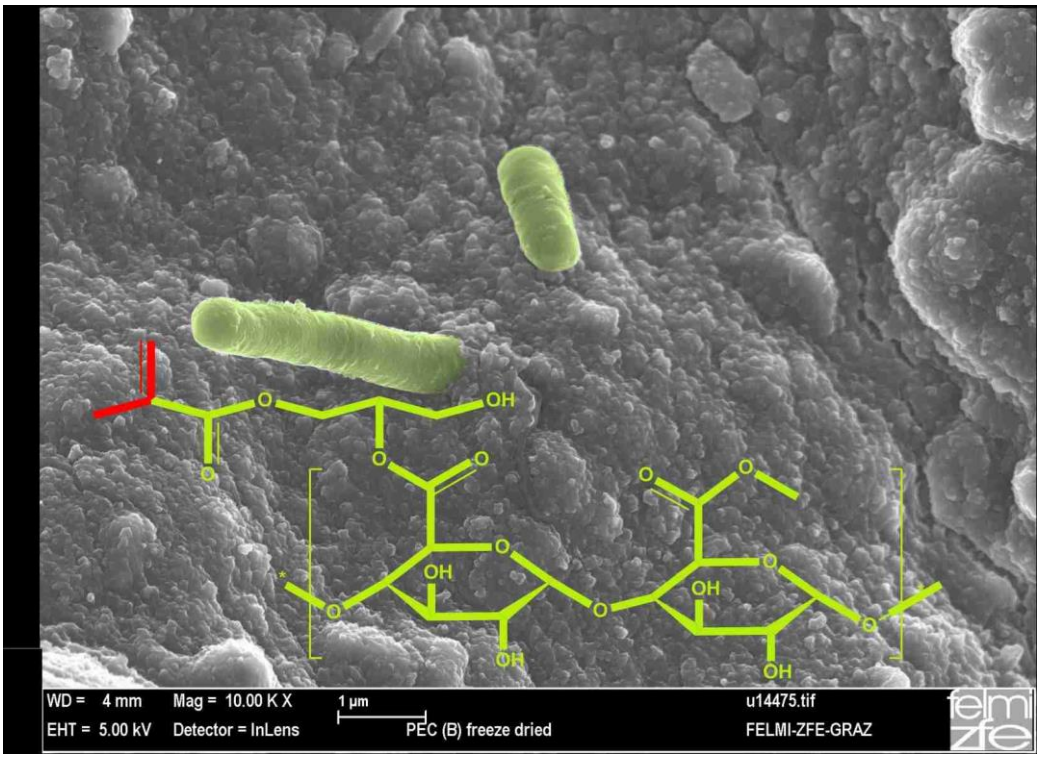

Figure 30: Hydrolysis of an enzyme-responsive polymer by *B. subtilis*, resulting in dye release. The polyglacturonic acid was modified with methacrylate.

Similarly, hydrogels consisting of methacrylated carboxymethylcellulose (CMC) as scaffold were hydrolysed by cellulases as trigger enzyme secreted by different Aspergillus species as well as Bacillus sp. [16]. An enzyme-dependent release of Alizarin was likewise obtained. Hydrogels with a mixture of CMC and PGA combine two enzyme substrates and allow a response to cellulases and pectinases. As representatives for contaminating/ pathogenic fungi *Aspergillus niger* and *Trichoderma epidermidis* and as bacterial example *Bacillus subtilis* were used to investigate the triggered release of Alizarin. A substrate combination enhances the sensitivity of the system [16].

6.4 Expert commentary

Detection of wound infection still constitutes a challenging task. An early and timely treatment of a wound bearing an emerging infection is probably the most important factor in wound management. The real time monitoring of the wound status potentially enables a more precise and less intense wound treatment. This could decrease the probability of emerging resistance.

To date, a variety of strategies and detection methods are published, ranging from electronic sensor technologies to bio-responsive polymers. The detection systems were primarily investigated in less complex environments, ranging from solutions of the respective trigger enzymes to artificial wound fluids. However, measurements directly in the wound bed are done in a complex medium (wound fluid) with interfering factors like high protein or fibrin concentrations that suppress the function of most detection systems. Nevertheless, the potential of enzyme-responsive systems for detection of infection was demonstrated in several studies using wound samples from various ulcers and postoperative wounds. Such detection systems constitute a promising step towards improved wound management. Fast, cheap and easy detection methods that do not require machinery or trained employees are also required for household devices, air conditioning systems and food packaging.

6.5 Five-year view

The developments in the past years indicate a trend towards point-of-care diagnostics enabling a fast and simple investigation of infection status. Several detection systems reported represent promising alternatives suitable for commercialization. However, many still need to be demonstrated to function in real environments rather than just in simulating media.

Electronic sensors are sensitive and specific, but still often susceptible to the interfering effects of the constituents of complex environments like wound beds. Furthermore the incorporation of electronics into devices increases cost and complexity like the need for wearable power sources.

In contrast, simple robust detection systems based on comparatively simple methods like enzyme-responsive polymer frameworks are both robust and cost effective. These could be a promising opportunity for incorporation into medical devices within the next five years. With the proof-of-concept now complete, the next stage of research is the robust application of these technologies in mass market products where they can potentially change the way microbial contamination impacts society.

6.6 References

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7 Lysozyme - Responsive Polymer Systems for Detection of Infection.

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There is a strong need for new point-of-care systems for the detection of wound infection. Overseen infections in chronic wounds induce severe complications, such as delayed healing and high risks for the patients, while time-consuming common gold and silver standard methods for infection assessment cannot be implemented in home care units. This study demonstrates for the first time the between correlation of lysozyme activity and silver-standard microbiological evaluation of wounds. Significantly higher (eight-fold increase; $P < 0.001$) lysozyme activity in infected wounds was in accordance with increasing bacterial burden of infected wound fluids. Moreover, a two-layer membrane-based test system was developed providing visible results on infection in a short time (30 minutes) while avoiding any intermediate steps such as centrifugation. In the first layer of the system, a size exclusion membrane (1.2–8 μm cut-off) retained labelled peptidoglycan while allowing only smaller fragments resulting from lysozyme hydrolysis to pass through. These fragments were then captured in a second layer, an anion-exchanging DEAE cellulose membrane, resulting in clearly visible colour changes. Colourmetric measurements demonstrated significant differences ($P < 0.001$) and six-fold higher delta E values between infected and non-infected wound fluids. This system allows a quick and straightforward determination of the status of a wound. The colourmetric readout refers to the increasing lysozyme activity in infected wound fluid.

7.1 Practical application

An enzyme-responsive polymer-based system for the detection of wound infection was developed. Elevated bacterial burden in wound fluids is correlated to an increase in the activity of lysozyme secreted by the human immune system in response to wound infection. The enzyme-responsive matrix is composed of dye-labelled peptidoglycan, resulting in coloured oligosaccharides upon hydrolysis by lysozyme. Embedded into a bilayer membrane system, lysozyme activity and consequently wound infection can be visualized. The first layer (a size exclusion membrane) contacts with wound fluid, leading to the release of coloured oligosaccharides, which are subsequently captured into the second layer (an ion exchanger membrane). This system allows simple and fast detection of wound infection.

7.2 Introduction

Wound infection is a common, but very serious complication during wound healing in chronic wounds, leading to augmented pain, prolonged hospital stays, and in case of an overlooked sepsis, to death. The normal wound healing is an overlapping and very complex process that involves the phases of inflammation, proliferation and remodelling. During these phases, microorganisms can always reach the state of colonization, but do not necessarily interfere with the process. “Critical colonization” is the point in which the host defences are unable to maintain a balance, resulting in delayed wound healing, a common occurring problem in chronic wounds. Infection results, when the invading organisms overwhelm the host defences, either by their sheer numbers or by impairing the host’s immunity [1,2].

Classical signs of infection like redness (*rubor*), heat (*calor*), swelling (*tumour*) and pain (*dolour*) are still involved in the evaluation of the current wound status. What is called the “clinical look” can be falsified due to neuropathy, vascular disease, venous disease, and impaired leukocyte function in diabetic patients [3, 4]. Different societies and working groups (IWGDF, IDSA) suggest new perceptions regarding the evaluation of a chronic wound, integrating not only the appearance but also the severity of an infection [5–7]. There were enhancements, but clinical signs are still seen controversial as criteria for diagnosing infection [8,9]. More precisely, but still disputed are the silver and gold standard techniques, the semi quantitative and quantitative microbiological investigations including biopsy. Weak points involve the lacking standardizations and the diversity in collecting the wound fluid (swabbing the wound bed) for further microbiological analyses and still controversial opinions concerning the microbiological burden leading to infection. Biopsy is not often carried out in clinical practice due to concerns over harm to the patient. [3,10,11]. Recently, new point-of-care detection systems were introduced to the market, including pH and odour sensors as well as temperature measurement devices. However, a focus has to be set on the wound fluid *per se.* It harbours enzymes of the direct immune answer of the body and therefore provides an insight into the local extracellular microenvironment of the wound reflecting the current status [12–14]. Hasmann *et al*. showed elevated enzyme levels in infected chronic wounds [15]. At this stage, save and simple one-step detection of enzyme activities without the need of various preperation steps of wound fluids (e.g. centrifugation) still remains a challenge. Here, a simplified but effective method was developed for lysozyme detection. Lysozyme, also termed muramidase or peptidoglycan N-acetylmuramoyl hydrolase, is an ubiquitous enzyme widely distributed in diverse organisms, such as bacteria, bacteriophages, fungi, plants, and animals [16]. It is well-known for its antibacterial properties but also exhibits antiviral, antitumor, and immune modulatory activities [17–19]. This makes it

a choice for the use as food preservative in milk, dairy products and fruits, as well as an active substance included in pharmaceutical preparations [20,21]. It is capable of hydrolysing glycosidic linkages between the N - acetylmuramic acid and N - acetylglucosamine residues of the bacterial cell wall peptidoglycan [22]. The assays for the determination of lysozyme activity are based on the hydrolysis of the *Micrococcus lysodeikticus* cell wall [23]. Besides the commonly used turbidimetric assay, *Micrococcus luteus* cells modified with Remazol brilliant blue R (RBB) were described by Ito *et al*. and Hardt *et al*. as a method more sensitive for the determination of the concentration of lysozyme in serum [24,25]. Here we correlated lysozyme activity with microbiological wound diagnostics to confirm the potential of the enzyme as a biomarker for wound infection. For simple detection of lysozyme in wound fluids, an enzyme responsive device consisting of a size exclusion and ion-exchanger membrane was investigated. Enhanced lysozyme activity and thereby infection was visualized by trapping of hydrolyzed blue dyed peptidoglycan oligomers in the capturing membrane.

7.3 Materials and methods

All chemicals if not explicitly stated were purchased from Sigma Aldrich, MO, USA.

7.3.1 Sample collection and preparation

Wounds from different ulcers and surgeries were examined by nurses and doctors. A questionnaire addressing the size, appearance (smell, temperature, and necrosis level), the secretion level, and the patients' data insured the documentation of the different wounds and delivered a preliminary evaluation of the wounds by „clinical look" judgment by wound specialists according to hospital guidelines. Permission to collect wound fluid was obtained from the Ethics Committee of the Medisch Spectrum Twente, Enschede.

7.3.2 Microbiological analyses

Before sample collection, wounds were cleaned with 0.9% NaCl (Sigma Aldrich, MO, USA) to remove superficial bacteria. Swabs were taken of the most contaminated and/or deep site of the wound bed and/or wound edges. The samples were analysed with MALDI TOF techniques, addressing the analyses of the occurring species, as well as gram staining dependent microscopy evaluations were performed to determine the bacterial load [26]. After gram staining, the slides were screened (magnification 1000x) and the bacterial count was reported. The semi quantitative reporting system was subdivided from + (<1) to +++ (>100) counts per ocular field [27–29]. By interpreting the received results, a division between infected and non-infected wound fluids was performed by the microbiologist. The following definitions were used: Infection: More than three different potential pathogenic microorganisms (ppmos), or the presence of one or more ppmos without general microbiological flora. Non - infected: no ppmos or ppmos in lower amount than general microbiological flora.

7.3.3 Biochemical analyses

For the biochemical analyses the wounds were swabbed with a nylon swab (Copa Microrhelogics, Brescia, Italy), diluted in sterile 0.9% sodium chloride and analysed for lysozyme activity and total protein load.

Protein Concentration:

The determination of the protein concentration of the used wound fluids was performed with a BCA kit (BCA Protein Assay Kit, Novagen, MA, USA). A working reagent was prepared by mixing 50 parts BCA solution with 1 part of 4% Cupric Sulfate. To determine the amount of

protein, 100 µL of the working solution was mixed with 100 µL of wound fluid. The purple colour formation was monitored over 60 minutes and measured at 562 nm using a platereader (Tecan infinite M200, Tecan Group Ltd., Männedorf, Switzerland). Wound fluids lacking measurable amount of protein were excluded from all further measurements and calculations to avoid false negative results.

Lysozyme Activity:

Lysozyme activity was directly measured in wound fluids using a modified method as described by Shugar, 1952 [23]. The assay is based on the determination of the lysis of *M. lysodeikticus* cells monitored photometrically at 450 nm. 1 mg/mL *M. lysodeikticus* cells were suspended in 0.9% NaCl. 290 µL of the suspension was incubated with 10 µL of sample or standard (Lysozyme from chicken egg white). The initial rate of increase in transparency was monitored every 60 seconds at 450 nm at room temperature (Tecan infinite M200, Tecan Group Ltd., Männedorf, Switzerland).

7.3.4 Labelling of peptidoglycan of *M. lysodeikticus* with Remazol Brilliant Blue R

A labelling procedure recently described by Hasmann *et al.* was slightly modified [15]. Briefly, 300 mg of *M. lysodeikticus* cell walls was suspended in 20 mL H_2O (15 mg/mL). 20 mL of a 10 mg/mL solution of Remazol Brilliant Blue R (RBB R) was prepared and added to the peptidoglycan suspension. Dyeing was carried out in a thermo shaker (Eppendorf, Hamburg, Germany), using 50°C for 30 minutes. Sodium sulphate (Na_2SO_4) was added to the incubating solution every 6 minutes to a total amount of 4 g Na_2SO_4. After this time period 2 mL of a sodium carbonate (Na_2CO_3) solution (100 mg/mL) was added and the incubation process was repeated for another 30 minutes. The reaction mixture was centrifuged for 10 min at 3692 g to remove excessive dyestuff. After discarding the supernatant, the remaining pellet was washed with 0.05 M potassium phosphate (pH 7) buffer until the supernatant was colourless. The last washing step was performed with H_2O bidest. The peptidoglycan was then immediately used for further filtration as well as degradation experiments.

For liquid measurements of the degradation dependent colour change, the pre - dyed peptidoglycan was pre-incubated as described below with different lysozyme activities. After a centrifugation step (10 min at 3692 g), the supernatant was transferred to a 96 well plate and measured at 600 nm (Tecan infinite M200, Tecan Group Ltd., Männedorf, Switzerland).

7.3.5 Enzyme dependent dye trapping system

The dyed peptidoglycan was diluted to a concentration to 10 mg/mL in a 0.1 M potassium phosphate (pH 7) buffer and incubated in equal volumes with lysozyme or wound fluid

reaching an end concentration of 5 mg/mL. Lysozyme was used in end concentrations of 100 U/µL 50 U/µL 25 U/µL 5 U/µL 0 U/µL. The different wound fluids (infected and non-infected) were diluted 1:20 in total in 0.1 M potassium phosphate (pH 7) buffer. The two step device consisted of one size exclusion membrane and one serial connected ion exchanging capturing membrane. The size exclusion membrane (Cellulose nitrate filter Satorius, Göttingen, Germany) was used in different porosities, ranging from 1.2 µm to 8 µm. Upon hydrolysis of the labelled peptidoglycan by lysozyme (30 minutes), only the small oligomeric coloured fragments were able to pass the size exclusion membrane and were then trapped in the weakly basic, anion exchange DEAE capturing membrane (Grade DE81 Ion Exchange Paper, circle, GE healthcare, Freiburg, Germany).. The colour changes on the surface of the capturing membrane were measured with a ColourLite sph850 spectrophotometer (ColourLite GmbH, Katlenburg-Lindau, Germany). As a reference a wetted capturing membrane was used. Based on the delta E values, calculations and statistical analyses were performed.

7.3.6 Statistical analyses

To compare the different enzyme activities in infected and non-infected wound fluids, two-sample t-tests assuming equal variances were performed. This method was also used for comparison of the delta E values measured on the capturing membrane. A P Value of less than 0.05 was considered as statistical significant.

7.4 Results

7.4.1 Activity of the lysozyme correlates with the bacterial burden

The wound fluids of 14 patients were collected in a hospital by nurses and doctors. A questionnaire ensured the proper documentation and characterization of the wounds, summarized in table 7. 36 per cent of the patients were female; the average age was 69 years. The patients showed different clinical pictures from diabetes to arterial diseases and rheumatism. Nearly 30% suffered from traumatic ulcers with an average wound size of 2.0 x 1.4 cm and duration of 45 days. 50 per cent of the wounds showed partial necrotic tissue; 57 per cent of the wounds had a moist wound bed and 30 % a foul smell. The results of the microbiological study showed 12 different potential pathogenic microorganisms (ppmos) namely *Klebsiella pneumoniae* ssp. *pneumonie, Proteus vulgaris, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Streptococcus agalactiae (*= Group B*), Pseudonomas aeruginosa, Haemolytic streptococcus Group G, Acinetobacter baumanii, Streptococcus constellatus, Streptococcus pyogenes (*Group A*), Streptococcus equisimilis (*Group C). The infected wounds harboured at least one or more ppmos showing a high bacterial burden in comparison to the non - infected wounds where only a common bacterial flora was observed, missing the ppmos. Figure 31 demonstrates the correlation of the bacterial burden in infected wounds and lysozyme activity, a neutrophil derived enzyme, related to the immune response. A major function of the neutrophils is to remove foreign material, therefore neutrophilic enzymes, like lysozyme, are present in the infected wound within 24 h [30]. Hasmann *et al*. (2011) showed that there is a statistical significant difference in the enzymatic activity of infected and not infected wound fluids [15]. Here we demonstrate the correlation of the increasing enzymatic activity to the bacterial burden to set the evidence that wound fluid is a highly reactive system displaying infections in wounds, when compared to silver standard methods. Lysozyme activity was found to be a statistic significant parameter differentiating infected and non-infected wounds (P values of 0.001 using a two-sample t-test assuming equal variances).

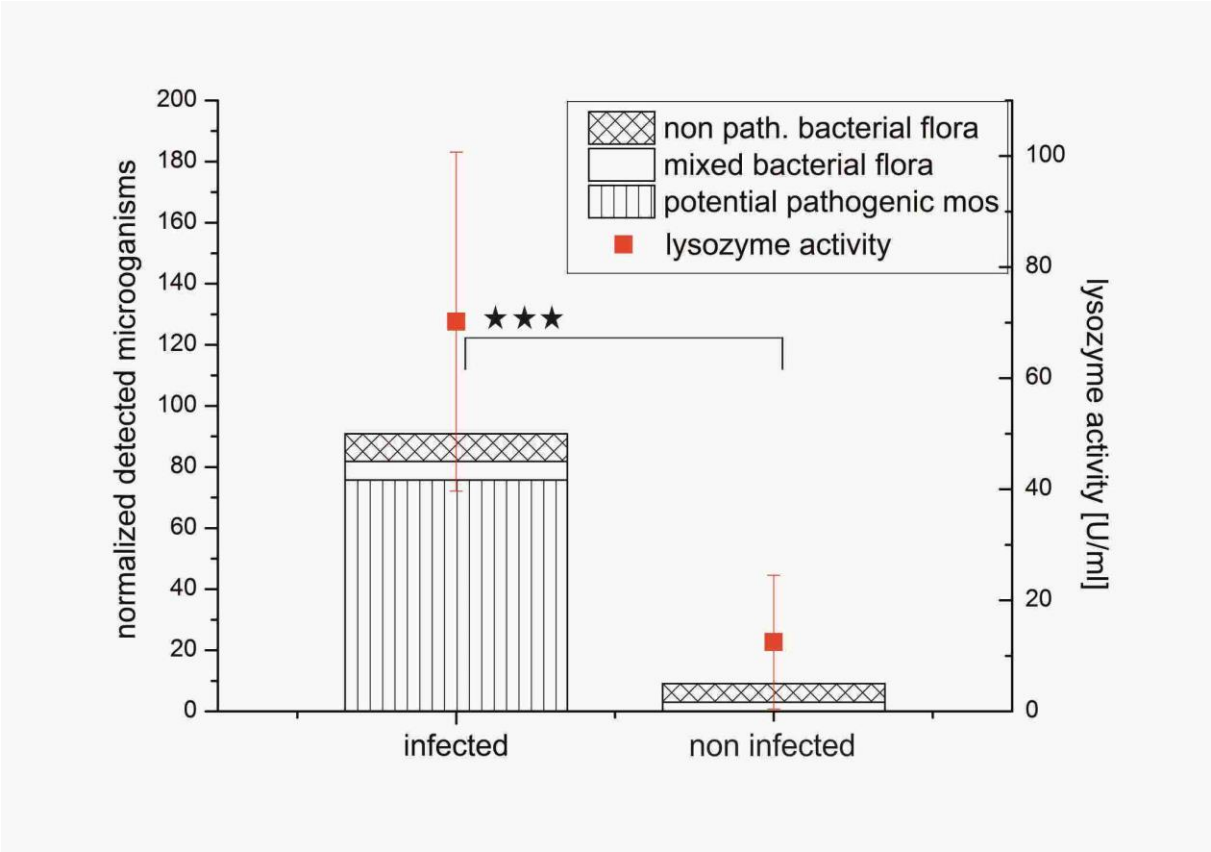

Figure 31: Lysozyme activity correlates with the increasing bacterial burden of infected wounds. 14 wound fluids from ulcer and post-operative wounds were swabbed and analysed microbiologically and with biochemical techniques. The bacterial load was determined by gram staining and counting, and the severity was displayed from "+" to "+++" for each organism. The potential pathogenic microorganisms (ppmos) were merged to one group as well as the mixed bacterial and the non-pathogenic flora. The percentage per group was calculated related to the infected and good healing (non-infected) wound fluids. The analyses of the lysozyme activity was done according to the method described in the materials and methods section and the percentage of the average results were presented in comparison to the outcome of the microbiological study. A correlation can be observed between the increasing lysozyme activity and bacterial count of potential pathogenic microorganisms. Statistical analyses point out these findings with P values of 0.001 (indicated by the asterisks in the figure) using a two-sample t-test assuming equal variances when comparing the enzyme activities from infected and non-infected (good healing) wound fluids.

Table 7: Patient characteristics: Summary of the collected information addressing the size, appearance (smell, temperature, and necrosis level), the secretion level, and the patients' sex and age of the observed wounds.

		count	average	mean	percent
Patient characteristics	Female gender	5			36
	Male gender	9			64
	Age in years		69.0	69.0	
	no disease	5			36
	diabetes	2			14
	Cardiac disease	0			0
	Pulmonary disease	1			7
	Arterial disease	1			7
	Venous disease	1			7
	Hypertension	1			7
	Rheumatism	1			7
	Inhaled corticosteroids	0			0
	Antibiotics last month	0			0
Wound characteristics	Arterial ulcer	1			7
	Venous ulcer	2			14
	Diabetic foot ulcer	2			14
	Traumatic ulcer	4			29
	Pressure ulcer	2			14
	Amputation wound	1			7
	Other	2			14
	Wound duration in days		45.3	42	
	Wound length in cm		2.0	1.5	
	Wound width in cm		1.4	1.0	
Wound appearance	Partial wound necrosis	7			50
	Serous exudate	7			50
	Ensanguined exudate	1			7
	Sanious exudate	2			14
	Ensanguined and sanious exudate	2			14
	Foul wound smell	4			29
	Dry wound bed	0			0
	Moist wound bed	8			57
	Wet wound bed	4			29

7.4.2 Lysozyme dependent device for infection detection

Lysozyme levels refer to the current wound status and permit a differentiation between non infected and infected wound fluids, as shown in figure 31. The hydrolysis of dyed peptidoglycan and the colour development can be monitored photometrically at 600 nm after a centrifugation step, pelleting the peptidoglycan particles from the supernatant (figure 3, supplementary OD measurement). To improve and simplify the detection method avoiding complex handling steps of wound fluids, a two layer membrane-system was developed containing labelled peptidoglycan as enzyme responsive matrix (see schematic presentation in figure 32). The first layer comprises a size exclusion membrane with different pore sizes ranging from 1.2 µm to 8 µm. The pore sizes selected to retain labelled *M. lysodeikticus* cell walls were selected slightly below and above the pore sizes normally used for microfiltration of bacteria. Only hydrolysed coloured peptidoglycan oligomers (dimers) were able to pass and were trapped by the anion capturing membrane. The blue colour development was thus only visibly upon substrate hydrolysis by lysozyme. The delta E values recorded from the surface of the capturing membranes, shown in figure 33, indicate significant distinction between infected and non-infected wound fluids (p values <0.001), as well as between infected wound fluids and low enzyme activities or blank measurements. This effect was seen for all different pore sizes of the size exclusion membrane. The highest colour development in the capturing membrane, attributed to infected wound fluids was seen at a pore size of 8 µm, whereas a pore size of 1.2 µm led to a reduction of the colour development of 50 %. A pore size of 3-5 µm provided the best results regarding the pure enzyme, and was also suitable for wound fluids, delivering statistical significant results.

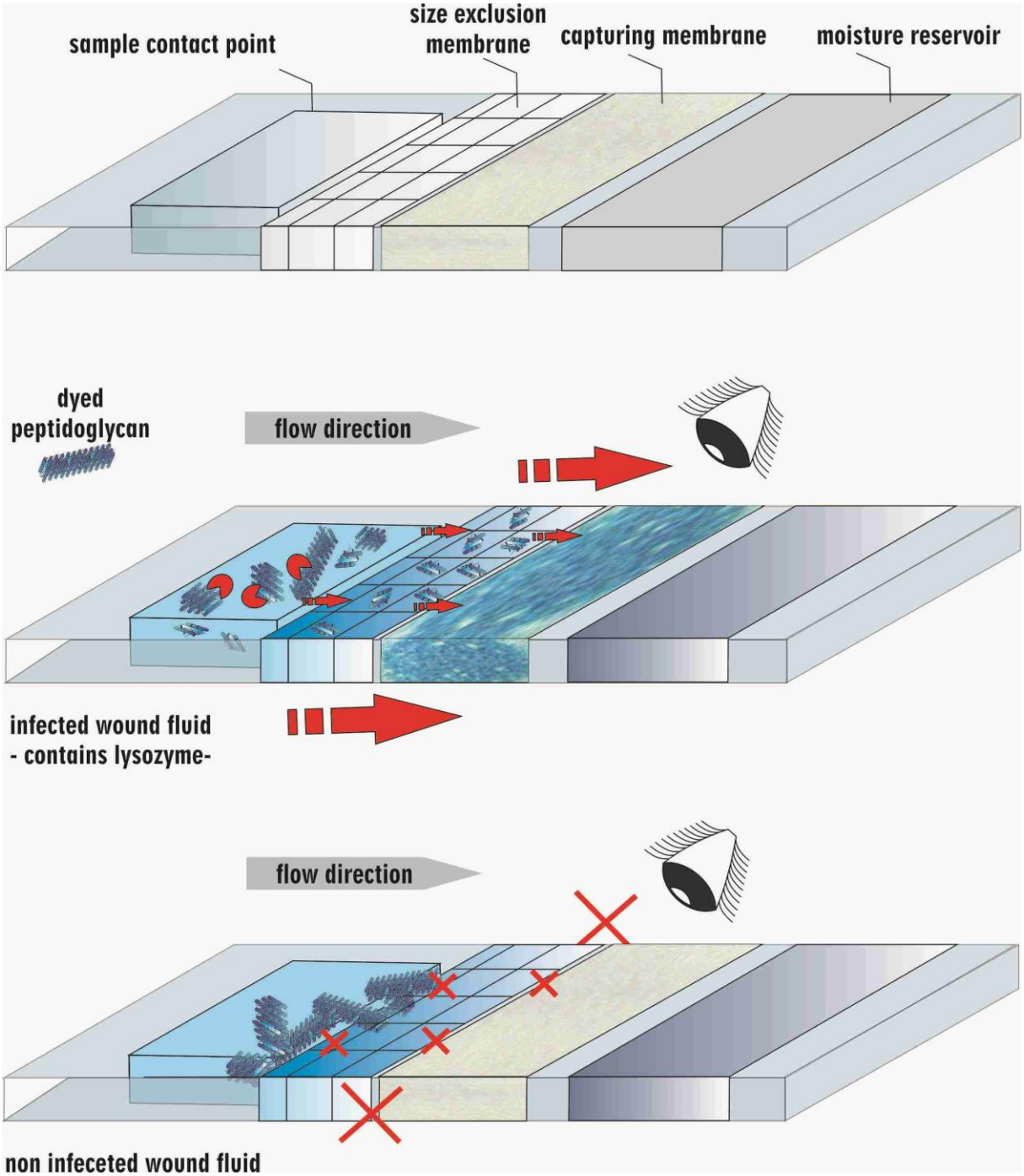

Figure 32: Schematic representation of the two membrane system applied in a lateral flow device. The system responds to hydrolysis of Remazol Brilliant Blue labelled peptidoglycan by lysozyme from infected wound fluids. This results in coloured oligomeric fragments, which can pass a size exclusion membrane, being subsequently trapped in a capturing membrane (anion exchanger membrane). The increasing enzyme activity is displayed due to augmented trapping of hydrolysed blue dyed peptidoglycan oligomers in the capturing membrane A moisture reservoir guarantees the continuous flow through to the capturing membrane.

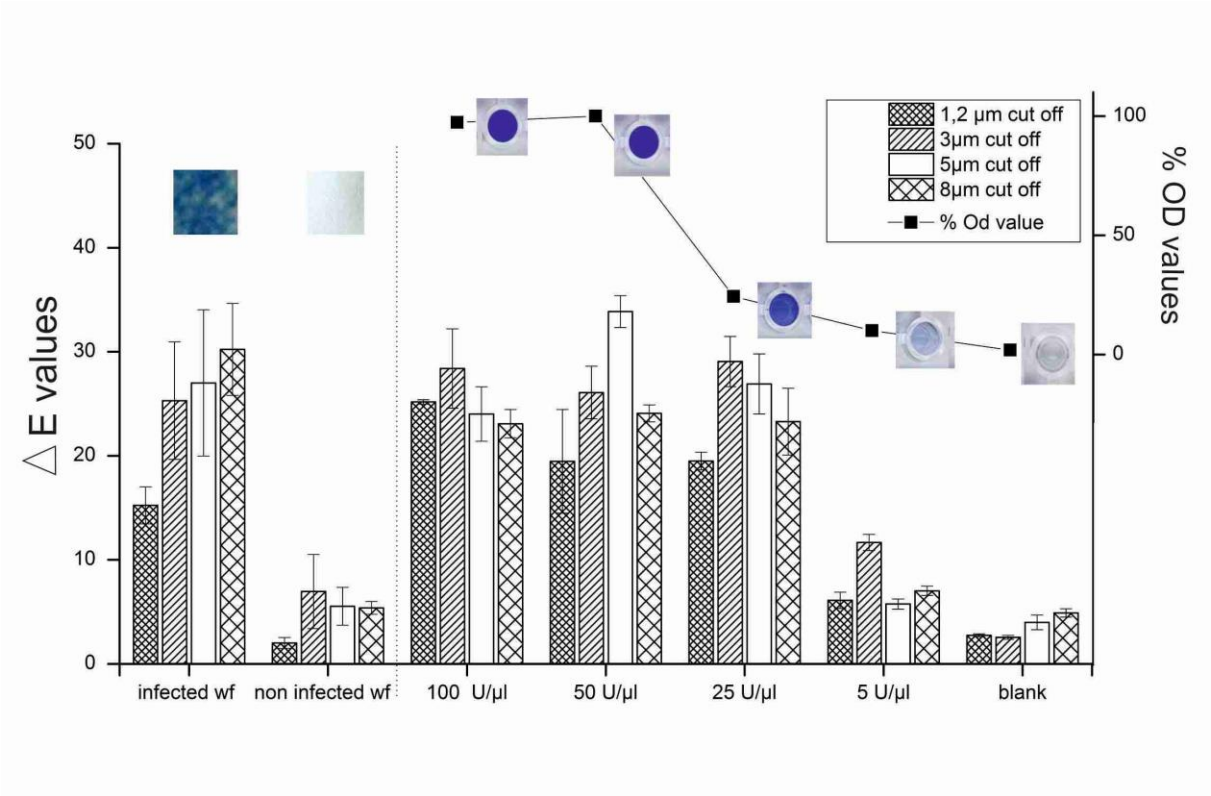

Figure 33: Trapping of coloured peptidoglycan fragments upon lysozyme hydrolysis. The colour differences (delta E values) recorded from the surface of the anion exchanger capturing membranes correlate to lysozyme activity and allow clear distinction of infected and non-infected wound fluids. Different pore sizes of the size exclusion membrane were used (1.2-8 µm) allowing the coloured peptidoglycan fragments to pass to the capturing membrane but retaining the non-hydrolysed peptidoglycan. In addition, samples were taken from the incubation mixture of coloured peptidoglycan with lysozyme and measured photometrically at 600 nm after centrifugation. Inlet photographs show the colour change of the capturing membrane of the system reacted with infected and non-infected wound fluid (left) and microtiter wells (right) containing coloured peptidoglycan after incubation with lysozyme and centrifugation.

7.5 Discussion

Despite the need for simple lysozyme tests in many areas, assay procedures for this enzyme e.g. based on turbidity changes of peptidoglycan solutions or on the release of coloured fragments of Remazol Brilliant Blue labelled peptidoglycan upon hydrolysis, rely on instrumental analysis or require separation steps (centrifugation). Several principles were further developed for higher throughput of samples, or improved regarding the detection limits [31]. Despite electrophoretic, chromatographic, and immune-enzymatic methods, different sensors have been developed on the basis of aptamers [32–39]. Analogous to protein-based antibodies, aptamers are nucleic acid-based molecules that can be selected to bind essentially to any molecule of choice [16,40–45].

Here, an all-in test system was developed which can be implemented in devices for wound infection without the need of external liquid handling steps. Hasmann *et al.* (2011) reported the potential of lysozyme as infection marker in chronic wounds with 12 fold increase of enzyme activity in infected wound fluids compared to non-infected wound fluids [15]. The evaluation of the wounds was done by clinical look investigations. Here, in addition we correlated lysozyme activity to the microbial burden as the silver standard for infection detection. With a novel membrane based system, a 6 fold increase in enzyme activity in infected wounds with a P value of 0.001 allows a clear distinction to non-infected wounds. Easy and fast diagnosis of infections in chronic wounds and surgical sites followed by adequate treatment is essential to reinforce the natural healing process and can be a life - saving issue. Lysozyme devices harbouring the enzyme embedded in agarose mono- or double layers were presented by Hasmann *et al* (2011) showing 4 fold increase of colour development in infected wound fluids.

Here, a two-section membrane based test system was developed, providing the basic concept of a future lateral flow device. In contrast to a two layer agarose gel based system suggested previously [15], the two-membrane based approach investigated here allows application as lateral flow device without restriction in terms of storage working at room temperature. The fast system (hydrolysis of the substrate within 30 minutes) involves a size exclusion and ion-exchanger membrane to avoid liquid handling steps (e.g. centrifugation) of potentially infectious fluids. Instead of immobilization of labelled peptidoglycan within the device, a size exclusion membrane was used. Thus, there is no risk of leaching of larger (non-hydrolysed) coloured molecules towards the capturing section allowing high sensitivity of the system. Consequently, this membrane retains dyed peptidoglycan, while fragments resulting upon hydrolysis by lysozyme are small enough to pass. In detail, we have previously shown by using LC-MS measurements that lysozyme can hydrolyse dyed

peptidoglycan even down to the respective dimers [15]. Trapping the hydrolysed substrate in the capturing membrane was performed using simple filter techniques known from RNA - protein separation protocols based on ionic interactions [46,47]. Correlating with standardized assay results, an increase of colour development up to 7 fold could be monitored comparing infected and non - infected wound fluids with P values of > 0.001.

Although a variety of analytic methods ranging from optical to chemical (i.e. pH) has been assessed for characterization of wounds, there is still no simple and fast device for the detection of wound infection available [48–52] PH sensors incorporated in bandages undergo a lot of challenges, from leaching into the wound to be sensitive to the pH range encountered in wounds. Also more complex devices like pH dependent swelling hydrogel sensors are not usable for the daily use, since moisture changes of the wound could lead to falsified results. Odour sensors, such as conducting polymer arrays made from substituted pyrroles, constitute a relatively cheap approach to gas analysis but suffer from sensitivity problems, sensor response time and interference by humidity. Examination of the bacteriology of wounds using culture independent molecular methods offers a more precise setup of the microorganisms of the wound than common culturing techniques. However, these methods require complex and time consuming working steps, well trained personal as well as machineries and are not prevalent in all medical service centres [5,53,54] . As shown in figure 1, the enzymatic activity of lysozyme correlates with the increasing bacterial burden. Enzymes of the natural host defence used as infection markers redundantise complex and time consuming wound diagnostics. This novel strategy introduces a fast and easy tool for infection determination in chronic wounds.

7.6 References

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8 Novel protease-based diagnostic devices for detection of wound infection

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A gelatinase based device for fast detection of wound infection was developed. Collective gelatinolytic activity in infected wounds was 23 times higher (P≤0.001) than in non-infected wounds and blisters according to the clinical and microbiological description of the wounds. Enzyme activities of critical wounds showed 12 fold elevated enzyme activities compared to non-infected wounds and blisters. Upon incubation of gelatine based devices with infected wound fluids, an incubation time of 30 minutes led to a clearly visible dye release. A 32 fold colour increase was measured after 60 minutes. Both, matrix metalloproteinase and elastase, contributed to collective gelatinolytic enzyme activity as demonstrated by zymography and inhibition experiments. The metalloproteinase inhibitor 1,10 phenanthroline (targeting matrix metalloproteinases) and the serine protease inhibitor PMSF (targeting human neutrophil elastase) inhibited gelatinolytic activity in infected wound fluid samples by 11% to 37% and 60% to 95%, respectively. S*taphylococcus aureus* and *Pseudomonas aerugionosa*, both known for gelatinase production, were isolated in infected wound samples.

8.1 Introduction

Wound infection is a severe complication during the wound healing process causing diagnostic and therapeutic problems [1,2]. Currently, detection of wound infection is based on evaluation of the well-known signs of inflammation like *rubor* (redness), *calor* (heat), *tumour* (swelling) and *dolour* (pain) by medical doctors [3]. Additionally, wound infection can be characterised by the identification of the causative organism(s) after wound swabbing [4]. Furthermore serum investigations identifying elevated white cell counts and elevated levels of serum C-reactive protein (CRP) can be performed. However, both methods are not suitable for rapid diagnosis of wound infection.

Additionally, the presence of neutrophils is a well-known marker for wound infection. The recruitment of neutrophils to the site of infection is one of the first events in wound repair [5] and takes place immediately after bacterial invasion. The major function of neutrophils is the removal of foreign material, bacteria, non-functional host cells and damaged matrix components, that may be present in the wound site [6,7].Excessive stimulation of neutrophils results in the release of proteolytic enzymes like gelatinase, elastase, cathepsin G as well as antimicrobial enzymes like myeloperoxidase and lysozyme [8] into the wound environment.

Therefore it is widely suggested that wound fluid has the potential to provide important biochemical information, which can be used as a diagnostic indicator. This information can be used for determining the overall status of a wound and for monitoring the progression of wound healing [8]. Recently the prognostic value of lysozyme [9], myeloperoxidase [10], elastase and cathepsin G [11] for wound infection was demonstrated.

Elevated protease activity, however, does not only seem to play an important role in predicting wound infection, but was also shown to be responsible for the non-healing of wounds in general. Different animal experiments and clinical studies suggest that especially matrix metalloproteases (MMPs) and human neutrophil elastase (HNE, a serine protease) contribute to this effect. Especially the excessive production of HNE by PMNs (polymorphonuclear leukocytes) leads to extensive pathological tissue destruction in a number of disorders, including delayed wound healing and chronic wounds [12-14]. A number of studies have found significantly elevated mean levels of HNE activity in pressure ulcers and in leg ulcers. Different studies show the potential of released elastase as indicator of uneventful wound healing and early inflammatory complications [15].

Excessive stimulation of neutrophils results in the release of proteolytic enzymes like gelatinase, elastase, cathepsin G as well as antimicrobial enzymes like myeloperoxidase and lysozyme into the wound environment [16, 17].

The extracellular matrix (ECM) is the largest component of the dermal skin layer being responsible for important properties like elasticity, tensile strength and compressibility. Recent data indicate that components of the ECM play an important role in normal wound healing and that the destruction of ECM components impairs healing [18].

For remodelling of ECM component the action of different enzymes is needed. The most important enzyme families are MMPs and serine proteases. Regarding MMPs, collagenases act on intact fibrillar collagen the gelatinases, can digest damaged fibrillar collagen; and stromelysins are able to degrade proteoglycans. Gelatinases seem to play an important role in the final stages of this process due to their ability to digest denatured collagen. But MMPs are not only important for removal of damaged skin, they are essential in many processes of the normal wound healing like angiogeneses, migration of cells, contraction of newly synthesised scar and scar remodelling [18, 19].

As excessive degradation of ECM by proteases can lead to tissue destruction or cellular invasions [21] elevated levels of matrix metalloproteinases (MMPs) and serine proteases are playing an important role in delayed wound healing [22- 25]. Therefore, proteolytic activity of MMPs is strictly controlled by different mechanisms.

Additionally to their involvement in physiological processes, MMPs have also been implicated in a variety of diseases including arthritis, periodontal disease, tissue ulcerations, tumour cell invasion and metastasis [21]. As they can modulate cytokine and chemokine levels [18] they also have important immunological functions and act as metabolic regulators. Moreover, MMPs play a key role in the host defence of bacterial infections as they can activate defensins and antibiotic peptides [26].

Apart from the human body, gelatinase as well as elastase are released by different microorganisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* during the infection process [27-29].

As there is a strong need for a fast prognostic tool by which the onset of clinical infection can be predicted early enough, we have investigated gelatinolytic activity (hence collectively resulting from both MMPs and bacterial proteases) as well as collagenolytic activity in different types of wounds. Especially enzyme levels in infected and non-infected wound fluids were compared to test the hypothesis that elevated gelatinolytic and collagenolytic activity can be used as markers for the early detection of wound infection prior to obvious clinical symptoms.

8.2 Materials and methods

8.2.1 Sample collection and preparation

Wound fluid from post-operative wounds, pressure ulcer wounds, malum perforens wounds and leg ulcer wounds was collected and analysed by biochemical techniques. Samples were taken with a nylon swab (Microrhelogics, Brescia, Italy), and diluted in NaCl (sodium chloride) buffer. Additionally, wound fluid from 5 blisters was taken as negative control. The criteria for the wound evaluation as infected/critical or non-infected were dependent on the „clinical look“, and the microbiological diagnostics. A questionnaire was prepared for the attending doctors to ensure the same evaluation parameters for every wound. According to the questionnaire, the wounds were labelled as infected or non-infected. Simultaneously, a microbiologist (Laboratorium Microbiologie Twente, Enschede, Netherlands) analysed the results of the wound swabbing cultures and, according to the counted microorganisms and resistances, described the wounds as infected, possibly infected (critically colonized) or non-infected. Wounds labelled as infected or non-infected were categorized due to the „clinical look. 8 out of the 29 wounds were described as infected by the attending medical doctors as well as by the microbiological analysis, while 8 wounds were labelled as non-infected by both. 8 wounds were judged as “critical“, which means possibly infected (critically colonized) due to the microbiological analyses. Patients studied were grouped and labelled with the following abbreviations followed by a number: i = infected wound; c=critical wound; n. i= non-infected wound; b = blisters. Permission to collect wound fluid was obtained from the Ethics Committee of the Medical University of Graz.

8.2.2 Microbiological study

Before sample collection, wounds were cleaned with 0.9% NaCl (Roth, Karlsruhe, Germany) to remove superficial bacteria. Swabs were taken of the most contaminated and/or deep site of the wound bed and/or wound edges. Samples were analysed by the “Laboratorium Microbiologie Twente” (Enschede, Netherlands). The swabs were streaked onto an agar plate containing blood and on containing three agars overlaid: blood, "chocolate" (=blood cooked prior pouring into the plate), and CLED (meant to detect gram negative bacteria). Both plates were incubated in a 5% CO_2 cabinet at 35°C for two days.

8.2.3 Determination of gelatinolytic/collagenolytic activity of wound fluid samples

The total gelatinolytic/collagenolytic activity in wound fluid samples was determined using the EnzChek gelatinase/collagenase assay kit (Invitrogen Life Tech Austria, Vienna, Austria). As substrates, labelled gelatine or collagen (DQ Gelatin and DQ Collagen Type IV Fluorescein Conjugate, Invitrogen Life Tech Austria, Vienna, Austria) were used. For the measurements, 80 µL of reaction solution were mixed with 20 µL substrate solution and 100 µL of diluted sample was added. For calibration, an enzyme preparation from *Clostridium histolyticum* (EnzChek gelatinase/collagenase assay kit, Invitrogen Life Tech Austria, Vienna, Austria) was used. The resulting fluorescence was measured at an excitation wavelength of 495 nm and at an emission wavelength of 515 in black 96 well plates Greiner- Sigma Aldrich, Vienna, Austria).

8.2.4 Gelatine beads

A suspension of gelatine (15%) (Roth, Karlsruhe, Germany) was heated for 15 minutes at 90°C and subsequently mixed with 5 mL NaOH (5.5 mM). The solution was then cooled to 40°C while stirring. After addition of 4 mL or 6 mL of GDGE (glyceroldiglycidyl ether) (Sigma Aldrich, Vienna, Austria), the solution was stirred at 40°C until a gel was formed, and placed in the oven for three days. The gelatine gels were pressed through a sieve with meshes of approximately 1 mm^2. Gels were then rinsed with water until neutral pH was reached, followed by at least 1 litre (2-3 times) of ethanol (100%) and at least 1 litre (2-3 times) of acetone. Finally, the gels were air-dried at room-temperature. For loading of the particles with a dye, 2.5 g of reactive blue (Sigma Aldrich, Vienna, Austria) was dissolved in 1.5 L of water with NaOH. Next, 10 g of gel particles obtained in the above mentioned synthesis were added and the solution was stirred for 90 minutes. Finally, the gel particles were rinsed with water until neutral pH was reached, followed by at least 1 litre (2-3 times) of ethanol (100%) and at least 1 litre (2-3 times) of acetone and dried at room temperature.

Incubation of beads was carried out as follows: 2 mg of beads were weight into 1.5 mL Eppendorf tubes. 100 µL 2x reaction buffer (EnzChek gelatinase/collagenase assay kit, Invitrogen Life Tech Austria, Vienna, Austria) and 100 µL of highly diluted sample or 100 µL NaCl (blank) were added, respectively. The beads were incubated at 37°C for 30 minutes to 72 hours. The beads were spinned down in a table centrifuge (2 minutes, 13.400 rpm). For determination of colour release, absorbance of the supernatant (100 µL) was measured at 600 nm at various time points. The supernatant was then refilled into the tubes and mixed with the beads to allow subsequent measurements. All measurements were repeated twice.

8.2.5 Inhibition Experiments

To relate the collective gelatinase activity in wounds to certain microbial or human enzymes, enzyme inhibition experiments were carried out. The effect of various inhibitors on gelatinase activity was determined by preincubating three different wound fluids with specific inhibitors for MMPs (1,10-Phenantroline monohydrate, gelatinase/collagenase assay kit, Invitrogen Life Tech Austria, Vienna, Austria; EDTA, Roth, Karlsruhe, Germany) and a serine protease inhibitor (PMSF, Sigma Aldrich,Vienna, Austria). For pre-incubation, 100 µL (10 mM final concentration) of different inhibitors were incubated for 30 minutes with 100 µL of diluted wound fluid samples. 100 µL of elastase (2 U/mL; Sigma Aldrich, Vienna, Austria) were used as a positive control for inhibition of serine protease via PMSF. After preincubation, 2 mg of gelatine beads were added. Measurements of gelatinase activity were carried out as described above. Wound fluids without any inhibitor were regarded as 100%.
Additionally, inhibition experiments were performed using the Enzcheck assay (Invitrogen Life Tech Austria, Vienna, Austria). Therefore, three different wound fluid samples were incubated with different inhibitors as described before. Enzyme activity was determined using the EnzChek gelatinase/collagenase assay kit. Wound fluids without any inhibitor were regarded as 100%.

8.2.6 Gelatin zymogram

To compare gelatinase levels in infected and non-infected wound fluids, qualitative zymographic analysis was performed using 7.5 % polyacrylamide gels containing 0.1% gelatine. Briefly, wound fluid samples were mixed 1:1 (v/v) with non-reducing sampling buffer (2.8 mL H_2O, 1 mL Tris-HCl (0.5 M, pH 6.8), 3.2 mL SDS (10% w/v), 0.5 mL bromphenol-blue (1%) and 7 mL glycerol, all Roth, Karlsruhe, Germany) and were incubated at room temperature for 10 minutes. Gels were loaded with 10 µL of samples. Electrophoresis was performed using a Mini Protean Cell (Biorad, Vienna, Austria) at constant 60 V for approximately 2 hours. Afterwards, gels were rinsed briefly with distilled water and then washed twice in renaturation buffer (5% w/v Triton X in dd. H_2O; Sigma Aldrich, Vienna, Austria) for 20 minutes to remove SDS and to allow the enzyme to reinstate. Incubation of gels was carried out overnight in incubation buffer (Tris-HCl, (50 mM, pH 7.5); $CaCl_2$ (10 mM); Roth, Karlsruhe, Germany) at 37°C. After incubation, gels were routinely stained with Coomassie blue (0.25% w/v; Roth, Karlsruhe, Germany) and distained in a solution containing 10% (v/v) acetic acid, 50% (v/v) dd. H_2O and 40% (v/v) ethanol. Gelatinase catalysed hydrolysis of gelatine led to observable clearing zones in a blue-stained background.

8.2.7 Statistical analysis

To compare the differences of enzyme activities in infected and non-infected wound fluids, unpaired t-test was performed. P values of less than 0.05 were considered as statistical significant.

8.3 Results and Discussion

8.3.1 Determination of gelatinolytic activity in wound fluid

In literature, only little is reported about gelatinolytic activities in infected wounds. Therefore, we have investigated the possible relationship between gelatinolytic activity and the presence of wound infection in different types of wounds. Using fluorescein labeled gelatin as substrate, significantly higher enzyme activities were detected in infected wounds compared to non-infected wounds. Gelatinolytic activity in infected wounds was 23 times higher ($P \leq 0.001$) than in non-infected wounds and blisters (Table 8 and Figure 34). These results match with the clinical and microbiological description of the wounds. Interestingly, enzyme activities of critical wounds showed 12 fold elevated enzyme activities compared to non-infected wounds and blisters, which could point out an upcoming infection. Additionally, collagenolytic activity using fluorescein labeled type I collagen was determined. Collagenolytic activity was significantly higher in infected (23 fold) and critical wounds (8 fold) compared to non-infected wounds and blisters.

Table 8: Gelatinolytic and collagenolytic activities in wound fluid samples: Gelatinolytic and collagenolytic activity (given as fluorescence intensity FI) was measured in infected, critical, non-infected wounds and blisters. There is a significant difference between enzyme activities of infected and non-infected wound fluids ($P<0.001$). (P-values for gelatinolytic activities: i – crit: 0.0098; i – n.i: 0.0001; i – b: 0.0000; for collagenolytic activities: i – crit: 0.0131; I – n.i: 0.0025; i – b: 0.0020)

Clinical description of the wound	Numbers of wounds	Gelatinase Activity [FI]	Collagenase Activity [FI]
Infected	8	26,893 ± 7,729	4,266 ± 2318
Critical	8	13,863 ± 8,469	1,348 ± 1,100
non-infected	8	1,410 ± 649	232 ± 55
Blisters	5	939 ± 574	76 ± 67

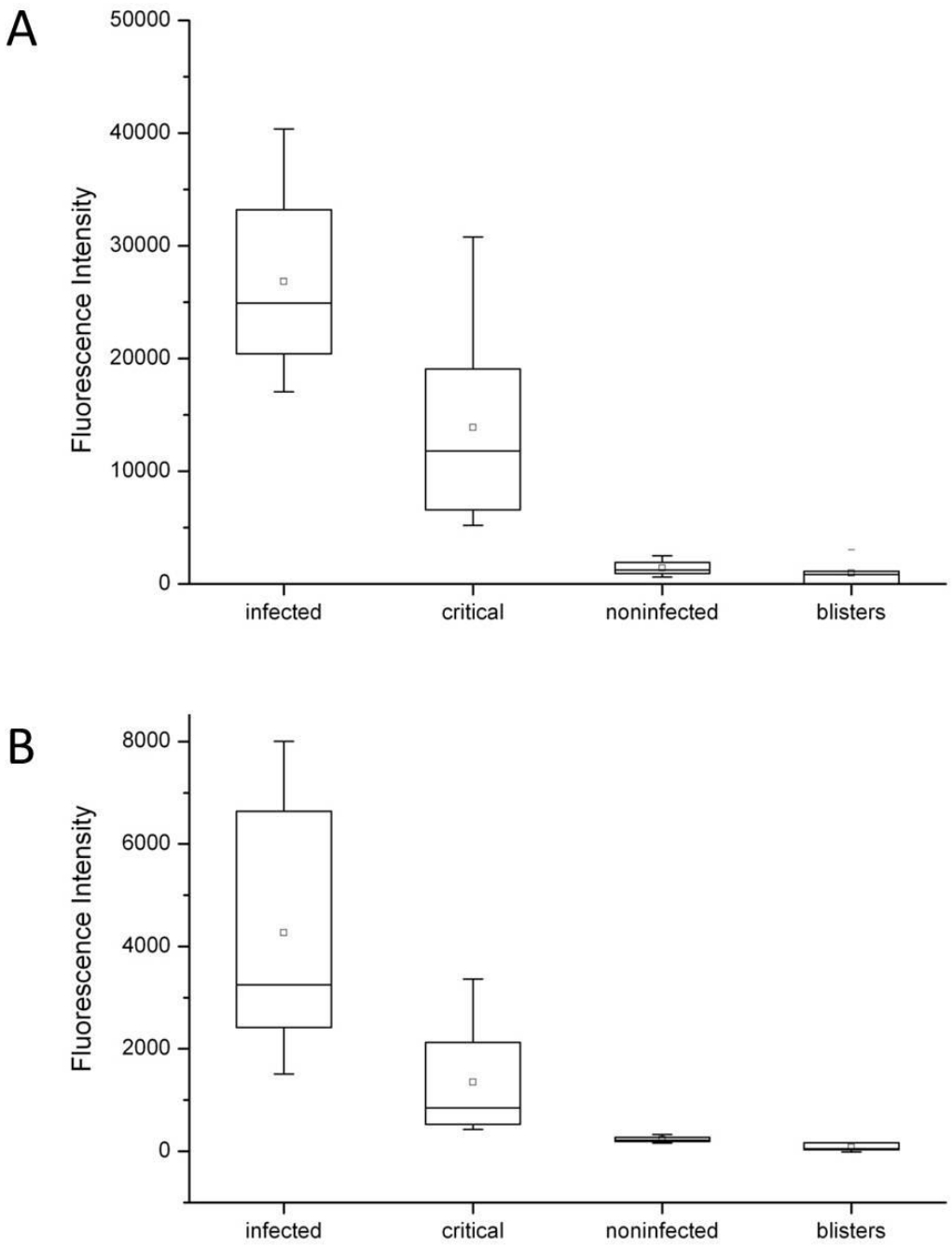

Figure 34: Gelatinolytic (A) and collagenolytic activities (B) in wound fluid samples. Gelatinolytic and collagenolytic activity (given as fluorescence intensity FI) was measured in 8 infected, 8 critical, 8 non-infected wounds and 5 blisters. There is a significant difference between enzyme activities in infected and non-infected wound fluids ($P<0.001$). (P-values for gelatinolytic activities: i – crit: 0.0098; i – n.i: 0.0001; i – b: 0.0000; for collagenolytic activities: i – crit: 0.0131; I – n.i: 0.0025; i – b: 0.0020)

It is well known that the chronicity of wounds is related to elevated levels of MMP`s.
The role of MMPs, especially of MMP-9/or MMP-2, has been implicated in the pathogenesis of several infectious diseases [30,31]. Several groups show, that delayed healing is characterized by an increase in matrix metalloproteinases (MMPs), a decrease in tissue inhibitors of MMPs (TIMPs), and a reduction in some growth factors. However, the mechanism of increased MMP-9 is uncertain. This is very likely, since MMP-9 is expressed mainly by neutrophils and macrophages, important cells for the inflammatory response [32].
While in the normal wound healing process the controlled degradation of the extracellular matrix allows the removal of damaged tissue [31], the excessive degradation of ECM by proteinases is supposed to be one factor for impaired wound healing. Components of the ECM play an important role in regulating and integrating many key processes of healing. It has been proposed that the prolonged inflammatory phase of chronic wounds causes increased levels of proteases such as MMPs, elastase, plasmin and thrombin, which destroy components of the ECM and damage the growth factors and their receptors that are essential for healing [18].
Additionally, extracellular proteolysis of gelatin can be ascribed to another group of proteases expressed by PMNs, namely serine proteinases [22]. Elevated levels of neutrophil-derived elastase play an important role in delayed wound healing and were recently documented in case of wound infection [11]. Our results clearly indicate the high value of these enzymes as possible diagnostic markers for infection and justify the further development of a simple diagnostic tool for easy detection of these elevated enzyme activities. As analysis of wound fluid is a non-invasive method which is assumed to provide powerful information for determining the overall status of a wound, a simple diagnostic tool was developed for detection of elevated proteolytic activity.

8.3.2 Investiagtion of gelatin beads for a diagnostic tool

For the development of a simple diagnostic tool, beads comprising gelatin with covalently bound dies were developed. Incubation with wound fluid was performed and the release of dye was determined spectrophotometrically after different time intervals (Figure 35). The concentration of the cross linker agent (GDGE) showed a clear effect on the colour release that could be observed even after one hour with the suitable concentration (Figure 35A).

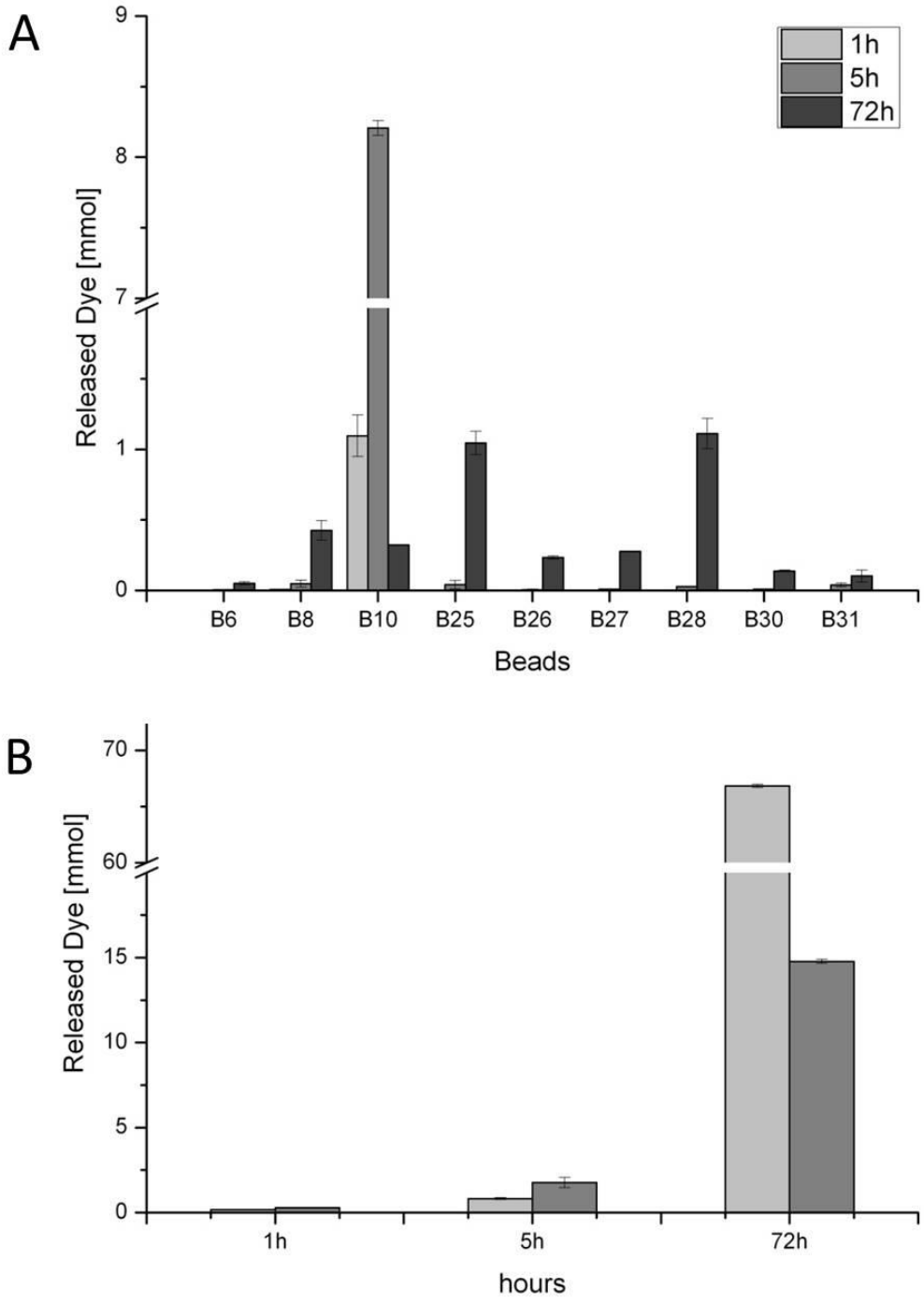

Figure 35: Release of dye from gelatine beads upon incubation with wound fluid or elastase for different time intervals. Different concentrations of cross linker lead to different release rates and concentrations of the dye, incubated with wound fluid (A). Detailed dye release study with one chosen gelatine bead (B) for an incubation interval over 72 hours with wound fluid and elastase.

A higher crosslink density (6 mL GDGE) led to a slower degradation of the matrix, as expected (data not shown). To monitor the release over a time period of 72 hours we used

wound fluid and elastase (Figure 35B). Incubation with infected, wound fluid samples led to a visible colouration of the supernatant already after 30 minutes. A clear difference in colour release between infected and non-infected (34 fold for 60 minutes) wound fluid samples was found (Figure 36). These results are in good accordance with the elevated gelatinolytic activities determined in infected wounds.

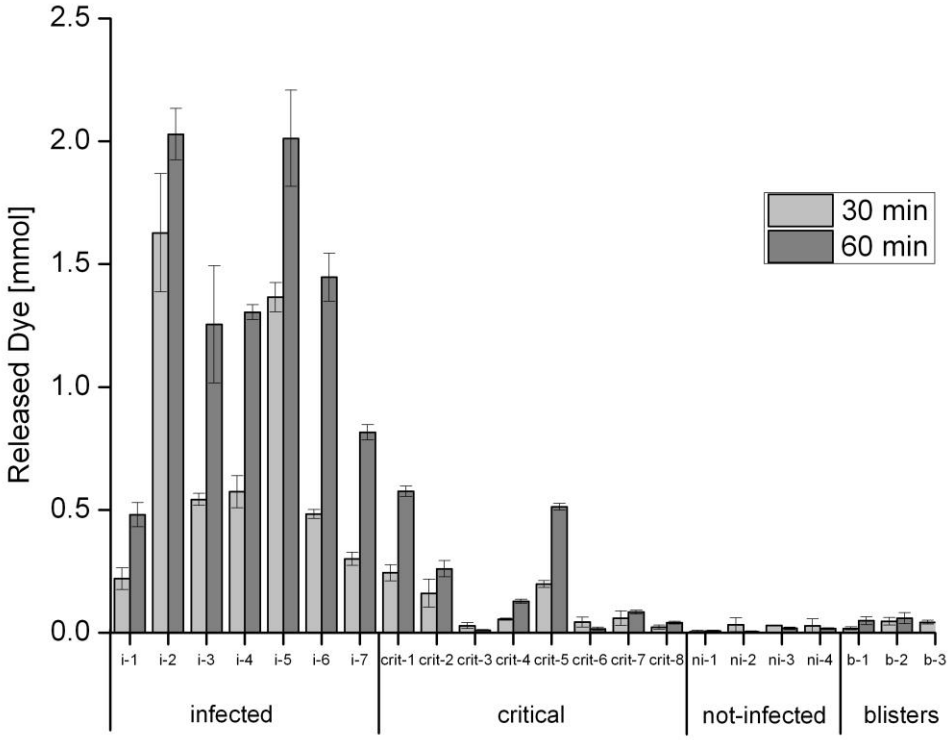

Figure 36: Release of dye from gelatine beads after incubation with wound fluid of infected, critical and non-infected wounds for 30 and 60 minutes. Additionally, blister fluid was used as a negative control. Released amount of dye is given in mmol.

Beads represent a suitable system especially for controlled release systems. Compared to a layer system, beads provide a bigger surface area with immobilized dye. This leads to a higher release output of dye (or dye fragments) in the same time period when incubated with enzyme or wound fluid.

Therefore it seems to be realistic that beads could be used for the construction of e.g. test stripes allowing diagnosis based on direct visual inspection. As stability is a crucial fact for application of the system, beads were tested after storage at +4°C and -20°C for three months. No loss of reactivity was found, indicating the suitability of the system for integration in a fast and easy to handle diagnostic system.

8.3.3 Differentiation of gelatinolytic activity

Besides MMPs and human neutrophil elastase, microbial elastase as well as other proteases of bacterial origin can contribute to the collective gelatinolytic activity in wound fluid. Thus, inhibition experiments and zymography were carried out to obtain detailed information about the contribution of the different proteases on gelatinolytic activity.

8.3.3.1 Inhibition Experiments

The influence of various types of inhibitors on gelatinolytic activity was investigated. Three different wound fluid samples of infected wounds were incubated with and without inhibitors for 2 hours and 5 hours. Inhibition was either determined by colour release of incubated beads or directly by measuring gelatinolytic activity with labeled gelatin. Elastase was used as a control.

The metalloproteinase (i.e. MMP) inhibitor 1,10-phenanthroline (Phe) partially inhibited gelatinolytic activity in infected wound fluid samples by 11% to 37% (Table 9). On the other hand, phenylmethlysulfonyl fluoride (PMSF), a serine protease inhibitor (targeting HNE) led to a reduction of gelatinase activity between 60% and 95%. In case of elastase used as a control, a 100% inhibition was found. As expected, the combination of Phe and PMSF led to a complete inhibition of gelatinase activity in wound fluid. These results clearly show the contribution of the different proteinases to gelatinolytic activity while their ratio is depended on the individual wound fluids.

Table 9: Inhibitory effect of phenantroline (Phe) and phenylmethlysulfonyl fluoride (PMSF) on the gelatinolytic activity in wound fluids within an incubation time of 5 hours. Shown values represent the inhibition (given in %) according to released dye (beads B) or measured gelatinolytic activity using the enzcheck assay (E)

Wound fluid samples		Inhibitors		
		Phe	PMSF	Phe/PMSF
sample 1	B	36.7	95.0	99.7
	E	11.0	71.2	99.7
sample 2	B	11.0	89.9	100.0
	E	11.2	61.6	100.0
sample 3	B	18.1	79.3	98.7
	E	0.0	59.5	98.7
elastase	B	0.0	100.0	100.0
	E	0.0	97.4	100.0

Protease inhibitors are classified by different systems identifying different families and superfamilies [33]. A commonly used inhibitor for metalloproteinases is 1,10-phenanthroline, which is lowering the concentration of metal by removing it from the enzyme active site. Indeed, incubation of elastase with 1,10-phenanthroline showed no inhibitory effect.
On the other hand, PMSF, a specific serine protease inhibitor, completely inhibited elastase activity. Serine protease inhibitors bind specifically and covalently to the active site serine residue in the serine protease. The inhibition of 60% to 95% of gelatinolytic activity in wound fluid samples clearly shows the high contribution of elastase to gelatinase activity measured in wound fluid. However, we recently could show that elastase is significantly elevated in infected wounds compared to non-infected wounds [11]. Therefore, the high influence of elastase on gelatinolytic activities in infected wounds is unexpected, but does not impair the value of the system when integrated into a diagnostic tool.

8.3.3.2 Zymography

For further characterization of proteases contributing to gelatinolytic activity in wound fluid, gelatin-zymography was carried out. Zymography techniques are used to detect proteolytic enzymes following electrophoretic separation in gel matrices. These methods are based on SDS-polyacrylamide gel that is co-polymerized with protein substrates. Proteins are degraded by the proteases which are restored during the incubation period in an enzyme reaction buffer after electrophoretic separation. Proteolytic activity can be visualized as clear zones where gelatin is degraded.
Zymograms were done under conditions optimal for members of the MMP class of enzymes (neutral pH in the presence of Ca^{2+}). Gelatinolytic activity could be identified only in infected wounds, while no clear zones were detected in non-infected wounds (Figure 37)

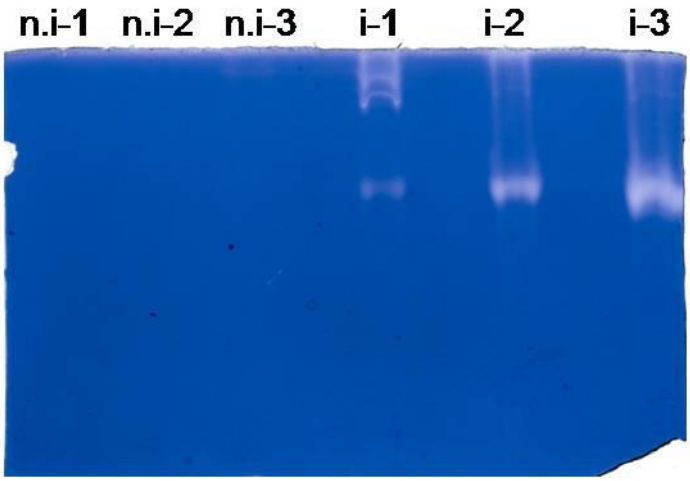

Figure 37: Zymogram of 3 non-infected (n.i-1, n.i-2, n.i-3) and 3 infected (i-1, i-2, i-3) wound fluid samples to compare gelatinolytic activity. Qualitative zymographic analysis was performed using polyacrylamide gels containing 0.1 % gelatine.

8.3.4 Microbiological study

For diagnosis of wound infection, microbiological analyses were performed of all investigated samples. *Staphylococcus aureus* was isolated in 7 samples out of 8, *Pseudomonas aerugionosa* in 1 and *Streptococcus spe*. in 2 samples. These data are in good accordance with previous studies by Ehrenkranz [34] and Murray [35]. Interestingly, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are both able to secrete gelatinases. As bacterial gelatinases degrade type IV collagen in the basement membrane and exposing underlying tissue, they may play a significant role in pathogenesis [36]. In addition, they are able to degrade partially denatured collagen fragments generated either by interstitial collagenase during normal connective tissue metabolism or by bacterial collagenase [36]. Extracellular proteases secreted by *Pseudomonas aeruginosa* include gelatinases such as MMPs (matrixmetalloproteinases) and other proteases such as elastase [37].The *P. aeruginosa* elastase has been reported to hydrolyze the 72-kD gelatinase to yield active forms during infection and wound healing. Additionally, some strains of *S. aureus* are able to secrete serine proteases like elastase [27]. As these bacterial strains are known to be very common in case of wound infection, contribution of bacterial proteases to overall gelatinolytic activity in wounds is likely.

Wound infection is one of the most common reasons for the non-healing of a wound, leading to death of the patient in the worst case. At the moment, there is no rapid diagnostic tool available for the indication of wound infection. Especially in home care, a diagnostic tool would be extremely helpful, as a fast prognostic aid could assist in predicting clinical infection of a wound before obvious clinical symptoms. Therefore we have determined gelatinolytic

and collagenolytic activities in different types of infected and non-infected wounds. As both enzyme activities are significantly elevated not only in infected wounds, but also in critical wounds, we have successfully developed an enzyme-responsive device for simple detection of elevated gelatinolytic activity in wound fluids. The data generated justify the evaluation of diagnostic parameters of gelatinase activity in a clinical study. Further on, this development could be integrated in a rapid and simple diagnostic system for detection of wound infection markers, thus allowing early stage warning of wound

8.4 References

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9 Fast Blue RR – siloxane derivatised materials indicate wound infection due to a deep blue colour development.

Under revision: D. Schiffer, G. Tegl, R. Vielnascher, H. Weber, R. Schöftner, H. Wiesbauer, E. Sigl, A. Heinzle, G. Guebitz: Fast Blue RR – siloxane derivatised materials indicate wound infection due to a deep blue colour development., *2015; Materials* 2015, *8*, 1-x manuscripts; doi:10.3390/ma80x000x

There is a strong need for simple and fast methods for wound infection determination. Myeloperoxidase, an immune system derived enzyme was found to be a suitable biomarker for wound infection. Hence, alkoxysilane derivatised Fast Blue RR was immobilized via simple hydrolytic polymerization. The resulting enzyme-responsive siloxane layers were incubated with myeloperoxidase, wound fluid or haemoglobin. The reaction was monitored via HPLC measurements and the colour development quantified spectrophotometrically. Myeloperoxidase was indeed able to oxidize immobilized Fast Blue RR leading to a blue coloured product. No conversion was detected in non-infected wound fluids. The visible colour changes of these novel materials towards blue enable an easy distinction between infected and non-infected wound fluids.

9.1 Introduction

Standard procedures for wound infection detection are time consuming (microbiology) or show limited reliability due to the judgement of the classical clinical signs, redness (*rubor*), heat (*calor*), swelling (*tumour*) and pain (*dolour*) or the detection of signals specific to secondary wounds [1–3]. Hence, alternative methods based on the assessment of biomarkers like metabolites, enzymes or microbes have been suggested [4]. New point-of-care devices should facilitate the diagnosis and treatment of chronic wounds [5]. Various enzymes of the immune system accumulate in the wound fluid during an infection, having a potential as biomarkers for infection detection [6]. Myeloperoxidase shows about ten times higher activities in infected wounds compared to non-infected wounds [7]. The potential of this enzyme was furthermore confirmed in a correlation study with silver standard wound diagnostics [7]. New substrates for myeloperoxidase were tested including Fast Blue RR (4-benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi (zinc chloride) salt) showing significant differences in colour development and indicating infections in wounds [8].

Fast Blue RR salt is commonly used for the detection of esterase and alkaline phosphatase activities in histochemical and colourimetric analysis [9]. In these reactions, naphthyl derivatives are applied as substrates and the enzymatic release of naphthol is followed via a coupling reaction with a diazonium salt such as Fast Blue RR. These reactions are usually performed in basic media and the formations of coloured derivatives take only a few minutes. Applications are found in assays for the determination of esterases as pro-drug target in prostate cancer, or to determine the content of alkylresorcinols (ARs) in ground and whole cereal grains [10,11].

Diagnostic devices in wound care should deliver fast results while being user friendly. Hence, simple handling of point-of-care devices is often achieved by immobilization of the biomarker detection chemistry onto materials. Obviously simple immobilization strategies are essential for large scale production of test strips. Alkoxysilane based biomarker substrates would allow the formation of functionalized siloxane layers on a large variety of material surfaces via simple hydrolytic polymerization. Such simple enzyme-responsive material surfaces could facilitate wound infection diagnosis and treatment.

9.2 Materials and methods

9.2.1 Functionalization of Fast Blue RR

Fast blue RR (N-(4-amino-2,5-dimethoxyphenyl) benzamide) was coupled to alkoxysilanes for immobilization. The coupling was performed according to Hasmann 2013 [8] and the molecular structure of the synthesized substrate was proven by 1H and 13C nuclear magnetic resonance (NMR). NMR spectra were measured on a Bruker Avance 3 at 300 MHz for protons and 75 MHz for 13C. The derivatised Fast Blue RR was dissolved in DMSO-d6. 16 scans were accumulated for 1H and 2048 scans for 13C.

9.2.2 Immobilization of derivatised Fast Blue RR

As a model surface for hydrolytic polymerization of alkoxysilane derivatised Fast Blue RR silica thin layer chromatography (TLC) plates were used. 1x1 cm squares were cut and overlaid with a 50 mM solution of derivatised Fast Blue RR dissolved in EtOH. The polymerization was performed for 24h at 105°C.

9.2.3 Transformation of the substrate

The transformation of alkoxysilane derivatised Fast Blue RR was carried out using pure myeloperoxidase (MPO, planta), infected and non-infected wound fluid (wf) of ulcer wounds and haemoglobin (Sigma). Wound fluids collected as described in Schiffer et.al. [6] MPO was diluted in potassium phosphate buffer (100mM; pH 7) to (end) concentrations of 20U/ mL; 10 U/mL; 5U/mL. The wound fluids were diluted in potassium phosphate buffer (100mM; pH 7) or sodiumacetate buffer (100mM; pH4), haemoglobin was used in end concentrations of 0.01 mM, 0.05 mM and 0.001 mM. The wound fluids and the haemoglobin dilutions were additionally measured at 405 nm to compare the haemoglobin content of the wf and the haemoglobin solutions. 10 µl of the respective solutions were mixed with 10 µl of 39.2 mM H2O2 solution and pipetted on the silica plates with the immobilized substrate. Solutions lacking H2O2 were used as blanks. Besides the visual inspection of the silica plates after 30 and 60 minutes, the colour changes on the surface of the silica plates were measured with a ColourLite sph850 spectrophotometer (ColourLite GmbH, Katlenburg-Lindau, Germany). As a reference, a wetted silica plate with immobilized substrate, but without enzyme was used. Based on the delta E values, calculations and statistical analyses were performed. To compare the different experimental setups regarding the statistical significance, two-sample

t-tests assuming equal variances were performed. A p value of less than 0.001 was considered as statistical significant.

9.2.4 HPLC sample treatment

The preparations for the HPLC samples were carried out as follows. The working solutions (1 ml) contained 20mM of the derivatised Fast Blue RR, 1.5 U/ ml MPO, 39.2 mM H2O2, 50mM NaCl in 100 mM potassium phosphate buffer (pH 7) or sodium acetate buffer (pH 4). As negative controls, all samples were also prepared leaving out MPO. The samples were incubated for 0/ 15/ 30/ 45/ 60/ 90/ 180/ 360 minutes. After these time points, 1 ml EtOH abs. was added and the acidic samples were adjusted to a pH of 7. A Carrez clarification was performed. 20µl of C1 solution (5.325 g of K4[Fe(CN)6] · 3 H2O, dissolved in water filled up to a volume of 50 mL) was added to the samples, put on the vortex for 1 minute, followed by addition of 20µl of C2 solution (14.400 g of ZnSO4 · 7 H2O, dissolved in water, filled up to a volume of 50 mL), 5 minutes shaking and an additional centrifugation step (15000 rpm) for 15 minutes. 500 µl of the samples were again mixed with 500 µl of EtOH abs. and purified using a 45 µm filter. The samples were then distributed (250µl each) in HPLC vials.

9.2.5 HPLC measurement

LC:

A LC 1260 pump (Agilent G1312B) was operated using 20mM ammonia formiate in water and acetonitrile as mobile phase. A gradient was set from 0% to 100% acetonitrile within 35 min in a 45 min method. The Column Poroshell 120 EC-C18 4.6*50mm 2.7 Micron (Agilent) was equilibrated at 40°C in a 1290 Infinity 2 TCC (Agilent G7116B) with 80% water 20mM ammonia formiate and acetonitrile for 60 min. The LC-MS grade water was purified by an ELGA PURELAB ultra (VWS) all other chemicals were supplied from Sigma Aldrich in LC-MS grade.

LC-ESI TOF :

The LC was coupled to a DAD (Agilent G4212B) and a Dual ESI G6230B TOF (Agilent). For the electrospray ionisation (operating in positive ion mode) a nebulizer was used, the dry gas flow was set to 8l/min and a pressure of 40 psig at 250°C was chosen. The fragmentor voltage was set to 200V, the skimmer at 65V, the octopole to a voltage of 750V and the reference masses were 121.0509 and 922.0098 m/z. Ions from 50 m/z to 3000m/z were acquired with the Agilent MassHunter Workstation (Version B06.01). The DAD signal at 254nm was also monitored by the Agilent MassHunter Workstation. A statistical calculation in

accordance to German industrial standard 32645 for the detection limit, detectability limit and limit of determination was performed. Significance was tested with p values less than 0.05.

9.3 Results and discussion

Recent studies confirmed the ability of myeloperoxidase (MPO) for infection detection in wounds [8]. Significant differences in enzyme activity comparing infected and non-infected wound fluids were observed, using the well-known substrate guaiacol suitable for spectrophotometric measurements. However, it is not possible to covalently immobilize guaiacol in way that it is still transformed by MPO. Hence, Fast Blue RR was covalently coupled to alkoxysilanes allowing simple formation of enzyme-responsive siloxane layers on a variety of surfaces. Figure 38 shows the coupling reaction and the final product which was analysed with NMR spectroscopy.

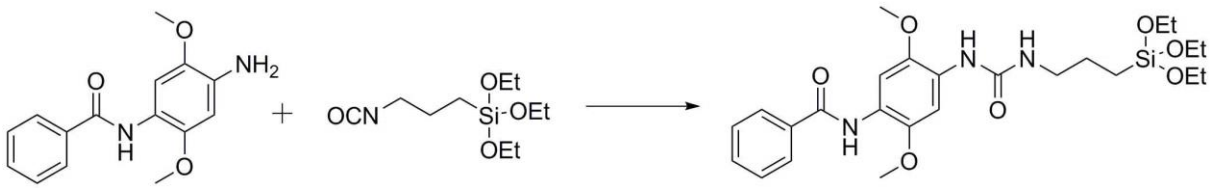

Figure 38: Derivatised Fast Blue RR: Fast blue RR was coupled to 3-(triethoxysilyl)propyl isocyanate. NMR measurements confirm the structure of the stable product

Simple hydrolytic polymerization of this enzyme-responsive substrate enables application in test strips for early-stage wound infection detection. Thereby it is essential to consider cross reactions with haemoglobin, likewise present in wound fluids. After immobilization of the substrate onto silica plates as model carrier, the reactivity was tested with pure enzyme (MPO), haemoglobin as well as with infected and non-infected wound fluids. A fast colour reaction towards blue (10 minutes-30 minutes) was recorded (Figure 2). The haemoglobin content of the wound fluid was determined prior to the incubation with the substrate and was found to be 0.007 mM. Figure 32 shows the blue colour development of the enzyme-responsive siloxane layer upon incubation. A significant difference in the delta E values was observed with infected and non-infected wound fluids with a p value of less than 0.001. The colour development in figure 32 shows the progression from 30 to 60 minutes. An incubation interval of 30 minutes already provides a statistically significant determination between infected and non- infected wound fluids. Cross-reactions with haemoglobin occur, but the delta E values show 60% less colour development in samples with a higher haemoglobin

content compared to the infected wound fluid sample. Significant differences, independent of the time interval are indicated in figure 39, representing p values of less than 0.001.

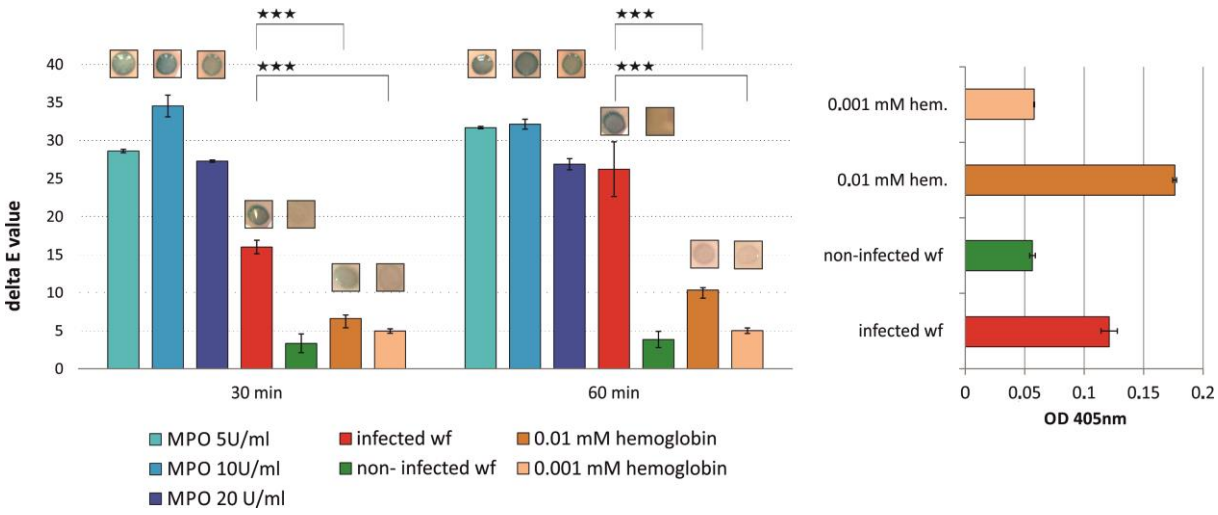

Figure 39: (a) Colour formation of immobilized alkoxysilane derivatised Fast Blue RR upon incubation with MPO and wound fluids: Alkoxysilane derivatised Fast Blue RR was polymerized and the resulting enzyme-responsive siloxane layer incubated with different enzyme activities of pure MPO, infected and non-infected wound fluids as well as different concentrations of haemoglobin over a time period of 30 and 60 minutes. The blue colour formation upon conversion of the substrate was quantified with a ColourLite sph850 spectrophotometer and is given as in delta E values. Two-sample t-tests assuming equal variances were performed, p-values equal or less than 0.001 were considered as significant (***). (b) The haemoglobin concentration of the infected and non-infected wound fluid was determined prior to the immobilization experiments.

9.3.1 LC ESI TOF

In order to investigate MPO oxidation alkoxysilane derivatised Fast Blue RR, LC-ESI-TOF experiments were conducted. Two different pH values were investigated comparing the reaction kinetics at physiological conditions and at acidic pH. Similar to other MPO substrates like guaiacol, LC-ESI-TOF indicated that oxidation of alkoxysilane derivatised Fast Blue RR led to the formation of a variety of oligomers [12]. Hence, the reaction was monitored based on consumption of alkoxysilane derivatised Fast Blue RR. For both pH values a substantial decrease within 60 min was observed by LC and ESI-TOF (Figure 40). An immediate Fast Blue RR consumption of alkoxysilane derivatised Fast Blue RR was observed at pH 4, however, similar results at both pH values were obtained for the subsequent time measurements. It is supposed the alkoxysilane derivatised Fast Blue RR is less stable under acidic conditions, which leads to a partial degradation of the substrate prior

to enzymatic conversion. The LC data shows a fast consumption of the enzyme responsive material with full conversion observed already after 6 h.

Both the colour reaction of the enzyme-responsive siloxane layer and LC-ESI TOF results reveal the suitability of derivatised Fast Blue RR being an effective system for a sensitive detection of elevated MPO activity after short time. The fast response within the first minutes of reaction time renders this detection system a promising candidate for the incorporation into point-of-care diagnostics. Not least the functional spacer facilitates the attachment on various surfaces.

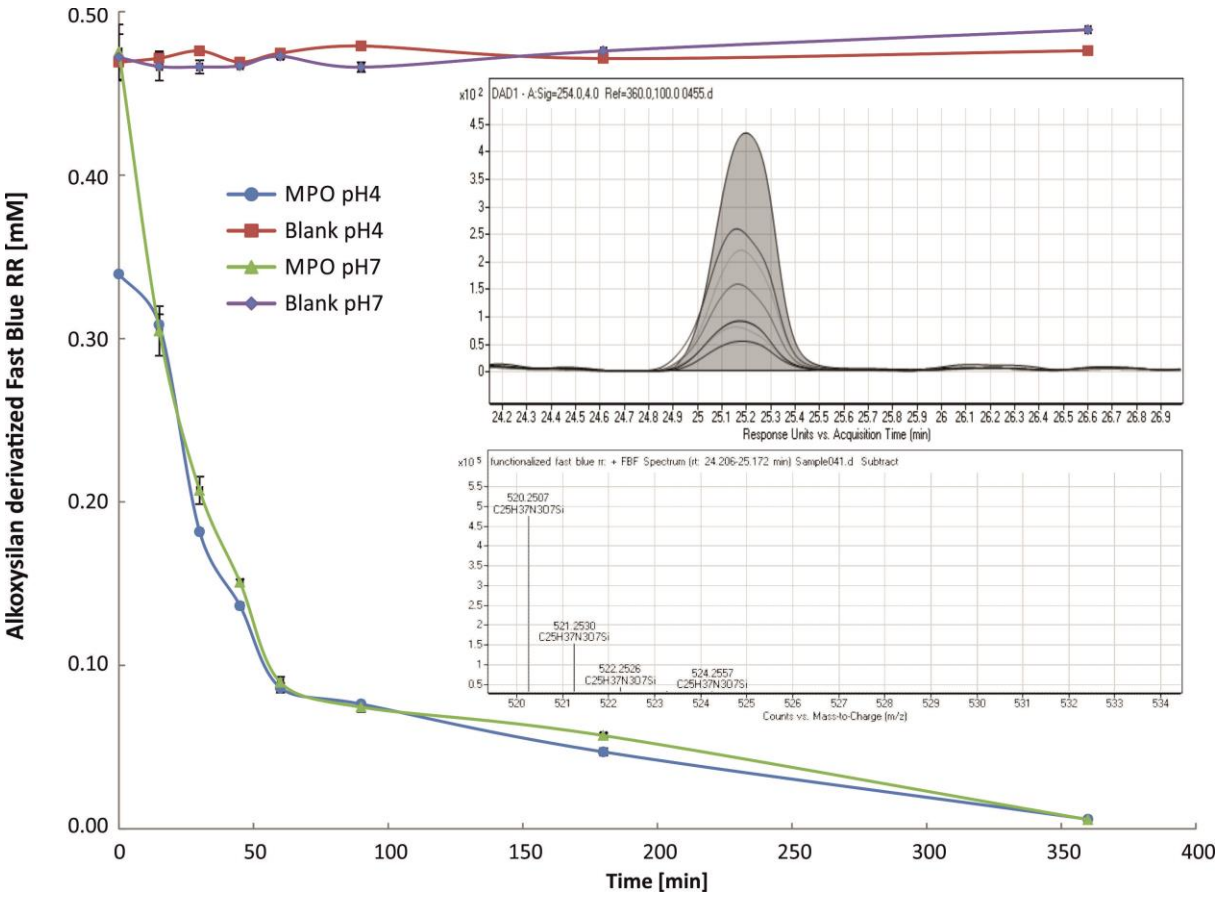

Figure 40: LC-MS TOF analysis of alkoxysilane derivatised Fast Blue RR conversion by MPO: A substantial conversion of substrate could be observed already after several minutes at both pH values. After 6 h incubation time no substrate could be detected anymore by LC-ESI TOF. The graphs inside the figure depict the decrease of substrate illustrated as the function the substrate mass being 520.25.

9.5 Conclusion

Fast Blue RR acts as a suitable marker for the detection of infection. Upon incubation with MPO or infected wound fluid, a blue colour development was observed on polymerized alkoxysilane derivatised Fast Blue RR layers. Cross reactions with haemoglobin occur but delta E values as well as the visual inspection confirm a negligible colour development compared to pure MPO solutions or infected wound fluids. LC-ESI TOF analyses further confirm the conversion of the substrate by MPO at different pH values. This derivatised substrate could facilitate the determination of infection in wounds.

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10 Conclusion

This work deals with the difficulties and controversies of proper and fast diagnosis of wound infection in chronic wounds and provides novel strategies for and easy-to-handle clinical infection detection. In practise, the commonly used - but error-prone method - for infection detection is the clinical description of the wounds based on its classical signs like redness (*rubor*), heat (*calor*), swelling (*tumour*), pain (*dolour*) and impairment of function (*functiolaesa*). The determination of the bacterial burden is known as the silver standard, but apart from that it is time consuming and requires trained staff and certain infrastructure. Biopsy is considered as the gold standard, but is hardly carried out in clinical practise, due to concerns over the harm of the patient and the likewise time consuming procedure. Consequently, there is a pressing need for point-of care diagnostics to facilitate wound status determination.

Comparing the progress towards biomarkers for infection published recently, three main groups of possible biomarkers for infection detection emerge – namely bacteria, enzymes / proteins and metabolites. The practicability of new strategies is varying, highly specific methods often need time consuming preparations, while fast and easy applications sometimes only cover few parameters.

The wound bed and the wound fluid per se harbour enzyme biomarkers, providing a revealing insight in the current wound status. The focus in this thesis was on the one hand on the biochemical determination of the enzyme activities compared to silver standard methods as well as on the development of enzyme - responsive systems that produce either electrochemical signals or colour reaction that both allows simple detection of infection.

A study including wound fluids of 75 patients suffering from ulcers, diabetic feet, acute wounds, decubitus wounds and blisters formed the basis for the biochemical analyses and the comparison of the enzyme levels with the microbiological results (silver standard) and the clinical description of the wounds in this thesis. The enzyme levels of human neutrophil elastase (HNE), myeloperoxidase (MPO) and lysozyme were measured spectrophotometrically, showing significant activity differences between infected and non-infected wound fluids. This was in accordance with the silver standard wound fluid diagnosis results distinguishing between the categories “infected” “possibly infected” and “non-infected” based on the presence of potentially pathogenic micro-organisms. On the other hand, only 35% of the results of the clinical look investigations were in accordance with these microbiological results. A precise detection of infection based on the three enzymes was possible in 85% of the samples, even though clinical look investigation did not predict an infection. Previously, this concept of a colour changing liquid enzyme assay as an application

for infection detection was patented (EP 09160557). This study confirmed the usability of these enzymes as infection markers based on these detailed scientific investigations.

Another part of the thesis focussed on the development an alternative strategy for the determination of elevated myeloperoxidase levels based on the principles of electrochemical sensors usually used for diabetic patients. Myeloperoxidase is a hydrogen peroxide consuming enzyme, the activity of which is commonly measured with guajacol. To eliminate supplemental substrate addition, the chlorination activity (the formation of hypochlorous acid) catalysed by this enzyme was investigated and compared to the peroxidation activity, showing concurrent activity increase in infected wound fluids. Based on this, an electrochemical sensor was designed, quantifying the consumption of hydrogen peroxide by myeloperoxidase based on amperometic principles (i.e. current loss). To enhance the storage ability and to avoid external addition of hydrogen peroxide, a second enzyme that provides the required hydrogen peroxide for the myeloperoxidase reaction was directly immobilised on the sensor. Namely glucose oxidase fulfilled these requirements, as it uses glucose as substrate, which was found in adequate concentrations in the wound fluids per se. Sensor systems for MPO activity measurements exist in other application areas using 3,3′,5,5′-Tetramethylbenzidine (TMB) as substrate that was, however, shown previously to interact with haemoglobin, possibly leading to false positive results in would infection determination. The chance to access the chlorination activity of the enzyme avoided cross reactions, resulting in significant differentiation of infected and non-infected wound fluids. An electrochemical precursor for infection detection was investigated, reducing redundant substrate supplements and working steps.

An electrochemical sensor is one example of an enzyme-responsive system for wound detection. Another concern of this thesis was the usage of enzyme responsive polymers as infection indicator. Various applications are described in the literature, including polysaccharides and proteins used as spheres, capsules and hydrogels as well as synthetic polymers composed of polyesters or polyamides used as films, or the polymerization of derivatised substrates on various materials. The common ground of these applications is that these substrates respond to enzymes leading to changes of their physical properties - and for our purpose, resulting in a visible colour change.

A method that could be combined with an enzyme – responsive carrier surface was investigated for lysozyme. The responsive matrix was composed of Remazol Brilliant Blue labelled peptidoglycan, resulting in the formation of coloured oligomers upon incubation with lysozyme or infected wound fluids. The simplified but effective concept consisted of a size exclusion and an ion- exchanger membrane, known from RNA-protein separation protocols.

Only hydrolysed coloured oligomers were able to pass the size exclusion membrane and were subsequently trapped by the anion capturing membrane. An occurring colour development up to seven-fold was monitored comparing infected and non-infected wound fluids. The all-in-one test concept was contemplated as simple as possible avoiding external liquid handling steps for the colour visualisation. An advantage compared to immobilisation of the substrate was the avoided leaching of any matrix components due to the consecutive membranes. An easy –to –handle method was developed providing the basic concept of a future lateral flow device. The visible deep blue colour development of the second membrane within 30 minutes facilitates the detection of infected wound fluids.

Despite the already mentioned enzymes, the applicability of the gelatinolytic enzyme activity was investigated and an application using dye releasing particles composed of gelatine was developed. Matrix metallo proteases (MMPs) and serine proteases are known to play a role in delayed wound healing. We tested the possibility of a relationship between the gelatinolytic activity and the presence of wound infection in infected and non-infected wound fluids using a fluorescein labelled gelatine as substrate. The gelatinolytic activity was 23 times higher in infected wound fluids. To obtain detailed information about the contribution of the different proteases, inhibition experiments using 1,10-phenantroline for MMPs and PMSF as serine protease inhibitor were carried out. Less inhibitory effect was seen for 1,10-phenantroline (11%) compared to PMSF (60-70%) depending on the individual wound fluids, a complete inhibition was observed by combining the inhibitors. As simple diagnostic tool, gelatine beads were loaded with reactive blue. In this approach the colour formation was achieved by the enzymatic degradation of the polymer and a subsequent release of the dye. Incubation with infected wound fluids let to a visible colourisation of the supernatant within 30 minutes, a 34-fold higher colour release was measured after 60 minutes. Easy to fabricate enzyme-responsive beads could be integrated in test strips due to their high output of dye release and their storage stability. Gelatine beads were tested after storage at +4°C and -20°C, without activity loss.

Apart from directly enzyme responsive natural polymers, functionalization of polymers with printable enzyme substrates was investigated for myeloperoxidase. Fast blue RR was recently discovered as suitable substrate for this enzyme. To create reactive enzyme-responsive material layers, the substrate was derivatised with alkoxysilan functionality for simple immobilisation via hydrolytic polymerisation leading to enzyme responsive siloxanes. After immobilisation on silica plates as a model carrier, the reactivity was tested with pure enzyme, haemoglobin and infected as well as non-infected wound fluids. An incubation interval of 30 minutes led to deep blue colour development on the surface of the carrier upon incubation with myeloperoxidase and infected wound fluid. Significant colour increase of 80%

was measured for infected wound fluid compared to non-infected wound fluid. Cross reactions with haemoglobin occurred, but the delta E values referring to the colour change measured with a spectrophotometer showed 60% less colour development in samples with a higher haemoglobin content (rarely found in wound samples) when compared to the infected wound sample. LC-ESI-TOF experiments confirmed the MPO oxidation of alkoxysilan derivatised Fast Blue RR due to a substantial decrease within 60 minutes. The fast and visible response of the material surface could facilitate the detection of an infection when used as external test strip during a bandage change in clinical practise. Simple printing of these substrates on various materials could enable the implementation of this easy-to-handle infection detection method in other already existing medical applications.

Wound diagnostic is still very challenging due to the few methods used in clinical practise. Many new ideas emerged during the last years focussing on different biomarkers for infection detection. Within this work we clearly demonstrates the potential of enzyme responsive materials for the simple and fast detection of myeloperoxidase, lysozyme, human neutrophil elastase as well as the gelatinolytic enzyme activity as suitable infection markers in wounds. We furthermore established different enzyme-responsive methods and approaches for each enzyme, suitable as stand-alone tool or for the incorporation in future medical devices.

11 List of Tables

12 List of Figures

13 Abbrevations

A

ADHP	10-acetyl-3,7- dihydroxyphenoxazine
AF	Auto fluorescence
Ag/AgCl	silver/silver chloride
APF	p- aminophenyl) fluorescein

C

CatG	cathepsin G
CCD	charge-coupled device
CLEAR	cross-linked ethoxylate acrylate
CLSM	confocal laser scanning microscopy
CMC	methacrylated carboxymethylcellulose
CMCS	Carboxymethyl chitosan
CPE	carbon paste electrode
CRP	C-reactive protein
C-SPEs	Carbon screen printed electrodes
CZE	cappillary zone electrophoresis

E

ECM	Extra cellular matrix
EIS	electrochemical impedance spectroscopy
ELISA	enzyme-linked immunosorbent assay
ESBL	extended spectrum β-lactamase
ESI-TOF	ElectroSpray Ionization - Time Of Fligh

F

Fast blue RR	(N-(4-amino-2,5-dimethoxyphenyl) benzamide)
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate

G

GMA	glycidylmethacrylate, glycidyl methacrylate
GOD	Glucose oxidase
GUV	Giant unilamellar vesicles

H

H_2O_2	Hydrogenperoxide
HNE	Human neutrophil elastase
HP	hairpin probe
HPF	39-(p-hydroxyphenyl) fluorescein
HPLC	high pressure liquid chromatography
HSL	N-3-oxo-dodecanoyl-l-homoserine lactone

I

IDSA	Infectious Disieases Society of America
IL-8	Interleukin 8
inf	infected
IWGDF Foot	The International Woking Group of Diabectic

K

kDa	kilo Dalton

L

LC	liquid chromatography
LFIA	lateral flow immunoassay
LYS	lysozyme

M

MALDI-TOF	Matrix assisted laser desorption i onization time-of-flight
MeOSuc-AAPV-pNA	N-methoxysuccinyl-AAPV-p-nitroanilide
mL	milliliter
mM	millimolar
MMP-8	Matrix metallo protease
MPO	Myeloperoxidase
MRSA	Methicillin-resistant S. aureus

N

n.i.	non-infected
Na_2CO_3	sodium carbonate
Na_2SO_4	Sodium sulphate
NaCl	sodium chloride
NGS	Next generation sequencing
NMR	nuclear magnetic resonance

P

PA	polyamide
PC	platelet concentrates
PCR	Polymer chain reaction
PDGF	platelet derived groth factor
PES	polyester
PG	Peptidoglycan
PGA	polygalacturonic acid
PGE	pencil graphite electrode
Phe	1,10-phenanthroline
PMN	Polymorphonulclear neutrophil
PMSF	phenylmethlysulfonyl fluoride
PNA	peptide nucleic acid
ppmos	potentially pathogenic microorganisms
PR3	proteinase 3
PRODIGI	Portable Real-time Optical Detection, Identification and Guidance for Intervention
PSiMc	porous silicon microcavity

Q

QCM	Quartz crystal microbalance

R

RBB	Remazol Brilliant Blue
RBB R	Remazol Brilliant Blue R
ROS	Reactive Oxygen Species
rt-PCR	Real-time PCR
$Ru(dpp)_3$	ruthenium (II)tris-(4,7-diphenyl-1,10-phenanthroline)

S

SD	signal drop
SIEFED	Specific Immunological Extraction Followed by Enzymatic Detection
SNP	Single Nucleotide Polymorphism
SP	signal probe
SPQC-IDE	piezoelectric quartz crystal-interdigital electrode
SPRI	plasmon resonance imaging
SWV	square wave voltammetry

T

TGF- β	Transforming growth factor beta
TIMP	Tissue inhibitor of matrix metallo protease
TMB	3,3-5,5-tetramethylbenzidine
TNF	tumour necrose factor

14 Publications

Papers

D. Schiffer, G. Tegl, R. Vielnascher, H. Weber, R. Schöftner, H. Wiesbauer, E. Sigl, A. Heinzle, G. Guebitz: *Fast Blue RR – siloxane derivatised materials indicate wound infection due to a deep blue colour development*., 2015; under revision/ minor revision

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Lebenslauf/CV

Persönliche Daten

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