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Diagnostic system for detection of wound infection based on novel immobilized enzyme substrates

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„DIE VORAUSSETZUNG FÜR WISSEN IST DIE NEUGIER"

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ABSTRACT

This work was performed within the European project called “Lidwine”. Research of this project is directed towards the prevention and detection of chronic wounds, focused on decubitus ulcers. The aim of this PhD thesis was the development of a diagnostic device for an early detection of wound infection directly in wound fluid. Bacterial infection of wounds is the main course of impaired wound healing and a severe complication regarding the normal wound healing process. In this case, a timely diagnosis of an incipient infection is the critical factor of a successful medical treatment. Therefore, different enzymes produced by the human immune system were selected and tested for their suitability as marker for wound infection. Activities of lysozyme, cathepsin G, elastase and myeloperoxidase were found to be elevated in case of infection. Based on these results different systems were investigated for identification of wound infection directly in wound fluid.

For lysozyme unstained and remazol brilliant blue stained *Micrococcus lysodeikticus* cells were incorporated into an agarose-matrix. Lysozyme activity was indicated by a decrease of turbidity of the layer. The sensitivity and specificity of lysozyme detection in wound fluid samples were enhanced by using remazol brilliant blue (RBB) labeled peptidoglycan incorporated into agarose layers or alginate beads. Digestion of these bacterial cell walls and colour release due to lysozyme activity was then correlated with the infection state.

For detection of myeloperoxidase activity, novel assays were established. As substrates guajacol, TMB, ABTS, Fast blue RR (4-benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi(zinc chloride) salt), crystal violet and leuco crystal violet were used. Additionally systematically substituted azo dyes with 2,7-dihydroxy-1-phenylazonaphtalene-3,6-disulfonic acid as basic structure was tested for their ability as being used as MPO substrate. For a better handling of the diagnostic device immobilisation of different MPO substrates was carried out. Crystal violet, 2,7-dihydroxy-1-(4-hydroxyphenylazo)naphtalene-3,6-disulfonicacid and Fast Blue RR (4-Benzoylamino-2,5-dimethoxybenzenediazonium chloride) were assessed for coupling to siloxanes for immobilisation.

Additionally the potential of human neutrophil elastase and cathepsin G for monitoring wound infection was assessed. These enzymes were determined directly in wound fluid. In a further step the immobilisation of specific chromogenic elastase and cathepsin G substrates on different supporting materials was carried out. This system provides a potential tool for distinction of infected and non infected wound fluids and could serve as possible application for online-monitoring of elastase and cathepsin G activity directly in bandages or dressings.

ZUSAMMENFASSUNG

Diese Arbeit wurde innerhalb des europäischen Projekts namens "Lidwine" durchgeführt. Dieses Projekt ist auf die Verhütung und Detektion von chronischen Wunden mit speziellem Fokus auf Dekubitus Wunden ausgerichtet. Das Ziel der Doktorarbeit ist die Entwicklung eines Diagnose-Kits zur Früherkennung von Wundinfektionen direkt in Wundflüssigkeiten. Bakterielle Infektion von Wunden ist der Hauptgrund von Wundheilungsstörungen und eine schwerwiegende Komplikation. Im Fall einer vorhandenen Infektion ist eine rechtzeitige Diagnose der entscheidende Faktor für eine erfolgreiche medizinische Behandlung. Deshalb wurden verschiedene Enzyme, die durch das menschliche Immunsystem produziert werden, ausgewählt und auf ihre Fähigkeit als Marker zur Detektion von Wundinfektion getestet. Es konnten erhöhte Aktivitäten von Lysozym, Cathepsin G, Elastase und Myeloperoxidase im Falle einer Infektion ermittelt werden. Basierend auf diesen Ergebnissen wurden verschiedene Systeme und Applikationen zur Identifizierung von Wundinfektionen direkt in der Wundflüssigkeit entwickelt und getestet.

Für Lysozym wurde zuerst ungefärbte *Micrococcus lysodeikticus* Zellen in einer Agarose-Matrix eingearbeitet. Lysozym-Aktivitäten wurden dabei durch Trübungsabnahme bestimmt. Die Sensitivität und Spezifität der Lysozym-Bestimmung direkt in Wundflüssigkeit wurde mit Hilfe von Remazol Brilliant Blau (RBB) markiertem Peptidoglycan, eingearbeitet in Agarose Schichten oder Alginatkügelchen, verbessert. Der Verdau dieser bakteriellen Zellwand und die Farbfreisetzung durch Lysozym Aktivität wurde mit dem Infektions-Status korreliert.

Zum Nachweis der Myeloperoxidase Aktivität wurden verschiedene Assays etabliert. Als Substrate wurden Guajacol, TMB (Tetramethylbenzidin), ABTS (2,2´-Azino-di-(3-ethylbenzthiazolin)-6-sulfonsäure, Fast Blue RR (4-Benzoylamino-2,5-dimethoxybenzendiazoniumsalz), Kristallviolett (LV) und Leuko Kristallviolett (LCV) verwendet. Zusätzlich wurden systematisch substituierte Azofarbstoffe mit 2,7-Dihydroxy-1-phenylazonaphtalene-3,6-disulfonsäure als Grundstruktur auf ihre Fähigkeit als MPO Substrat getestet. Für ein besseres Handling des Detektions-Kits wurden verschiedenen Substraten MPO immobilisiert. Kristallviolett, 2,7-Dihydroxy-1-(4-

hydroxyphenylazo)-naphthalin-3,6-disulfonsäure und Fast Blue RR (4-Benzoylamino-2,5-dimethoxybenzenediazoniumsalz) wurden mit Siloxanen erfolgreich gekoppelt.

Zusätzlich wurde das Potenzial von humaner Neutrophil Elastase und Cathepsin G zur Detektion von Wundinfektion untersucht. Diese Enzyme wurden direkt in Wundflüssigkeit bestimmt. In einem weiteren Schritt wurde die Immobilisierung von spezifischen chromogenen Elastase und Cathepsin G Substraten auf unterschiedlichen Materialien durchgeführt. Dies würde eine so genannte Online-Überwachung der Wunde direkt auf die Wundauflage oder im Verband ermöglichen.

PREAMBLE

The thesis is divided into seven chapters.

Chapter 1 is splitted into two parts. The first part provides background information about the topic of wound healing reviewing literature. Particular attention is given to problems concerning chronic wounds and wound infection. Additionally common methods for the detection of wound infection are illuminated. Furthermore the role of neutrophils in our immune system is explained. Then enzymes which can be used as neutrophil markers like lysozyme, myeloperoxidase, elastase and cathepsin G are described in detail. The second part of chapter 1 deals with smart materials responding to enzymes as triggers. The main focus is put on the controlled release of substances due to enzyme activity.

Chapter 2 highlights the motivation behind the current work and explains the aim in detail.

Chapters 3, 4 and 5 describe different systems for diagnosing wound infection, based on 4 different enzymes, namely lysozyme, myeloperoxidase, elastase and cathepsin G.

The final chapter 6 gives a summarized conclusion of the current work and explores the possible application of the results.

The thesis ends with the appendix, including the list of publications and the acknowledgement.

1

GENERAL INTRODUCTION

1 Project

The doctoral thesis was carried out within the “Lidwine-Project”. This is an EU-funded integrated project, focused on the development of multifunctional medical textiles to prevent wounds on the one hand and to stimulate wound healing on the other hand. Research of this project is directed towards prevention and diagnosis of infected chronic wounds like “decubits ulcer”. Decubitus comes from the latin word “*decumbere*”, which means to lie down. Generally, decubitus ulcer is a pressure sore or what is commonly called a "bed sore". It can range from a very mild pink colouration of the skin, which disappears in a few hours after pressure is relieved on the area, to a very deep wound extending through bones into internal organs. The main cause of decubitus wounds is impaired blood supply and tissue malnutrition due to prolonged pressure. Decubitus often occurs when patients are fixed in position for a long time period [1].

The work of the University of Technology within this project is the development of a diagnostic tool for diagnosing infection directly in wound fluid.

2 Wound Healing

Wound healing, or wound repair, is a complex and dynamic process of repairing broken skin or tissue after injury [2,3]. When the skin is broken a set of complex biochemical events take place, in a closely orchestrated cascade to repair the damage [4]. Normal wound healing is the restoration of anatomic and functional integrity of the broken skin [5]. After healing, only a scar with little fibrosis reminds of the injury [3,4]. The normal wound healing process can be spitted into 3 different phases: the inflammatory-, proliferative- and remodelling-phase which overlap in time [3,4]. Healing in acute wounds is based on the coordinated events of different cellular activities comprising phagocytosis, chemotaxis, mitogenesis, collagen synthesis and the synthesis of other matrix components, and should be finished within 3 months [6,7].

Wound healing is a very complex biological process and therefore very susceptible to interruption [8]. Various causes including infection, old age, diabetes and venous or arterial diseases, are leading to the formation of chronic, non-healing wounds [6]. In contrast to acute wounds, these chronic wounds fail to heal in a timely and orderly sequence [4,6]. Skin ulcers are the most common type of chronic wounds, including diabetic foot ulcers, pressure ulcers and venous stasis ulcers [4,9]. Chronic ulceration can affect any anatomical region, like the occiput, scapula, sacrum and heels [6]. However, the most common site of involvement is the sacrum and the lower limb [6]. Several studies found out that approximately 1% of the population suffer from chronic leg ulcers [10-12]. According to different surveys, 70-85% of ulcers are associated with venous hypertension and venous insufficiency followed by diabetes and arterial diseases [6,13,14].

Different physiological and mechanical factors have a negative impact on the healing progress of wounds [9]. Systemic factors include advanced age, malnutrition and diabetes while foreign bodies, tissue maceration and ischemia belong to local factors which result in a chronic wound that fails to heal [15]. Besides these systemic and local factors, the imbalance of growth factors and enzymes are important in chronic wounds [15,16]. Wound healing in general is regulated by a great number of different bio-molecules, including cytokines, growth factors and enzymes. In a chronic wound, the normal process of healing is disrupted at one or more points, mostly in the inflammatory- or proliferative-phase [6,17]. Due to their importance in the healing process, alterations of these key factors contribute to the bad healing behaviour of chronic wounds [6,8]. Compared to acute wounds, levels of growth factors are reduced in chronic wounds [8]. In addition, certain growth factors seem to be degraded by proteases

which are secreted mainly by macrophages and polymorphonuclear leucocytes [18-20]. The production and activity of proteases are tightly regulated in the normal wound healing process [6]. Increased proteinase activity in non-healing wounds due to over-expression, leads to excessive destruction of the extracellular matrix [8]. Diverse studies found elevated levels of various proteases in chronic wound fluids [18,21]. Cathepsin G and neutrophil elastase, which is able to degrade fibronectin, have also been observed to be significantly higher in chronic wounds [22]. Fibronectin is an essential protein involved in the remodelling of the ECM.

Additionally, bacterial infection is the most common cause for chronic wounds [16]. Bacteria in wounds delay or even halt normal wound healing through diverse mechanisms. Persistent production of inflammatory mediators, metabolic wastes and toxins and the activated state of neutrophils producing free oxygen radicals are only a few examples of such mechanisms [23,24]. Furthermore, bacteria compete for essential nutrients and available oxygen with macrophages and fibroblasts [25].

Generally, the presence of microbes in wounds can be divided into 4 different stages [26]:

- Contamination: presence of non-replicating microorganisms within a wound without any host reaction
- Colonization: presence of replicating microorganisms without any host reaction
- Critical colonization: no invasive infection but release of toxins causes a delay in wound healing without manifest signs of inflammation
- Infection: presence of replicating microorganisms with an associated host reaction

It is widely believed, that all wounds are contaminated to some degree by bacteria of the normal skin flora - non-replicating microorganisms are always present. In contrast, colonized wounds contain replicating microorganisms which do not damage the host [16]. Wound infection is defined by the presence of replicating microorganisms leading to a host response [27]. The development of wound infection depends on different factors. Generally, infection occurs when invading bacteria achieve dominance over the host´s immune system [28]. Wound infection is more likely when a critical number of bacteria ($>10^5$ per gram of tissue) is exceeded [6,29]. 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 [6,29]. Besides, the nature of the wound and different individualized factors such as the patient´s general health condition determine whether infection will take place [28].

3 Methods for Detection of Infection – State of the Art

Detection of wound infection is beset with difficulties and is a real challenge for the attending physician [28,30]. Undiagnosed and untreated wound infection delays the healing process and can implicate serious consequences for the patient like osteomyelitis and extends human suffering [31-33]. In the worst case infection can even lead to death after an occurring sepsis. Not only patient´s pain and suffering, but also the enormous costs for extended hospitalization pose a big problem. Wound infection extends the hospital stay for at least 10 days [34]. An early and timely diagnosis of an incipient infection is crucial since it would allow the initiation of treatments in early phases. This would drastically reduce the formation of severe infections. Currently, wound infection is not diagnosed until infection is already evident. As a consequence, it is much more difficult to treat the infection and the patient is more likely to suffer a negative outcome. As with most diseases, an early treatment is more likely to be less expensive and more effective than if the disease is at an advanced stage [35]. As shown by name [35], the risk of death increases in case of delayed treatment. Every hour of delay decreases the survival by 7.6 % [36]. At the moment there exists not a single, definitive diagnostic test to identify wound infection. The clinician must rely on a combination of laboratory and clinical information to make the diagnosis [35].

The most practical way for identifying wound infection relies mainly on the judgement of the classical signs of infection [25,37]:

- Rubor (redness)
- Calor (increased heat)
- Tumor (swelling)
- Dolor (pain)

These indicators are related to the inflammatory process. Problematic using these signs for assessment of wound infection is the fact that they can diminish or alter in some cases [28]. Up to now, clinical signs and symptoms are the main source of information for clinicians to determine treatment.

Surface Swab Cultures

If wound infection is suspected, the most common technique for evaluation of wound infection is surface wound swabbing [38]. Swabs are taken and micro-organisms are identified by cultivation. Results of this analysis are available not before 1 to 3 days. Since all wounds are

contaminated or colonized, information about the kind of species present in wounds is not a reliable indication for wound infection [28]. According to *Enoch et al.* [6], critical factors for infection are: high levels of bacterial content, bacteria capable of altering their phenotypic and genotypic characteristics, presence of multi-drug resistant organisms and biofilm formation. Furthermore, the virulence of pathogens is important, namely the production of endotoxins and exotoxins, which can be very harmful for the host. As the usage of swabs can only give information about the kind of bacteria and about the approximate number of the bacterial load, identification of bacterial infection by microbiological methods is not a real diagnosis for infection. Furthermore, the method is expensive and takes at least three days. However, bacterial swabs can provide necessary information about antibiotic resistances and changes of the microbial flora [28].

Quantitative Tissue Cultures

The bacterial load of a wound can be measured through invasive tissue biopsy [28]. This technique is not regularly undertaken since it is labour-intensive. Additionally different studies have shown that wounds can heal despite high bacterial loads [39].

Serum Investigations

If infection is suspected, blood tests very often provide an indication of following medical treatment. Besides the determination of the amount of white blood cells (leukocytes), the concentration of C-reactive protein (CRP) is measured. CRP belongs to the family of acute phase proteins and is widely used for determination of bacterial infection [40]. CRP has a very short reaction time of only 6 hours [41]. In case of tissue injury of bacterial infection, CRP-levels can rise up to 10^3-fold. CRP-determinations have widely displaced the determination of erythrocyte sedimentation rate (ESR) since it is less sensitive and has a time lag of more than one week [42]. However, raised CRP levels are usually measured in blood which requires collection of blood and analysis in a clinical lab.

As already explained, that neither the bacterial load nor the isolation of predominate microorganisms present in wounds can provide an assessment if the wound is infected or not. The only crucial factor determining wound infection is the host itself. Healthy persons can resist higher bacterial loads than persons with a general weak health condition [28].

To sum up, all these methods have in common that they are time-consuming and cannot be used in home-care or for quick assessment during change of bandages. In many situations,

these methods would take too long for diagnosing infections. On the other hand, inspection of odour and appearance of the wound requires experienced doctors and can very often seen when the infection is already manifested [43].

4 Neutrophils

Neutrophil granulocytes generally referred to as neutrophils are the most abundant type of white blood cells in humans [44,45]. 50% to 60% of all circulating leukocytes are neutrophils [46]. The name “neutrophil granulocyte” derives from typical staining patterns in hematoxylin and eosin stained preparations, where these cells can be identified very easily by their light pink coloured cytoplasmic compartments. In the 1980s it was Elias Metschnikoff who firstly recognized that specialized phagocytic cells are able of ingesting bacteria [47]. Later on, Paul Ehrlich was the first who named these cells neutrophil granulocytes. Metschnikoff and Ehrlich were awarded the nobel prize in the year 1908 for discovering neutrophils and their pioneering groundwork [48].

4.1 Biology of Neutrophils

Neutrophils are terminally differentiated cells, incapable of cell division [46,49]. Together with eosinophils and basophils, they belong to the group of polymorphonuclear cells [44,45]. Neutrophils can be characterized by their multilobulated nuclei. They mature in the bone marrow before being released into the blood stream, where they only spend 4 to 10 hours [46].

4.2 Neutrophils and Host Defense

Neutrophils are part of the non-specific natural immune system and play a crucial role in the host defense against invading microorganisms. Once the skin is wounded or infection occurs, neutrophils are recruited and are one of the first cells present at site of infection or injury [46]. They arrive approximately 24 hours after injury [50]. In case of infection, neutrophil differentiation can increase up to 10 fold [46]. The main task of neutrophils is the decontamination of the wound bed by killing of invading pathogens [51]. Neutrophils engulf invading microorganisms into a phagosome, which fuses with intracellular granules to form a phagolysosome. In this organelle, microorganisms are killed using different mechanisms [49].

The importance of neutrophils is underscored, since individuals suffering from neutropenia or neutrophil dysfunction have a high incidence of bacterial infections [52,53].

4.3 Neutrophil Microbiocidal Mechanisms

Neutrophils use different strategies for killing invading microorganisms. The microbiocidal pathways of neutrophils can be divided into oxygen-dependent and oxygen-independenet reactions [54,55]:

Oxygen-dependent Mechanisms

Oxygen-dependent mechanisms can be grouped into myeloperoxidase-independent and myeloperoxidase-dependent reactions. The oxygen-dependent myeloperoxidase-independent mechanism is also called “respiratory burst”, since there is a 50- to 100-fold increase in oxygen consumption during phagocytosis. After activation, the NADPH oxidase complex is bound to the membrane of the phagosome, producing superoxide anion ($O_2^{\cdot-}$) (Formula 1).

Formula 1 $$NADPH + 2O_2 \rightarrow NADPH^+ + 2O_2^{\cdot-} + H^+$$

As shown in Formula 2, superoxide dismutase then converts the produced oxygen to hydrogen peroxide [49].

Formula 2 $$2H^+ + O_2^{\cdot-} \rightarrow 2H_2O_2 + O_2$$

During this process, very reactive oxygen species (ROS) that kill bacteria are generated [46,47,56].

In the oxygen-dependent myeloperoxidase-dependent mechanism, microbes can be killed by halogenations of hydrogenperoxide metabolites, catalyzed by the enzyme myeloperoxidase. Azurophilic granules fuse with the phagosome, allowing myeloperoxidase being released into the phagolysosome. MPO makes then use of the formed H_2O_2 (Formula 3) and available halide ions (X^-) like Cl^- to produce hypochlorite (HOCl), a very toxic compound [46,47,49,56].

Formula 3 $$X^- + H_2O_2 + H^+ \rightarrow HOX + H_2O$$

Additionally, hypochlorite can break down into singlet oxygen ($^1O_2$) [57].

Oxygen-independent Mechanisms

At sites of insufficient oxygen supply like in abscesses or sites of inadequate blood supply, oxygen-independent mechanisms play an important role. In case of anaerobic conditions, products of the respiratory burst are not available [58]. In this case, pathogens are trapped in so called phagolysosomes, which are formed through fusion of a phagosome with a lysosome. The low pH within the phagolysosome avoids the growth of many different bacteria species of the one hand and increases the activities of enzymes contained in the phagolysosome. Toxic enzymes, proteins and antibacterial substances are used for the killing of invaded pathogens [49]. These effector-molecules include cationic proteins (like cathepsin), lysozyme, lactoferrin and proteolytic and hydrolytic enzymes.

Additionally, neutrophils can protect themselves from the toxic oxygen intermediates by detoxification [46,47,56]. Since neutrophils contain catalase and gluthathione, hydrogenperoxide for example can be converted to water and oxygen [57].

Besides the killing of pathogens by phagocytosis and the release of antimicrobial substances [59], neutrophils are able of kill bacteria extracellular, without phagocytosis [60]. Brinkmann *et al.* demonstrated that activated neutrophils can produce extracellular fibers for killing of bacteria [61]. These neutrophil extracellular taps (NETs) are composed of chromatin, DNA, histones and granule proteins such as neutrophil elastase and cathepsin G [60,62,63]. These NETs bind to gram-positive and gram-negative bacteria [60] and kill them by a high local concentration of neutrophil elastase [64]. This specialized function can also aid in focusing the activity of neutrophil elastase against ECM tissue remodelling and prevent the risk of diffuse tissue damage [64].

4.4 Neutrophils in Wound Healing

Neutrophilic inflammation is defined by the infiltration of neutrophils to inflamed tissue and is a kind of inflammatory response. During this inflammatory process, chemotactic factors direct the recruitment of neutrophils to the sites of infection or injury. When neutrophils have received these signals, it takes them only a few minutes to arrive there. There exist a number of human diseases being involved in such reaction [65]. Bacterial infection is the prime example of this inflammatory response [66].

5 Neutrophil Markers

Different enzymes produced by neutrophils, which can be used as triggers for the design of a diagnostic system of detection of infection, are described more in detail.

5.1 Lysozyme

Lysozyme (EC 3.2.1.17), also known as muramidase or 1,4 N-acetylmuramidase, is a cationic protein consisting of a single chain of 130 amino acid residues with four disulfide bonds [67,68]. Characteristics of lysozyme are shown in Table 1.

Table 1: Characteristics of lysozyme

Enzyme Characteristics	Lysozyme
EC Number	3.2.1.17
Systemic Name	Peptidoglycan N-acetylmuramoylhydrolase
Synonyms	Muramidase 1,4-N-Acetylmuramidase
Abbreviation	Lys
Protein Characteristics	129 Amino acids 14.65 kDa pI: 10.5
pH-Optimum	6.0-9.0

Lysozyme has a molecular weight of 14 kDa and catalyses the hydrolysis of ß(1-4) glycosidic bonds between N-acetylmarmic acid and N-acetelglucsoamine [67,68]. These two sugars are the structural components of bacterial peptidoglycan, the major component of the bacterial cell wall [69]. Lysozyme was first described by Alexander Fleming in 1922 [70]. He proposed that nonpathogenic microorganisms fail to cause disease, because they are very sensitive to lysozyme-mediated killing [70]. Lysozyme exhibits microbiocidal effect against gram positive

species, while gram-negative species are resistant to enzymatic degradation by this enzyme [71]. Gram-negative bacteria shield their peptidoglycan layer by an outer membrane [72]. In addition to its lytic activity directed towards gram positive species, lysozyme is able to kill gram negative bacteria not by lysis of their peptidoglycan murein-sacculus, but by an alternative, non-enzymatic mechanism [68]. Lysozyme can function as a non-specific innate opsonin by binding to the bacterial surface, thus reducing the negative charge and facilitating therefore phagocytosis of the bacterium before opsonins from the acquired immune system arrive [73].

Lysozyme is located in the primary and secondary granules of polymorphonuclear neutrophils (PMN) and the major secretory product of macrophages [74,75]. It is also produced by epithelial cells [68].

Lysozyme is abundant in a number of mammalian secretory products like tears, salvia, human milk and mucus, as well as in tissues [76,77]. The highest lysozyme levels have been found in tears, gastric juice and mothers` milk [77].

It is well known that any deviation of the lysozyme concentration from the normal level in serum or urine is related to diseases. Elevated levels of lysozyme in serum and synovial fluids are described in case of some diseases. For example, a high level of lysozyme was observed in the urine of patients with monocytic leukaemia [78]. Serum levels may be raised in chronic granulomatous inflammatory disorders including tuberculosis and sarcoidosis [79,80]. These observations indicate that the measurement of the concentration of lysozyme in serum or urine is useful for the diagnosis or screening of diseases. In addition, different clinical studies have suggested that increase of this enzyme is a serum marker of active chronic inflammation [81,82,83].

5.2 Neutrophil Serine Proteases (NSPs)

Human neutrophil elastase (HNE: EC 3.4.21.37) and cathepsin G (CatG: EC 3.4.21.20) are two members of serine proteases of the chymotrypsin family [84,85]. Characteristics of these enzymes are illustrated in Table 2.

Table 2: Characteristics of neutrophil serine proteases from Human PMNs

Enzyme Characteristics	Human Leukocyte Elastase	Cathepsin G
EC number	3.4.21.37	3.4.21.20
Synonyms	Neutrophil elastase Granulocyte elastase Lysosomal elastase Neutrophil elastase	Chymotrypsin-like protease Neutral Protease
Abbreviation	HNE, NE	CatG, CATG
Protein Characteristics	218 Amino acids 30 kDa pI > 9	235 Amino acids 28.5 kDa pI ~ 12
pH-Optimum	8.0-8.5	~ 8.0
Substrate Specificity	Val-Xaa > Ala-Xaa	Aromatic amino acids, preferentially in P1-position
Endogenous Inhibitors	α1-Proteinase inhibitior α_2-Macroglobulin SLPI Elafin MNEI	α1-Proteinase inhibitior α_1-Anti-chymotrypsin SLPI MNEI

SLPI secretory leukocyte protease inhibitor

MNEI monocyte neutrophil elastase inhibitor

They are both cationic, single glycoproteins which are mainly stored in azurophilic granules of PMNs [85]. Their activities rely upon a catalytic triad of aspartate, histidine and serine residues [84,85]. Electrons are shifted from the carboxyl group of aspartate to the oxygen of serine, enabling now serine to attack the peptide bonds [86]. The major role of NSPs intracellular within phagolysosomes is the degradation of intracellular pathogens and cell

debris [59]. Released NSPs are responsible for extracellular proteolytic processes at sites of inflammation outside the cell [59,87,88]. There, NSPs are part of the regulation-network of the innate immune system, inflammation and infection [89-91]. Neutrophil elastase and cathepsin G are endo-proteolytic enzymes and therefore they are able of breaking down a great number of different extracellular proteins [92].

Serine proteases play a crucial role in physiological and pathological processes. This is highlighted by the fact, that serine protease inhibitors make up more than 10% of all plasma proteins [93].

Human Leukocyte Elastase (HNE)

Neutrophil elastase (EC 3.4.21.37) referred to as ELA-2 or leukocyte elastase belongs to the chymotrypsin family of serine proteases and is expressed by polymorphonuclear (PMN) leukocytes [94,95]. HNE is a 33-kDa, highly cationic glycoprotein with an isoelectric point between pH 10-11 [93]. It consists of a single peptid chain of 218 amino acids with two aspargine-linked carbohydrate side chains [93]. HNE favours to cleave peptide bonds which are carboxyterminal to small, hydrophobic residues [93]. There exists a number of isoforms that have different extents of glycosylation [94,95]. The protein is synthesized as a preproenzyme of 267 amino acids [96]. For processing and activation of HNE this protein is post-translationally processed at both the N- and C-termini [96]. Characteristic therefore is the removement of the N-terminus Ser–Glu dipeptide which is leading to a change in the protease conformation and acquisition of catalytic activity [96]. The mature HNE is stored in the azurophil granules in its active from until extruded into phaolysosomes or released into the extracelluluar environment [64].

HNE is one of the key molecules of the innate immune system, acting especially against gram-negative bacteria but also against spirochaetes and fungi [97,98,99].

In response to inflammatory stimuli, neutrophils are recruited via the circulating blood to the site of infection. Following activation, neutrophils engulf and kill encountering bacteria by attacking them with antimicrobial proteins, proteolytic enzymes and reactive oxygen species [100,101]. One of those antibacterial compounds released into the phagolysome engulfing the invading bacteria is neutrophils elastase [102]. HNE is released into the phagolysome surrounding the pathogen, and attacks the outer membrane protein, OmpA. Belaaouaj *et al.*

showed, that in gram-negative bacteria, HNE is leading to loss of structural integrity and in consequence to bacterial death [52].

Besides the involvement in killing of pathogens by membrane degradation, catalytically active neutrophil elastase is found at the plasma membrane of activated neutrophils [103]. After activation, neutrophil elastase is released out-side the cell with some portion remaining bound at the neutrophil plasma membrane [103]. Additionally, HNE is able to regulate the activity of different enzymes [104]. For instance, HNE can activate the proenzymatic form of matrix metalloproteinase-9 [105].

Since neutrophile elastase is one of the most destructive enzymes in the body, multiple protease inhibitors, such as α_1-antitrypsin, α_2-macroglobulin and secretory leukocyte protease inhibitor, tightly regulate the activity of neutrophil elastase under physiological conditions [106,107]. HNE has a broad substrate spectrum. It is able of digesting nearly every type of matrix protein (elastin, fibronectin, proteoglycans, heparin and collagens) as well as key plasma proteins like different clotting factors, immunoglobulins, cytokines, adhesion molecules and components of the complement system [106,108,109]. The balance between elastase and its inhibitors is essential for protection against unregulated tissue destruction and maintaining tissue integrity [110]. At sites of infection, activity of neutrophils elastase can be explained by an imbalance between its elevated levels and that of endogenous protease inhibitors [108,111].

However, at inflammatory sites, neutrophil elastase is able to escape from regulation by different mechanisms [106]. Unregulated elastase can then induce the release of pro-inflammatory cytokines, such as interleukin-6 and interleukin-8, leading to destruction of tissue [112,113]. Elastin which, together with collagen, determines the mechanical properties of connective tissue, has the unique property of elastic recoil, and is mainly distributed in lungs, arteries, skin and ligaments [114].

Elastase is involved in the pathogenesis of a number of inflammatory disorders [108]. Heightened activities of elastase are known for diseases like rheumatoid arthritis, hereditary emphysema, chronic obstructive pulmonary disease, adult respiratory distress syndrome, ischemia/reperfusion injury, emphysema, cystic fibrosis and tumor progression [115, 116].

Furthermore, elevated elastase levels are described at the very beginning of infection and were therefore selected as an enzyme for the diagnostic device for detection of wound infection. This suggests that the detection of elastase in wound fluid could give early stage

warning of wound infection, before obvious clinical signs of infection are present. And indeed, elastase activity is used as predictor and indicator of inflammatory diseases [117].

Cathepsin G

Cathepsin G (CatG) is a serine protease, sharing 37% sequence homology with HNE [118]. Like HNE, it consists of only one single peptide chain of 235 amino acid residues [118]. CatG has chymotrypsin- and trypsin-like activity and is active at a very broad pH range, while the optimal working pH is around pH 8 [119]. The active side of the catalytic triade is formed, that bonds of the proteins are hydrolzyed after aromatic and positively charged residues in P1 position [120]. Together with human leucocyte elastase and proteinase 3, cathepsin G is a major content of the azurophilic granules in neutrophils and is released at sites of infection or injury by activated neutrophils [121,122]. Active CatG is not only located in azurophilic granules, but is also found as membrane bound-form on the surface of neutrophils after degranulation [103,123].

CatG plays a major role in a variety of processes, including platelet activation, microbicidal activity, conversion of angiotensin I to angiotensin II and cleavage of clotting factors [124-127]. Additionally CatG is able to inactivate cytokines like interleukin-1 (IL-1), interleukin-8 (IL-8) and tumour necrosis factor α (TNF-α) [128,129]. Cathepsin G possesses a broad substrate specificity including substrates like collagen, fibronectin, proteglycans and elastin [128,129].

Furthermore, CatG is involved in the pathogenesis of chronic diseases like emphysema and cystic fibrosis [130].

5.3 Myeloperoxidase (MPO)

Myeloperoxidase (EC 1.11.1.7) is a heme-containing alkaline protein, belonging to the enzyme class of peroxidases. Characteristics of this enzyme are shown in Table 3.

Table 3: Characteristics of myelopyeroxidase

Enzyme Characteristics	Myeloperoxidase
EC Number	1.11.1.7
Systemic Names	Hydrogenperoxide oxidreductase
Synonyms	Lactoperoxidase
Abbreviation	MPO
Protein Characteristics	1162 Amino acids 150 kDa pI: 9.2
Cofactor	Häm

Native Myeloperoxidase exists as a covalently bound tetrameric complex (150 kDa) with two dimers connected by a disulfide bond. Each dimer comprises a glycosylated β-heavy subunit (59 kDa) and an unglycosylated light α–subunit (14 kDa) [131-134]. Each dimer is covalently bound to a prosthetic heme group. Up to now, 3 isoforms have been identified in neutrophils which differ only in their size [135]. MPO is mainly stored in azurophilic granules of PMNs and released in case of leukocyte activation and degranulation.

It is highly concentrated in human neutrophils, comprising more than 5% of the dry cell weight [136]. In lower concentration, this enzyme is also found in the granules of monocytes [137].

MPO possess two different kinds of enzyme activities, namely peroxidise activity and chlorination activity [137]. MPO catalyzes the production of hypochlorous acid by convertion of hydrogen peroxide and chloride-ions [138]. Hypochlorous acid is the most powerful bactericidal oxidant produced by neutrophils [47].

MPO is involved in the pathogenesis of numerous inflammatory diseases [139]. Elevated levels of MPO are a clear indicator of inflammatory processes as already described for otitis media, chronic sinusitis and in peritoneal fluid during genital inflammation [140-142]. Additionally, heightened MPO levels are known in patients with coronary cardiovascular events and in patients with chest pain [143].

6 Smart Materials Responding to Enzymes as Triggers

“Smart” or so called “intelligent” materials are able to change their properties in response to changes in their environment [144]. Different environmental stimuli are listed in Table 4.

Table 4: Environmental stimuli

Mode of Action	Stimuli
Physical	Temperature
	Ionic strength
	Solvents
	Radiation
	Electric fields
	Mechanical stress
	High pressure
	Sonic radiation
	Magnetic fields
Chemical	pH
	Specific ions
	Chemical agents
Biochemical	Enzyme substrates
	Affinity ligands
	Other biological agents

In recent years, an immense amount of interest has been increasingly placed on biomaterials that change properties in response to enzyme stimuli. Using enzymes as triggers is advantageous due to their high specificity of catalyzed reactions, mild working conditions and

their key roles in many biological pathways [145]. Therefore, the high specificity of enzymes can be exploited for the design of smart materials, allowing a communication between environment and the material. Enzymes used as triggers can impart bio-responsive properties to materials containing specific elements susceptible to modification by these biocatalysts. With this concept, controlled release of functional molecules such as drugs, antimicrobial substances or perfumes amongst a variety of other materials can be designed. Especially in the field of medicine, smart materials could pave the way to selectively remove or deliver drugs, adapted to the course of disease.

6.1 Controlled Release Systems and Mechanisms

Controlled release (sustained or triggered) can be achieved by combining a polymer with a drug or other active agents in such a way that the active agent is released from the material in a predesigned and reproducible manner [146]. Smart materials are constructed in a way that they respond to triggers (e.g. enzymes) allowing a controlled release of active agent. Especially controlled release and drug delivery have grown in importance in the last years. Controlled drug release systems are directed to improve the effectiveness of the therapy [147]. Providing control over the drug delivery can be a crucial factor especially in cases when traditional oral or injectable drug formulations cannot be used [148]. A more effective therapy can be achieved while the potential for both under- and overdosing can be reduced [149]. Despite the improved efficacy and increased therapeutic activity, using controlled-delivery systems have further advantages like the maintenance of drug levels within a desired range and reduced toxicity [149,150]. The convenience for fewer and more effective drug-administrations increases patient compliance [151].

Especially in the therapy of cancer and the treatment of many endocrinological disorders, controlled drug delivery is of great importance [152].

The ideal drug delivery system is inert, biocompatible, mechanically strong, comfortable for the patient, capable of achieving high drug loading, safe from accidental release, simple to administer and remove, and easy to fabricate and sterilize [148,149].

The release profile of the active agent or drug can be very versatile. The active agent can be released constantly over a long time period or can be delivered at a specific time. Drug delivery over an extended duration is advantageous for drugs that are easily metabolized and rapidly eliminated after administration [150].

Generally, there exist three different mechanisms by which active agents are released from the delivery system: diffusion, degradation (erosion) and swelling followed by diffusion (Figure 1).

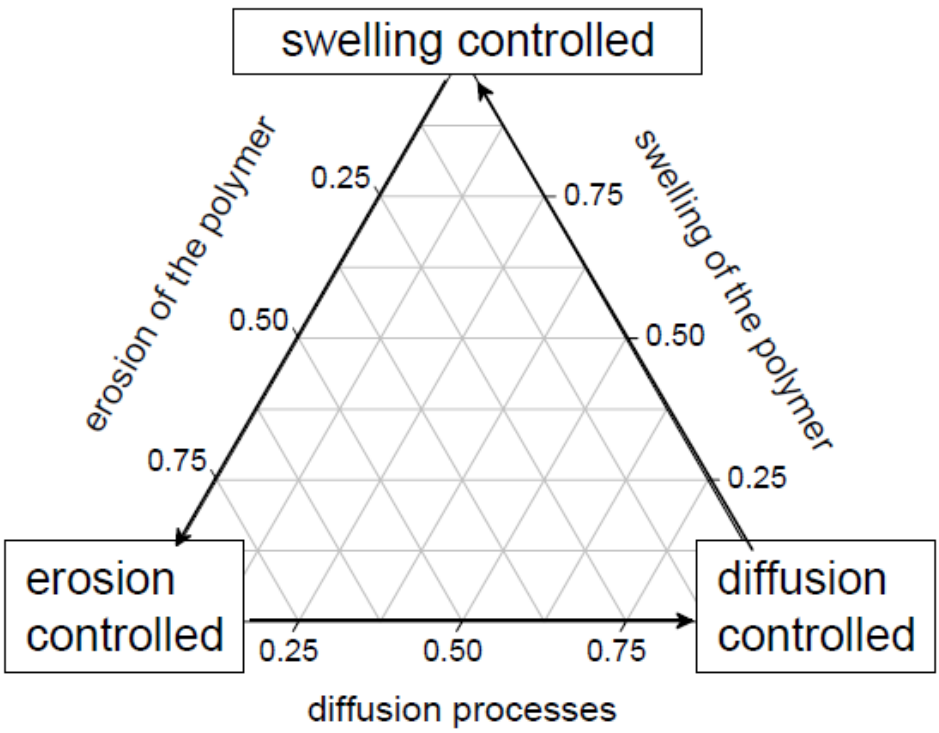

Figure 1: Possible drug release mechanism from degradable polymer devices (modified from Winzenburg [146])

Many of the controlled release systems are designed to create a sustained release like diffusion or swelling [153-156].

Diffusion Controlled Release

Diffusion is one of the key mechanisms used to control the drug release especially from pharmaceutical devices [157]. In diffusion controlled release systems the drug or active agent is released into the surrounding medium by permeation from the device [158]. In general, controlled release devices can be divided into matrix-systems and reservoir systems [159]. In matrix systems, the drug is directly dispersed and uniformly distributed in a porous network [160,150]. Matrix systems include swellable and non-swellable polymers [161]. Drugs or other active agents have to pass the way through pores and cavities to exit the device [150,160].

Since matrix systems follow Higuchi´s model they lead to non-uniform release rates [162]. This disadvantage can be eliminated using so called reservoir drug delivery devices. The active agent is surrounded by a permeable, inert membrane, allowing a fairly constant drug delivery [158]. Only the surrounding layer is effecting and limiting the release [149]. An uniform and invariable thickness of this layer ensures stable diffusion rates [149].

Swelling Controlled Release

In case of swelling controlled drug release systems, active agents are dispersed in glassy polymers [160]. Hydrogels are able to respond to alterations of the environmental conditions with changes of their swelling behavior, network structure, permeability and mechanical strength [163]. The release of the active substance is a combination of water-absorption and desorption of the drug [164]. The rate-controlling factor is the ability of the polymer to resist the increase in volume and change in shape [165]. This system can lead to absorption of solvent therefore to expansion of the polymer, resulting in different release kinetics [158].

Erosion Controlled Release

Diffusion and swelling controlled mechanisms are based on polymers which do not alter their chemical structure. The opposite is the case for erosion controlled release devices. These polymers are biodegradable and can be degraded due to natural biological processes [149]. Erosion controlled release can be divided into two different mechanisms: surface- and bulk-erosion. Surface-erosion occurs when the rate of water penetration is lower than the rate of polymer degradation. Only the outer surface of the polymer is degraded, and not the core-material [166]. Bulk-erosion occurs when the water permeation is faster than the rate of degradation. Consequently, polymers in the bulk are degraded [150,166]. For most biodegradable polymers both mechanisms occur at the same time [167]. The extend is regulated by the chemical structure of the polymer backbone [168].

7 References

1. C. Bansal, R. Scott, D. Stewart, and C.J. Cockerell, Decubitus ulcers: A review of the Literature, Int.J.Dermatol. 44 (2005) 805-810.

2. D.T. Ngyen, D.P. Orgill, and G.F. Murphy, Chapter 4: The Pathophysiologic Basis for Wound Healing and Cutaneous Regeneration, in: Biomaterials For Treating Skin Loss, CRC Press (US) & Woodhead Publishing (UK/Europe), Boca Raton/Cambridge, 2009. pp. 25-57.

3. M. Mercandetti and A.J. Cohen, Wound healing, healing and repair, http://emedicine.medscape.com/article/1298129-overview (2008)

4. W.K. Stadelman, A.G. Digenis, and G.R. Tobin, Physiology and Healing Dynamics of Chronic Cutaneous Wounds, Am.J.Surg. 176 (1998) pp. 26S-38S.

5. W. Jeffery, Introduction to Care of Chronic Wounds, Am.J.Surg. 176 (1998) pp. 1S-2S.

6. S. Enoch and P. Price, Cellular, Molecular and Biochemical Differences in the Pathophysiology of Healing between Acute Wounds, Chronic Wounds and Wounds in the Wlderly. Worldwidewounds.com., (2004)

7. R.A.F. Clark, Wound Repair: Overview and General Considerations, In: The Molecular and Cellular biology of Wound Repair, Plenum Publishing Company, USA, 1996.

8. G.S. Schultz and B.A. Mast, Molecular Analysis of the Environment of Healing and Chronic Wounds: Cytokines, Proteases, and Growth Factors, Wounds 10 (1998) pp. 1F-9F.

9. J. de la Torre and J.A. Chambers, Wound healing, chronic wounds, http://emedicine.medscape.com/article/1298452-overview (2008)

10. M.J. Callam, C.V. Ruckley, D.R. Harper, and J.J. Dale, Chronic Ulceration of the Leg - Extent of the Problem and Provision of Care, Br.Med.J. 290 (1985) pp. 1855-1856.

11. O. Nelzen, D. Bergqvist, and A. Lindhagen, The Prevalence of Chronic Lower-Limb Ulceration has been Underestimated: Results of a Validated Population Questionnaire, Brit.J.Surg. 83 (1996) pp. 255-258.

12. O. Nelzen, D. Bergqvist, A. Lindhagen, and T. Hallbook, Chronic Leg Ulcers - An Underestimated Problem in Primary Health-Care among Elderly Patients, J.Epidemiol.Community Health 45 (1991) pp. 184-187.

13. M.J. Callam, D.R. Harper, J.J. Dale, and C.V. Ruckley, Chronic Ulcer of the Leg - Clinical History, Br.Med.J. 294 (1987) pp. 1389-1391.

14. D.A. Simon, F.P. Dix, and C.N. Mc Collum, Clinical Review: Management of Venous Leg Ulcers, Br.Med.J. 328 (2004) pp. 1358-1362.

15. K.G. Harding, H.L. Morris, and G.K. Patel, Healing Chronic Wounds, Br.Med.J. 324 (2002) pp. 160-163.

16. A. Deodhar and R. Rana, Surgical Physiology of Wound Healing: A review, J.Postgrad.Medicine 43 (1997) pp. 52-56.

17. G.S. Lazarus, D.M. Cooper, D.R. Knighton, D.J. Margolis, R.E. Pecoraro, G. Rodeheaver, and M.C. Robson, Definitions and Guidelines for Assessment of Wounds and Evaluation of Healing, Arch.Dermatol. 130 (1994) pp. 489-493.

18. D.R. Yager, L.Y. Zhang, H.X. Liang, R.F. Diegelmann, and I.K. Cohen, Wound Fluids from Human Pressure Ulcers Contain Elevated Matrix Metalloproteinase Levels and Activity Compared to Surgical Wound Fluids, J.Invest.Dermatol. 107 (1996) pp. 743-748.

19. M. Wlaschek, D. Peus, V. Achterberg, W. Meyer-Ingold, and K. Scharffetter-Kochanek, Protease Inhibitors Protect Growth Factor Activity in Chronic Wounds, Brit.J.Dermatol. 137 (1997) pp. 646-646.

20. S.J. Leibovich and R. Ross, Role of Macrophage in Wound Repair - Study with Hydrocortisone and Antimacrophage Serum, Am.J.Pathol. 78 (1975) pp. 71-99.

21. M.A. Avidano, C.S. Cotter, S.P. Stringer, and G.S. Schultz, Analysis of Protease Activity in Human Otitis Media, Otolaryngol.Head Neck Surg. 119 (1998) pp. 346-351.

22. F. Grinnell and M.F. Zhu, Fibronectin Degradation in Chronic Wounds Depends on the Relative Levels of Elastase, Alpha 1-Proteinase Inhibitor, and Alpha-2-Macroglobulin, J.Invest.Dermatol. 106 (1996) pp. 335-341.

23. G. Dow, A. Browne, and R.G. Sibbald, Infection in Chronic Wounds: Controversies in Diagnosis and Treatment., Ostomy.Wound Manage. 45 (2010) pp. 23-40.

24. M. Laato, J. Niinikoski, C. Lundberg, and B. Gerdin, Inflammatory Reaction and Blood Flow in Experimental Wounds Inoculated with Staphylococcus aureus, Eur.Surg.Res. 20 (1988) pp. 33-38.

25. D. Krasner, Chronic Wound Care: A Clinical Source Book for Professionals, Health Management Publications, 1997.

26. M. Ayton, Wound care: wounds that won´t heal, Nurs.Times 81 (1985) pp. 16-19.

27. N. Bergstrom, M.A. Bennet, and C.E. Carlson, Treatment of Pressure Ulcers: Clinical Practice Guideline, U.S. Department of Health and Human Services. Public Health Service, Agency for Health Care Policy and Research. AHCPR Publication No. 95-0652., Rockville, MD, 1994.

28. R.G. Sibbald, H. Orsted, G.S. Schultz, P. Coutts, and D. Keast, Preparing the Wound Bed 2003: Focus on Infection and Inflammation, Ostomy.Wound Manage. 49 (2003) pp. 23-51.

29. J.P. Heggars, Assessing and Controlling Wound Infection, Clin.Plast.Surg. 30 (2003) pp. 23-35.

30. K.F. Cutting, C.Ed, and R.J. White, Criteria for Identifying Wound Infection - Revisited, Br.J.Community Nurs. 9 (2004) pp. S6-S16

31. D.P. Lookingbill, S.H. Miller, and R.C. Knowles, Bacteriology of Chronic Leg Ulcers, Arch.Dermatol. 114 (1978) pp. 1765-1768.

32. C.S. Bryan, C.E. Dew, and K.L. Reynolds, Bacteremia Associated With Decubitus Ulcers, Arch.Intern.Med. 143 (1983) pp. 2093-2095.

33. J.E. Galpin, A.W. Chow, A.S. Bayer, and L.B. Guze, Sepsis Associated with Decubitus Ulcers, Am.J.Med. 61 (1976) pp. 346-350.

34. P.J. Miller, B.M. Farr, and J.M. Gwaltney, Economic Benefits of an Effective Infection Control Program. Case study and Proposals, Rev.Infect.Dis. 121 (1989) pp. 284-288.

35. R.P. Dellinger, M.M. Levy, J. Bion, M.M. Parker, and R. Jaeschke, Surviving Sepsis Campaign Guidelines for Management of Severe Sepsis and Septic Shock, Int.Care.Med. 36 (2008) pp. 296-327.

36. A. Kumar, D. Roberts, K.E. Wood, B. Light, J.E. Parrillo, S. Sharma, R. Suppes, D. Feinstein, S. Zanotti, L. Taiberg, D. Gurka, A. Kumar, and M. Cheang, Duration of Hypotension before Initiation of Effective Antimicrobial Therapy is the Critical Determinant of Survival in Human Septic Shock, Crit.Care Med. 34 (2006)

37. N.A. Stotts and T.K. Hunt, Managing Bacterial Colonization and Infection, Clin.Geriatr.Med. 13 (1997) pp. 565-573.

38. H. Rode, I.D.O. Vale, and A.J.W. Millar, Infection is one of the Commonest Causes of Death in Burn Patients, Particularly those with Extensive Damage, Cont.Med.Edu. (2008)

39. J.A. Steer, R.P.C. Papini, A.P.R. Wilson, D.A. MCatGrouther, and N. Parkhouse, Quantitative Microbiology in the Management of Burn Patients. II. Relationship between Bacterial Counts Obtained by Burn Wound Biopsy Culture and Surface Alginate Swab Culture, with Clinical Outcome Following Burn Surgery and Change of Dressings, Burns 22 (1996) pp. 177-181.

40. P.R. Katz, S.I. Gutman, G. Richman, J. Karuza, W.R. Bartholomew, and J. Baum, Erythrocyte Sedimentation Rate and C-Reactive Protein Compared in the Elderly, Clin.Chem. 35 (1989) pp. 466-468.

41. N.G. Adams, Diagnostic Use of C-Reactive Protein in Bacteraemic Emergency Department Patients, Emerg.Med.Australas. 17 (2005) pp. 371-375.

42. F. Van Lente, The Diagnostic Utility of C-Reactive Protein, Hum.Pathol. 13 (1992) pp. 12-15

43. R.H. Yolken, Enzymic Analysis for Rapid Detection of Microbial Infection in Human Body Fluids: An Overview, Otolaryngol.Head Neck Surg. 27 (1981) pp. 1490-1498.

44. V. Witko-Sarsat, P. Rieu, B. Descamps-Latscha, P. Lesavre, and L. Halbwachs-Mecarelli, Neutrophils: Molecules, Functions and Pathophysiological Aspects, Lab.Invest. 80 (2000) pp. 617-653.

45. C. Nathan, Neutrophils and Immunity: Challenges and Opportunities, Nat.Rev.Immunol. 6 (2006) pp. 173-182.

46. J.A. Smith, Neutrophils, Host Defense, and Inflammation: a Double-Edged Sword, J.Leukocyte Biol. 56 (1994) pp. 672-686.

47. M.B. Hampton, A.J. Kettle, and C.C. Winterbourn, Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing, Blood 92 (1998) pp. 3007-3017.

48. S.H.E. Kaufmann, Immunology's Foundation: the 100-Year Anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff, Nat.Immunol. 9 (2008) pp. 705-712.

49. A. Mayer-Scholl, P. Averhoff, and A. Zychlinsky, How Do Neutrophils and Pathogens Interact?, Curr.Opin.Microbiol. 7 (2004) pp. 62-66.

50. J.E. Park and A. Barbul, Understanding the Role of Immune Regulation in Wound Healing, Am.J.Surg. 187 (2004) pp. S11-S16.

51. J.V. Dovi, A.M. Szpaderska, and L.A. DiPietro, Neutrophil Function in the Healing Wound: Adding Insult to Injury?, Thromb.Haemost. 92 (2004) pp. 275-280.

52. A. Belaaouaj, Neutrophil Elastase-Mediated Killing of Bacteria: Lessons from Targeted Mutagenesis, Microbes Infect. 4 (2002) pp. 1259-1264.

53. S. Schimpff, W. Satterlee, V.M. Young, and A. Serpick, Empiric Therapy with Carbenicillin and Gentamicin for Febrile Patients with Cancer and Granulocytopenia, N.Engl.J.Med. 284 (1971) pp. 1061-1065.

54. R.K. Root and M.S. Cohen, The Microbicidal Mechanisms of Human Neutrophils and Eosinophils, Rev.Infect.Dis. 3 (1981) pp. 565-598.

55. P. Elsbach and J. Weiss, A Reevaluation of the Roles of the O2-Dependent and O2-Independent Microbicidal Systems ofPhagocytes, Rev.Infect.Dis. 5843-53. (1983) pp. 843-853.

56. The Microbial World: A look at All Things Small, http://www.microbiologytext.com/ (2006)

57. G. Mayer, Immunology, Chapter 1: Innate (Non-Specific) Immunity, http://pathmicro.med.sc.edu/ghaffar/innate.htm (2007)

58. B. L. Wetherall, H. Pruul, and P.J. McDonald, Oxygen-Independent Killing of Bacteroides Fragilis by Granule Extracts from Human Polymorphonuclear Leukocytes, Infect.Immun. 43 (1984) pp. 1080-1084.

59. P.M. Henson, J.E. Henson, C. Firrschen, D.L. Bratton, and D.W.H. Riches, Degranulation and Secretion by Phagocytic Cells, In: J.I. Gallin, I.M. Goldstein, and R. Snyderman(Eds.), Inflammation - Basic Principles and Clinical Correlation, Raven Press, New York, 1992. pp. 511-540.

60. W.L. Lee and S. Grinstein, The Tangled Webs That Neutrophils Weave, Science 303 (2004) pp. 1477-1478.

61. V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhmann, D.S. Weiss, Y. Weinrauch, and A. Zychlinsky, Neutrophil Extracellular Traps Kill Bacteria, Science 303 (2004) pp. 1532-1535.

62. D.F. Bainton, Distinct Granule Populations in Human Neutrophils and Lysosomal Organelles Identified by Immuno-Electron Microscopy, J.Immunol.Methods 232 (1999) pp. 153-168.

63. N. Borregaard and J.B. Cowland, Granules of the Human Neutrophilic Polymorphonuclear Leukocyte, Blood 89 (1997) pp. 3503-3521.

64. F. Chua and G.J. Laurent, Neutrophil Elastase: Mediator of Extracellular Matrix Destruction and Accumulation, Proc.Am.Thorac.Soc. 3 (2006) pp. 424-427.

65. H.L. Malech and J.I. Gallin, Current Concepts: Immunology. Neutrophils in Human Diseases, New.Engl.J.Med. 317 (1987) pp. 687-694.

66. F. Dallegri and L. Ottonello, Tissue Injury in Neutrophilic Inflammation, Inflamm.Res. 46 (1997) pp. 382-391.

67. P. Jolles and J. Jolles, What's New in Lysozyme Research? Always a Model System, Today as Yesterday, Mol.Cell.Biochem. 63 (1984) pp. 165-189.

68. T. Ganz, Antimicrobial Polypeptides in Host Defense of the Respiratory Tract, J.Clin.Invest. 109 (2002) pp. 693-697.

69. D.C. Phillips, The Three-Dimensional Structure of an Enzyme Molecule, Sci.Am. 215 (1966) pp. 78-90.

70. A. Fleming, On a Remarkable Bacteriolytic Element Found in Tissues and Secretions, Proc.Roy.Soc.Ser.B. 93 (1922) pp. 306-317.

71. R.T. Ellison and T.J. Giehl, Killing of Gram-Negative Bacteria by Lactoferrin and Lysozyme, J.Clin.Invest. 88 (1991) pp. 1080-1091.

72. L. Leive, The Barrier Function of the Gram-Negative Envelope, Ann.NY Acad.Sci. 235 (1974) pp. 109-129.

73. G.D. Gómez and J.L. Balcázar, A Review on the Interactions Between Gut Microbiota and Innate Immunity of Fish, FEMS Immunol.Med.Microbiol. 52 (2008) pp. 145-154.

74. S. Gordon, J. Todd, and Z.A. Cohn, In Vitro Synthesis and Secretion of Lysozyme by Mononuclear Phagocytes, J.Exp.Med. 139 (1974) pp. 1228-1248.

75. E.M. Cramer and J. Breton-Gorius, Ultrastructural Localization of Lysozyme in Human Neutrophils by Immunogold, J.Leukoc.Biol. 41 (1987) pp. 242-247.

76. M. Klockars and S. Reitamo, Tissue Distribution of Lysozyme in Man, J.Histochem.Cytochem. 23 (1975) pp. 932-940.

77. J. Hankiewicz and E. Swierczek, Lysozyme in Human Body Fluids, Clin.Chim.Acta. 57 (1974) pp. 205-209.

78. E.F. Osserman and D.P. Lawlor, Serum and Urinary Lysozyme (Muramidase) in Monocytic and Monomyelocytic Leukemia, J.Exp.Med. 124 (1966) pp. 921-52.

79. R.S. Pascual, J.B.L. Gee, and S.C. Finch, Usefulness of Serum Lysozyme Measurement in Diagnosis and Evaluation of Sarcoidosis, New.Engl.J.Med 289 (1973) pp. 1074-1076.

80. H. Tomita, S. Sato, R. Matsuda, Y. Sugiura, H. Kawaguchi, T. Niimi, S. Yoshida, and M. Morishita, Serum lysozyme Levels and Clinical Features of Sarcoidosis, Lung 177 (1999) pp. 161-7.

81. I. Torsteinsdottir, L. Hakansson, R. Hallgren, E. Gudbjornsson, N.G. Arvidson, and P. Venge, Serum Lysozyme: A Potential Marker of Monocyte/Macrophage Activity in Rheumatoid Arthritis, Rheumatol. 38 (1999) pp. 1249-1254.

82. O. Selroos and M. Klockars, Serum Lysozyme in Sarcoidosis - Evaluation of Its Usefulness in Determination of Disease Activity, Scand.J.Respir.Dis. 58 (1977) pp. 110-116.

83. C.W.G. Turton, E. Grundy, G. Firth, D. Mitchell, B.G. Rigden, and M.Turner-Warwick, Value of Measuring Serum Angiotensin I Converting Enzyme and Serum Lysozyme in the Management of Sarcoidosis, Thorax 34 (1979) pp. 57-62.

84. B. Korkmaz, T. Moreau, and F. Gauthier, Neutrophil Elastase, Proteinase 3 and Cathepsin G: Physicochemical Properties, Activity and Physiopathological Functions, Biochim. 90 (2008) pp. 227-242.

85. B. Barrick, E.J. Campbell, and C.A. Owen, Leukocyte Proteinases in Wound Healing: Roles in Physiologic and Pathologic Processes, Wound Repair.Regen. 7 (1999) pp. 410-422.

86. W. Bode, E. Meyer, and J.C. Powers, Human Leukocyte and Porcine Pancreatic Elastase: X-ray Crystal Structures, Mechanism, Substrate Specificity, and Mechanism-Based Inhibitors, Biochem. 28 (1989) pp. 1951-1963.

87. A. Janoff and J. Scherer, Mediators of Inflammation in Leukocyte Lysosomes. IX. Elastinolytic Activity in Granules of Human Polymorphonuclear Leukocytes, J.Exp.Med. 128 (1968) pp. 1137-1155.

88. M. Baggiolini, J. Schnyder, U. Bretz, B. Dewald, and W. Ruch, Cellular Mechanisms of Proteinase Release from Inflammatory Cells and the Degradation of Extracellular Proteins, Ciba Found.Symp. 75 (1979) pp. 105-121.

89. U. Bank and S. Ansorge, More than Destructive: Neutrophil-Derived Serine Proteases in Cytokine Bioactivity Control, J.Leukoc.Biol. 69 (2001) pp. 197-206.

90. C.C. Taggart, C.M. Greene, T.P. Carroll, S.J. O'Neill, and N.G. McElvaney, Elastolytic Proteases: Inflammation Resolution and Dysregulation in Chronic Infective Lung Disease, Am.J.Resp.Crit.Care 171 (2005) pp. 1070-1076.

91. O. Wiedow and U. Meyer-Hoffert, Neutrophil Serine Proteases: Potential Key Regulators of Cell Signalling During Inflammation, J.Intern.Med. 257 (2005) pp. 319-328.

92. J.G. Bieth, Leukocyte Elastase, In: A.J. Barret, N.D. Rawlings, and J.F. Woessner(Eds.), Handbook of Proteolytic Enzymes, Academic, San Diego, CA, 1999. pp. 1517-1523.

93. C.A. Owen and E.J. Campbell, The Cell Biology of Leukocyte-Mediated Proteolysis, J.Leukoc.Biol. 65 (1999) pp. 137-150.

94. J.G. Bieth, In Vivo Significance of Kinetic Constants of Protein Proteinase Inhibitors, Biochem.Med. 32 (1984) pp. 387-397.

95. K. Ohlsson and I. Olsson, The Neutral Proteases of Human Granulocytes. Isolation and Partial Characterization of Granulocyte elastases, Eur.J.Biochem. 42 (1974) pp. 519-527.

96. A. Lindmark, A.M. Persson, and I. Olsson, Biosynthesis and Processing of Cathepsin G and Neutrophil Elastase in the Leukemic Myeloid Cell Line U-937, Blood 76 (1990) pp. 2374-2380.

97. A.A. Belaaouaj, R. McCarthy, M. Baumann, Z. Gao, T.J. Ley, S.N. Abraham, and S.D. Shapiro, Mice Lacking Neutrophil Elastase Reveal Impaired Host Defense against Gram Negative Bacterial Sepsis, Nat.Med. 4 (1998) pp. 615-618.

98. R. Garcia, L.Gusmani, R. Murgia, C. Guarnaccia, M. Cinco, and G. Rottini, Elastase Is the Only Human Neutrophil Granule Protein that alone is Responsible for In Vitro Killing of Borrelia Burgdorferi, Infect.Immun. 66 (1998) pp. 1408-1412.

99. J. Tkalcevic, M. Novelli, M. Phylactides, J.P. Iredale, A.W. Segal, and J. Roes, Impaired Immunity and Enhanced Resistance to Endotoxin in the Absence of Neutrophil Elastase and Cathepsin G, Immunity 12 (2000) pp. 201-210.

100. P. Elsbach and J. Weiss, Oxygen-Independent Antimicrobial Systems of Phagocytes, In: J.I. Gallin, I.M. Goldstein, and R. Snyderman(Eds.), Inflammation - Basic Principles and Clinical Correlation, Raven Press, New York, 1992. pp. 603-636.

101. S.J. Klebanoff, Oxygen Metabolites from Phagocytes, In: J.I. Gallin and R. Snyderman(Eds.), Inflammation - Basic Principles and Clinical Correlation, Lippincott Williams & Wilkens, Philadelphia, PA, 1992. pp. 721-768.

102. A. Belaaouaj, K.S. Kim, and S.D. Shapiro, Degradation of Outer Membrane Protein A in Escherichia coli Killing by Neutrophil Elastase, Science 289 (2000) pp. 1185-1187.

103. C.A. Owen, M.A. Campbell, P.L. Sannes, S.S. Boukedes, and E.J. Campbell, Cell Surface-Bound Elastase and Cathepsin G on Human Neutrophils: A Novel, Non-Oxidative Mechanism by which Neutrophils Focus and Preserve Catalytic Activity of Serine Proteinases, J.Cell.Biol. 131 (1995) pp. 775-789.

104. R.E. Young, M.B. Voisin, S. Wang, J. Dangerfield, and S. Nourshargh, Role of Neutrophil Elastase in LTB4-Induced Neutrophil Transmigration In Vivo Assessed with a Specific Inhibitor and Neutrophil Elastase Deficient Mice, Br.J.Pharmacol. 151 (2007) pp. 628-637.

105. C. Delclaux, C. Delacourt, P. D'Ortho, V. Boyer, and A. Harf, Role of Gelatinase B and Elastase in Human Polymorphonuclear Neutrophil Migration across Basement Membrane, Am.J.Respir.Cell.Mol.Biol. 14 (1966) pp. 288-295.

106. K. Kawabata, T. Hagio, and S. Matsuoka, The Role of Neutrophil Elastase in Acute Lung Injury, Eur.J.Clin.Pharmacol. 451 (2002) pp. 1-10.

107. J. Travis and G.S. Salvesen, Human Plasma Proteinase Inhibitors, Annu.Rev.Biochem. 52 (2003) pp. 655-709.

108. S.J. Weiss, Tissue destruction by neutrophils, N.Engl.J.Med. 320 (1989) pp. 365-376.

109. R.L. Vender, Therapeutic Potential of Neutrophil-Elastase Inhibition in Pulmonary Disease, J.Investig.Med. 44 (1996) pp. 531-539.

110. P.S. Hiemstra, Novel Roles of Protease Inhibitors in Infection and Inflammation, Biochem.Soc.T. 30 (2002) pp. 116-120.

111. K. Kawabata, A.R. Moore, and D.A. Willoughby, Impaired Activity of Potease Inhibitors towards Neutrophil Elastase Bound to Human Articular Cartilage, Ann.Rheum.Dis. 55 (1996) pp. 248-252.

112. M. Bedard, C.D. McClure, and N.L. Schiller, Release of Interleukin-8, Interleukin-6, and Colony-Stimulating Factors by Upper Airway Epithelial Cells: Implications for Cystic Fibrosis, Am.J.Respir.Cell.Mol.Biol. 9 (1993) pp. 455-462.

113. H. Nakamura, K. Yoshimura, N.G. McElvaney, and R.G. Crystal, Neutrophil Elastase in Respiratory Epithelial Lining Fluid of Individuals with Cystic Fibrosis Induces Interleukin-8 Gene Expression in a Human Bronchial Epithelial Cell Line, J.Clin.Invest. 89 (1992) pp. 1478-1484.

114. B. Siedle, S. Cisielski, R. Murillo, B. Löser, V. Castro, C.A. Klaas, O. Hucke, A. Labahn, M.F. Melzig, and I. Merfort, Sesquiterpene Lactones as Inhibitors of Human Neutrophil Wlastase, Bioorg.Med.Chem. 10 (2002) pp. 2855-2861.

115. K.A. Elsaid, G.D.Jay, and C.O.Chichester, Detection of Collagen Type II and Proteoglycans in the Synovial Fluids of Patients Diagnosed with Non-Infectious Knee Joint Synovitis Indicates Early Damage to the Articular Cartilage Matrix, Osteoarthr.Cartilage 11 (2003) pp. 673-680.

116. G.M. Tremblay, M.F. Janelle, and Y. Bourbonnais, Anti-Inflammatory Activity of Neutrophil Elastase Inhibitors, Curr.Opin.Investig.Drugs 4 (2003) pp. 556-565.

117. J.A.B. Groeneveld, P.G.H.M. Raijmakers, E.C. Hack, and L.G. Thijis, Interleukin 8-Related Neutrophil Elastase and the Severity of the Adult Respiratory Distress Syndrome, Cytokine 7 (1995) pp. 746-752.

118. G. Salvesen, D. Farley, J. Shuman, A. Przybyla, C. Reilly, and J. Travis, Molecular Cloning of Human Cathepsin G: Structural Similarity to Mast Cell and Cytotoxic T Lymphocyte Proteinases, Biochem. 26 (1987) pp. 2289-2293.

119. T. Burster, H. Macmillan, T. Hou, B.O. Boehm, and E.D. Mellins, Cathepsin G: Roles in Antigen Presentation and Beyond, Mol.Immunol. 47 (2010) pp. 658-665.

120. M. Wysocka, A. Legowsk, E. Bulak, A. Jaskiewicz, H. Miecznikowska, A. Lesner, and K. Rolka, New Chromogenic Substrates of Human Neutrophil Cathepsin G Containing Non-Natural Aromatic Amino Acid Residues in Position P(1) Selected by Combinatorial Chemistry Methods, Mol.Divers. 11 (2007) pp. 93-99.

121. M. Molino, N. Blanchard, E. Belmonte, A.P. Tarver, C. Abrams, J.A. Hoxie, C. Cerletti, and L.F. Brass, Proteolysis of the Human Platelet and Endothelial Cell Thrombin Receptor by Neutrophil-Derived Cathepsin G, J.Biol.Chem. 270 (1995) pp. 11168-11175.

122. M.A. Parry, T. Myles, J. Tschopp, and S.R. Stones, Cleavage of the Thrombin Receptor: Identification of Potential Activators and Inactivators, Biochem.J. 320 (1996) pp. 335-341

123. C.M. Maison, C.L. Villiers, and M.G. Colomb, Proteolysis of C3 on U937 Cell Plasma Membranes. Purification of Cathepsin G, J.Immunol. 147 (1991) pp. 921-926.

124. M.A. Selak, M. Chignard, and J.B. Smith, Cathepsin G is a Strong Platelet Agonist Released by Neutrophils, Biochem.J. 251 (1988) pp. 293-299.

125. W.M. Shafer, J. Pohl, V.C. Onunka, N. Bangalore, and J. Travis, Human Lysosomal Cathepsin G and Granzyme B Share a Functionally Conserved Broad Spectrum Antibacterial Peptide, J.Biochem. 266 (1991) pp. 112-116.

126. C.F. Reilly, D.A. Tewksbury, N.M. Schechter, and J. Travis, Rapid Conversion of Angiotensin I to Angiotensin II by Neutrophil and Mast Cell Proteinases, J.Biol.Chem. 257 (1982) pp. 8619-8622.

127. P.T. Turkington, Cathepsin G, a Regulator of Human Vitamin K, Dependent Clotting Factors and Inhibitors, Throm.Res. 67 (1992) pp. 147-155.

128. R.E. Abbott, C.J. Corral, D.M. MacIvor, X.Lin, T.J. Ley, and T.A. Mustoe, Augmented Inflammatory Responses and Altered Wound Healing in Cathepsin G-Deficient Mice, Arch.Surg. 133 (1998) pp. 1002-1006.

129. D.M. MacIvor, S.D. Shapiro, C.T.N. Pham, A. Belaaouaj, S.N. Abraham, and T.J. Ley, Normal Neutrophil Function in Cathepsin G-Deficient Mice, Blood 94 (1999) pp. 4282-4293.

130. P.I. Bird, Regulation of Pro-Apoptotic Leucocyte Granule Serine Proteinases by Intracellular Serpins, Immunol.Cell.Biol. 77 (1999) pp. 47-57.

131. S.J. Klebanoff, Myeloperoxidase, Proc.Assoc.Am.Physicians 111 (1999) pp. 383-389.

132. R. Fenna, J. Zeng, and C. Davey, Structure of the Green Heme in Myeloperoxidase, Arch.Biochem.Biophys. 316 (1995) pp. 653-656.

133. P.C. Andrews and N.I. Krinsky, The Reductive Cleavage of Myeloperoxidase in Half, Producing Enzymatically Active Hemi-Myeloperoxidase, J.Biol.Chem. 256 (1981) pp. 4211-4218.

134. M.R. Andersen, C.L. Atkin, and H.J. Eyre, Intact Form of Myeloperoxidase from Normal Human Neutrophils, Arch.Biochem.Biophys. 214 (1982) pp. 273-283.

135. S.O. Pember, S.M. Fuhrer-Krüsi, K.C. Barnes, and J.M. Kinkade jr, Isolation of Three Native Forms of Myeloperoxidase from Human Polymorphonuclear Leukocytes, FEBS Letters 140 (1982) pp. 103-108.

136. J. Schultz and K. Kaminker, Myeloperoxidase of the Leucocyte of Normal Human Blood. I. Content and Localization, Arch.Biochem.Biophys. 96 (1962) pp. 465-467.

137. G. Deby-Dupont, C. Deby, and M. Lamy, Neutrophil Myeloperoxidase Revisited: It's Role in Health and Disease, Intensivmed. 36 (1999) pp. 500-513.

138. D.C. Dale, L.Boxer, and W.C. Liles, The Phagocytes: Neutrophils and Monocytes, Blood 112 (2008) pp. 935-945.

139. S.J. Klebanoff, Myeloperoxidase: Friend and Foe, J.Leukoc.Biol. 77 (2005) pp. 598-625.

140. J. Alegre, J. Jufresa, C. Aleman, R. Segura, L. Armadans, R.N. Marti, C. Cervera, and T. Fernandez de Sevilla, Pleural Fluid Myeloperoxidase as a Marker of Infectious Pleural Effusions, Eur.J.Intern Med.12 (2001) pp. 357-362.

141. D. Hurst and P. Venge, Levels of Eosinophil Cationic Protein and Myeloperoxidase from Chronic Middle Ear Effusion in Patients with Allergy and/or Acute Infection, Arch.Otolaryngol.Head Neck Surg. 114 (1996) pp. 531-544.

142. G.A.B. Edelstam, O. Lundkvist, P. Venge, and T.C. Laurent, Hyaluronan and Myeloperoxidase in Human Peritoneal Fluid During Genital Inflammation, Inflammation 18 (1994) pp. 13-21.

143. M.L. Brennan, M.S. Penn, F. Van Lente, V. Nambi, M.H. Shishehbor, R.J. Aviles, M. Goormastic, M.L. Pepoy, E.S. McErlean, E.J. Topol, S.E. Nissen, and S.L. Hazen, Prognostic Value of Myeloperoxidase in Patients with Chest Pain, New.Engl.J.Med. 349 (2003) pp. 1595-1604.

144. Biomaterials Science: An Introduction to Materials in Medicine, Elsevier Academic Press, London, 2004.

145. R.V. Ulijn, Enzyme-Responsive Materials: A New Class of Smart Biomaterials, J.Mater.Chem. 16 (2006) pp. 2217-2225.

146. G. Winzenburg, C. Schmidt, S. Fuchs, and T. Kissel, Biodegradable Polymers and their Potential Use in Parenteral Veterinary Drug Delivery Systems, Adv.Drug.Delivery Rev. 56 (2004) pp. 1453-1466.

147. R. Langer, Drug Delivery and Targeting, Nature 392 (Supp) (1998) pp. 5-10.

148. M. Brannon-Peppas, Polymers in Controlled Drug Delivery, Med.Plastics Biomater. (1997)

149. M. Asif, M.S. Arayne, N. Sultana, and F. Hussain, Fabrication of Nanoparticles within Polymeric Pores for Controlled Release of Drug, Pak.J.Pharm.Sci.19 (2006) pp.73-84

150. K.E. Uhrich, S.M. Cannizzaro, R.S. Langer, and K.M. Shakesheff, Polymeric Systems for Controlled Drug Release, Chem.Rev. 99 (1999) pp. 3181-3198.

151. C.S. Brazel and N.A. Peppas, Temperature- and pH-Sensitive Hydrogels for Controlled Release of Antithrombotic Agents, Mater.Res.Soc.Symp.Proc. 331 (1994) pp. 211-216.

152. D.R. Karsa and R.A. Stephanson, Encapsulation and Controlled Release, The Royal Society of Chemistry, Cambridge, England, 1993.

153. K.D. Bagshawe, C.J. Springer, F. Searle, P. Antoniw, S.K. Sharma, R.G. Melton, and R.F. Sherwood, A Cytotoxic Agent can be Generated Selectively at Cancer Sites, Br.J.Cancer 58 (1988) pp. 700-703.

154. J.L. Merlin, Encapsulation of Doxorubicin in Thermosensitive Small Unilamellar Vesicle Liposomes, Eur.J.Cancer 27 (1991) pp. 1026-1030.

155. S.J. Risch and G.A. Reineccius, Encapsulation and Controlled Release of Food Ingredients, American Chemical Society, Washington, DC, 1995.

156. M.H. Vingerhoeds, H.J. Haisma, M. Vanmuijen, R.B.J. Vanderijt, D.J.A. Crommelin, and G. Storm, A New Application for Liposomes in Cancer-Therapy - Immunoliposomes Bearing Enzymes (Immuno-Enzymosomes) for Site-Specific Activation of Prodrugs, FEBS Letters 336 (1993) pp. 485-490.

157. L.T. Fan and S.K. Singh, Controlled Release: A Quantitative Treatment (Polymers, Properties and Applications), Springer-Verlag, Berlin, 1989.

158. R.W. Baker, Controlled Release of Biologically Active Agents, John Wiley & Sons, New York, 1987.

159. R. Langer, Polymeric Delivery Systems for Controlled Drug Release, Chem.Eng.Commun. 6 (1980) pp. 1-48.

160. D.L. Wise, Handbook of Pharmaceutical Controlled Release Technology, Marcel Dekker Inc., New York, 2000.

161. X. Huang and C.S. Brazel, On the Importance and Mechanisms of Burst Release in Matrix-Controlled Drug Delivery Systems, J.Control.Release 73 (2001) pp. 121-136.

162. R.W. Korsmeyer and N.A. Peppas, Macromolecular and Modeling Aspects of Swelling-Controlled Systems, In: T.J. Roseman and S.Z. Mansdorf(Eds.), Controlled Release Delivery Systems, Marcel Dekker Inc., 1983. pp. 77-8.

163. A.M. Lowman and N.A. Peppas, Hydrogels, In: E. Mathiowitz(Eds.), Encyclopaedia of Controlled Drug Delivery, John Wiley & Sons, 1999. pp. 397-418.

164. K.V.R. Rao and K.P. Devi, Swelling Controlled-Release Systems - Recent Developments and Applications, Int.J.Pharm. 48 (1988) pp. 1-13.

165. J.A. Bouwstra and H.E. Junginger, Hydrogels, In: J. Swarbrick and J.C. Boylan (Eds.), Encyclopaedia of Pharaceutical Technology, Marcel Dekker, 1983. pp. 77-90.

166. J. Siepmann and A. Göpferich, Mathematical Modeling of Bioerodible, Polymeric Drug Delivery Systems, Adv.Drug Deliv.Rev. 48 (2001) pp. 229-247.

167. D.L. Gilbert, Collagen Macromolecular Drug Delivery Systems, (1988)

168. R.M. Mainardes and L.P. Silva, Drug Delivery Systems: Past, Present, and Future, Curr.Drug Targets (2004) pp. 449-455.

2

AIM

The aim of the PhD thesis was the development of a diagnostic device for an early detection of wound infection directly in wound fluid. Bacterial infection of wounds is the main course of impaired wound healing and a severe complication regarding the normal wound healing process. Timely diagnosis of an incipient infection is the critical factor of a successful medical treatment. Since common methods for diagnosing infection are all together time-consuming, physicians are waiting for a method that promises to detect infection in a fast way and, what is more important, prior to obvious clinical symptoms. At the moment, infection is diagnosed by evaluating the typical signs of infection like *rubor*, *colour*, *tumor* and *dolor*. For diagnosing wound infection long experience of the attending doctor is necessary. Additionally, the determination of bacteria present in the wound does not give evidence of an occurring infection because the immune system of a healthy host is more capable of antagonizing infection than that of an affected host. Currently often expensive hit and miss approaches are taken, without providing results. Moreover complications can be prevented and quality of life for patients can be improved, as the time to healing is shortened.

For diagnosing wound infection different enzymes which are associated with an inflammatory response of the human body were chosen. Enzyme levels should be detected directly in wound fluid. It is widely assumed that wound fluid has the potential to provide important biochemical information which can be used as a diagnostic indicator providing information for determining the overall status of a wound and for monitoring the progression of wound healing. Furthermore, wound fluid can be used to monitor specific parameters that have been

identified as prognostic indicators or are targets of a particular therapy. Therefore, different enzymes produced by the human immune system were selected and tested for their ability as marker for wound infection. Based on these results a diagnostic tool for identification of wound infection directly in wound fluid should be investigated.

This device should provide information that will support the clinician to rule out certain issues and to choose effective treatment. Such a diagnostic device tool would allow early intervention with suitable treatment and would reduce dramatically clinical intervention and the use of antibiotics.

3

NOVEL PEPTIDOGLYCAN BASED DIAGNOSTIC DEVICES FOR DETECTION OF WOUND INFECTION

A new concept for a fast diagnostic tool for wound infection based on lysozyme and elastase triggered release of dye from a peptidoglycan matrix was investigated. The matrix consisted of alginate/agarose and peptidoglycan covalently labelled with Remazol Brilliant Blue (RBB). Lysozyme activity in post operative wounds and decubitus wound fluids was significantly elevated upon infection (4830 ± 1848 U $ml^{-1)}$ compared to non infected wounds (376 ± 240 U ml^{-1}). Consequently, incubation of 8% (w/v) labelled agarose/peptidoglycan blend layers with infected wound fluid samples for 2 hours at 37°C resulted in a 4 fold higher amount of dye released than measured for non-infected wounds. For alginate/peptidoglycan beads a 7 fold higher amount of dye was released in case of infected wound fluid samples compared to not-infected ones. Apart from lysozyme, proteases (i.e. gelatinase MMP-9 and elastase) were detected in wound fluids (e.g. using Western blotting). When dosed in ratios typical for wounds, a slight synergistic effect was measured for peptidoglycan hydrolysis (i.e. dye release) between lysozyme and these proteases. Incubation of a double layer system consisting of stained and non-stained peptidoglycan with infected wound fluids resulted in a colour change from yellow to blue thus allowing simple visual detection of wound infection.

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1 Introduction

Wound infection has been shown to impair wound contraction in both acute and chronic wounds [1,2]. However, currently detection of wound infection is based on evaluation of the well known signs of inflammation like *rubor* (redness), *calor* (heat), *tumor* (swelling) and *dolor* (pain) by medical doctors and/or time consuming procedures requiring special machinery [3]. There is currently no rapid diagnostic tool available for the indication of wound infection which would especially be helpful in home care of chronic ulcer patients. Chronic ulceration can affect any anatomical region - the most common site is the lower limb. The estimated prevalence of active leg ulceration in europe is at least 0.1 - 0.3 percent [4,5]. Wound healing involves a complex interaction between epidermal and dermal cells, the extracellular matrix, controlled angiogenesis, and plasma derived proteins - all coordinated by an array of cytokines and growth factors. This dynamic process is classically, but somewhat artificially, divided into three overlapping phases - inflammation, proliferation, and remodelling [6]. Inflammation plays a pivotal role in all phases of the normal wound-healing response following the initial injury. The inflammatory response will act to recognize and eliminate potential pathogens and foreign material [7].

Infection has been defined as the product of the entrance, growth, metabolic activities, and resultant pathophysiologic effects of microorganisms in the patient’s tissues [8]. When this combination of events results in excessive amounts of bacteria in tissue, the balance or equilibrium is upset and infection ensues. High levels of bacteria not only result in infection but have been demonstrated to impair every process of the wound healing scheme [9,10]. Consequently, early detection of wound infection is essential in wound care.

Wound infection can be characterised by the identification of the causative organism(s) after wound swabbing [11]. Serum investigations involve small amounts of blood being obtained from the patient to identify elevated white cell counts and elevated levels of serum C-reactive protein (CRP), a protein normally not found in the serum, but present in many acute inflammatory conditions and with necrosis. However, both methods are not suitable for rapid diagnosis of a chronic wound infection [12].

Furthermore, neutrophils are a predominant cell marker in the wound within 24 hours after injury. The major function of the neutrophils is to remove foreign material, bacteria and non-functional host cells and damaged matrix components that may be present in the wound site

[13,14]. Bacteria release chemical signals, attracting neutrophils, which ingest them by the process of phagocytosis [15]. Enzymes and other constituents of human neutrophil granules are amongst others myeloperoxidase, defensins, elastase, cathepsin G, phospholipase A2 and lysozyme which are partly released [16].

One of the biological functions of lysozyme is believed to be a self-defense against infection by bacteria [17]. Lysozyme (EC 3.2.1.17), known as muramidase or N-acetylmuramide glycanhydrolase, is an enzyme capable of hydrolysing glycosidic linkages between the *N*-acetylmuramic acid and *N*-acetylglucosamine residues of the bacterial cell wall peptidoglycan [18]. Many of the assays for the determination of lysozyme activity are based on the hydrolysis of the *Micrococcus lysodeikticus* cell wall and are thus a function of muramidase activity of the enzyme [19]. Beside the common used turbidimetric assay, *M. luteus* cells modified with remazol brilliant blue R (RBB-R) were described by Ito *et al.* [20] and Hard *et al.* [21]. This method provides a more sensitive method applied for the determination of the concentration of lysozyme in serum, being accurate enough for screening many samples [20].

It is well known that any deviation of the lysozyme concentration from the normal level in serum or urine is related to diseases. For example, a high level of lysozyme was observed in the urine of patients with monocytic leukaemia [22]. Serum levels may be raised in chronic granulomatous inflammatory disorders including tuberculosis and sarcoidosis [23,24]. These observations indicate that the measurement of the concentration of lysozyme in serum or urine is useful for the diagnosis or screening of diseases. In addition, these clinical studies have suggested that increase of this enzyme is a serum marker of active chronic inflammation [25-27].

It is widely assumed that wound fluid has the potential to provide important biochemical information which can be used as a diagnostic indicator providing information for determining the overall status of a wound and for monitoring the progression of wound healing. Furthermore, wound fluid can be used to monitor specific parameters that have been identified as prognostic indicators or are targets of a particular therapy [28-30].

As there is a strong need for a fast prognostic aid which would assist in predicting clinical infection of a wound prior to obvious clinical symptoms, we have determined lysozyme levels in different types of chronic wounds. Based on these data we have investigated different enzyme responsive devices for simple detection of this enzyme and, as a consequence, the development of a rapid and simple diagnostic system for detection of wound infection.

2 Materials and Methods

2.1 Sample Collection and Preparation

Fluid from 10 post-operative wounds, eight decubitus ulcer wounds and eight blisters were collected and analyzed by biochemical techniques. Exudate samples were collected directly from exudating wound surface by using needles or a spoon before the daily treatment. Four samples were collected during V.A.C (Vacuum Assisted Closure) therapy in special canisters without starch. The samples were centrifuged three times at 420 g for 20 minutes to remove cells and tissue material and stored at -20°C until analyzed. 13 wounds out of these 26 wounds were described as clinically infected by the attending doctors. The patients studied so far were grouped and labeled with the following abbreviations (followed by a number): I = infected wound; n.i = non infected wound. Permission to collect wound fluid was obtained from the Ethics Committee of the Medical University of Graz.

2.2 Enzyme Activities Measured Directly in Wound Fluid

Lysozyme Activity

Lysozyme activity was directly determined in wound fluids using a modified method as described by Shugar (1952) *et al.,* [19]. The assay is based on the determination of the lysis of *Micrococcus lysodeikticus* cells monitored turbidimetrically at 450 nm. *Micrococcus lysodeikticus* cells were suspended in 0.1 M KH_2PO_4 buffer (pH 7.0) to obtain an approximate concentration of 0.05% (w/v). 10 µl of sample or enzyme standard solution were added to 290 µl of this suspension to start the reaction. The initial rate of increase in transparency was monitored every 60 seconds at a wavelength of 450 nm and a temperature of 25°C.

Gelatinase Activity

Gelatinase activity was measured with a commercial gelatinase kit (Enzcheck Gelatinase/Collagenase Assay Kit). As substrate gelatin (DQ Collagen Type IV Fluorescein Conjugate, Invitrogen) was used. For measurement, 80 µl of reaction solution were mixed with 20 µl substrate solution and 100 µl diluted sample or collagenase from *Clostridium histolyticum.* The resulting fluorescence was measured at an excitation wavelength of 495 nm and at an emission wavelength of 515 nm, in black 96 well plates.

Elastase Activity

Elastase activity is determined by measuring the cleavage of the chromogenic substrate N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (MeOSuc-AAPV-pNA) as already described by Trengove *et al.,* [31]. A solution of 1 mM MeOSuc-AAPV-pNA (diluted in DMSO) in 0.1 M HEPES buffer (pH 7.4, containing 0.5 M NaCl) is used. To 5 µL sample (wound fluid) or commercial elastase (0.1 U/ml till 1 U/ml), 100 µL of the substrate solution is added. Substrate degradation is continuously monitored by measuring the increasing absorbance at 405 nm every 60 seconds at a temperature of 30°C. The initial speed of the reaction (linear range) is used for calculations.

2.3 Zymography

Zymography was carried out according to an adapted and modified method of Ito *et al.,* [20] and Hard *et al.,* [21]. Polyacrylamide gel electrophoresis was performed using 10.0% (w/v) separating gels and 10% (w/v) stacking gels, comprising 4% (w/v) peptidoglycan. Samples were mixed 1:1 (v/v) with non reducing sampling buffer (2.8 ml H_2O, 1 ml 0.5 M Tris-HCl pH 6.8, 3.2 ml SDS (10% w/v), 0.5 ml bromphenol-blue (1% (w/v)) and 7 ml glycerol) and were incubated at room temperature for 10 minutes. Gels were loaded with 10 µl of sample. Electrophoresis was performed using a Mini Protean Cell (Biorad) 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) for 20 minutes to remove the SDS and to allow the enzyme to renature again. Incubation of gels was carried out over night in incubation buffer (phosphate buffer: 100 mM; pH 7.5) at 37°C. After incubation, gels were routinely stained with Coomassie blue (0.25% w/v) and destained in a solution containing 10% (v/v) acetic acid, 50% (v/v) dH_2O and 40% (v/v) ethanol. Digestion of peptidoglycan was detected as a clearing zone in a dark blue background. The procedure was optimized with commercial lysozyme (5000 to 313 U/ml) which was also used as a standard. After optimisation, infected (i) and non-infected (n.i) wound fluid samples were measured. Additionally, the activity of commercial collagenase was investigated.

2.4 Labelling of Micrococcus lysodeikticus Cells

Labelling of bacterial cells was based on a standard all-in dyeing process. Briefly, a solution of 50 mg *Micrococcus lysodeikticus* (ML) cell wall was suspended in 0.5 ml Remazol Brilliant

Blue R (RBB) solution (0.5% w/v in dd. H_2O). Sodium sulphate (Na_2SO_4, 2.5% (w/v)) and sodium carbonate ($NaCO_3$1% w/v) was dissolved in deionised water and directly added to the suspension of RBB-PG-solution with the equal volume. Dyeing was carried out in a thermoshaker (Eppendorf), using following thermal gradient: 25°C for 10 minutes and 65°C for 5 minutes. The reaction mixture was centrifuged for 5 minutes at 11357 g to remove non bound dyestuff. The supernatant was discarded and the pellet was washed with deionised H_2O until the supernatant was colourless. The amount of dyestuff not being bound to peptidoglycan was determined to be 2.91 mg. The RBB content was therefore calculated to be 14% (w/w).

2.5 Preparation of Agarose/Peptidoglycan Layers

Agarose/Peptidoglycan Based Mono-Layers

I.) Unstained Agarose/Peptidoglycan Based Mono-Layers

Unstained agarose/peptidoglycan blend layers were prepared as follows. Agarose was used as matrix for the blends and was dissolved in phosphate buffer (100 mM; pH 7.0) by heating in the microwave oven. Thereafter, different concentrations of peptidoglycan, ranging from 15 to 50 mg (0.15 – 0.50% (w/w)) were suspended in the 10 g of agarose solution (60°C) and were mixed properly. Preparation of the blends was directly carried out in 96 well plates. Therefore, different volumes of hot agarose-peptidoglycan suspension (60 µl to 150 µl) were directly transferred into the wells and were allowed to polymerize over night. This protocol was optimized regarding incubation temperature, layer thickness and peptidoglycan-concentration. Optimization was carried out regarding the increase of transparency of the matrix upon incubation with commercial lysozyme.

II.) Stained Agarose/Peptidoglycan Based Mono-Layers

Stained agarose/peptidoglycan blend layers were prepared similarly by using stained peptidoglycan prepared as described above. For optimisation different stained peptidoglycan-concentrations ranging from 0.4-40% (w/w), as well as different agarose-concentrations ranging from 0.25-1.00% (w/v) were used upon incubation with commercial lysozyme.

Agarose/Peptidoglycan Based Double-Layers

A double-layer system was prepared directly in microtiter plates comprised of stained and unstained agarose/peptidoglycan. For this approach, 100 µl PG-RBB-agarose of suspension

(50 mg PG-RBB / 2.5g Agarose, 2% (w/v)) was covered with 50 µl unstained PG-agarose suspension (50 mg PG-RBB / 2.5 g Agarose 2% (w/v)).

2.6 Stained Alginate/Peptidoglycan Based Beads

Peptidoglycan-loaded alginate blend beads were prepared as follows. Briefly, an aqueous solution comprising 0.75% (w/v) alginate was prepared by stirring the solution at 25°C for approximately 4 hours. Before use, the solution was left to stand to remove disturbing trapped air bubbles. Thereafter RBB stained peptidoglycan (8% (w/v)) was added and the suspension mixed. Beads were prepared at room temperature by dropping a hot PG-RBB-alginate solution into 500 ml of an agitated calcium chloride solution (5% (w/v)), using a peristaltic pump equipped with a needle. The formed beads were left in the calcium solution for 30 minutes and were then rinsed with deionised water. Beads were stored till further use at 4°C. The RBB content of the beads was calculated to be 20 ng RBB per g beads.

2.7 Enzymatic Hydrolysis of Stained and Unstained Agarose/Peptidoglycan Layers and Alginate/Peptidoglycan Beads

In order to optimize composition and preparation conditions of agarose/peptidoglycan layers and alginate/peptidoglycan beads, in vitro-degradation experiments were performed with commercial lysozyme from chicken white egg (AppliChem) at 37°C and shaking at 350 rpm. Additionally, the influence of commercial neutrophil-elastase (Sigma), collagenase Type IV (GIBCOTM Invitrogen) and matrixmetalloproteinase MMP-9 (Sigma) was investigated.

2.8 Enzymatic Hydrolysis of Agarose/Peptidoglycan Mono- and Double-Layers

Unstained Agarose/Peptidoglycan Based Mono-Layers

Different amounts of polymer solution were used per well and dried over night in the fridge. 200 µl of enzyme solution (lysozyme or wound fluid samples diluted 1:10 with buffer) were added to the polymer for incubation. Degradation of peptidoglycan due to lysozyme activity was monitored as increase in transparency, measured at 450 nm. Measurements took place directly in the reaction plate for 120 minutes.

Stained Agarose/Peptidoglycan Based Mono-Layers

RBB- stained agarose/peptidoglycan layers casted in microtiter plates were incubated at 37°C with 200 µl of lysozyme (ranging from 312 up to 5000 U/ml) or wound fluid samples diluted 1:10 with buffer. Every 15 minutes 100 µl of sample was taken, transferred into a microtiter-plate and measured at 600 nm. All measurements were repeated 6-fold.

2.9 Enzymatic Hydrolysis of Alginate/Peptidoglycan Beads

The release of dye-PG-fragments with different lysozyme activities was tested. Therefore, 9 beads (79.39 mg +/- 8.34 mg) of Remazol Briliant Blue stained beads were incubated at 37°C with 200 µl lysozyme (5000; 2500; 1250, 625, 312 and 0 U/ml) in eppendorf tubes at 350 rpm. In addition to lysozyme, the effect of collagenase, elastase and MMPs on the hydrolysis of agarose/peptidoglycan beads was tested. Therefore, RRB-PG beads were incubated with lysozyme (250 U/ml) together with collagenase (10 U/ml), elastase (10 U/ml) and MMPs respectively. The release of soluble blue fragments into the supernatant was measured at 600 nm at given time intervals. All measurements were repeated 6-fold. Additionally, similar experiments were performed with wound fluid samples diluted 1:10.

2.10 Longterm-Stability Tests of Alginate/Peptidoglycan Beads and Agarose/Peptidoglycan Mono-Layers

The stability of RBB-stained alginate/peptidoglycan beads and agarose/peptidoglycan blend layers in microtiter plates which were stored at 4°C was determined. Microtiter plates and beads which were aliquoted in eppendorf tubes were stored at 4°C. Every week, beads and layers were incubated with 200 µl of lysozyme (10,000 U/ml) at 37°C. After 30 minutes of incubation, OD_{600} was measured.

2.11 Direct Infusion Mass Spectrometry Analysis

To investigate the influence of staining of *Micrococcus lysodeikticus* cells on lysozyme catalysed hydrolysis reaction products were analysed with an LC/MS (liquid chromatography-mass spectrometry) in direct injection mode. The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with an electrospray ionisation operated in positive and negative ion mode and the electrospray voltage was set to 3500 V. Dry gas flow was set to 9 l min^{-1} with a temperature of 350°C, nebulizer set to 40 psi. Maximum

accumulation time was fixed to 300 ms and the loading of the trap was controlled by the instrument with an ICC of 30000. Data was collected for 2.5 min/sample between 50 and 400 Da/e in a single scan with a scan rate of 0.02 min per scan. For analysis, the recorded mass data were averaged. 50 mg stained and unstained PG was incubated in 1.5 ml phosphate buffer with lysozyme (6666 U/ml). Incubation was carried out at 37°C for 12 hours. The supernatants were transferred into glas vials and vials were placed in the autosampler of the LC system for automation of the MS injection.

2.12 Western Blot

Western blot analysis was performed for detecting matrix metalloproteinase MMP-9 in wound fluid. Proteins were separated via sodium dodecyl sulphate – polyacrylamid gel electrophoresis (SDS-PAGE) by molecular weight. They were then transferred to a membrane and detected by protein-antibody interaction. The ECL^{TM} Western blotting Analysis System was provided by Amersham.

For SDS-PAGE 7.5% SDS gels (0.75 mm) were used. Per sample 5-50 µg protein were applied. Samples were mixed with 2.5 µl 10x-sample buffer and were diluted with ddH_2O to a volume of 25 µl. Wound fluids as well as purified MMP-9 (gelatinase B, 92 kDa type IV collagenase, *Chemicon* Gelatinase kit) and standards (broad range, low range) were heated at 95°C for 10 minutes. 10 µl of sample solution and 8 µl of standard solution were loaded respectively. Electrophoresis was conducted at a voltage of 150 V (73 mA) with the BIO-RAD apparatus.

After separation, proteins on the gels were blotted onto the membrane in the blotting cell (BIO-RAD) with 350 mA for 1 hour. Non-specific binding sites were blocked in 0.5% non-fat dried milk over night in the fridge. After washing, incubation with the primary antibody solution (*Chemicon* Gelatinase kit, diluted 1:5000) for 90 minutes was performed. After extensive washing of the membranes, they were incubated with peroxidise-conjugated goat anti rabbit IgG (1:15000). Protein bands were detected using the ECL plus western blotting detection system (Amersham Pharmacia Bitech UK Ltd) with subsequent exposure to X-ray films.

2.13 Statistical Analysis

To compare the differences of lysozyme activity in infected and non infected wound fluids, paired t-test was performed. $P \leq 0.005$ was said to be statistical significant.

3 Results

3.1 Enzyme Activity in Wound Fluids

Lysozyme activities of infected and non infected wounds were determined according to Shugar *et al.,* [19] while commercial lysozyme was used as standard in this procedure. By the attending doctors, 13 wounds out of 26 were labeled as non infected wounds (n.i), while 13 wounds were described as clinically infected (i). As shown in Table 5, lysozyme activity in infected wound fluid was significantly higher than in non- infected wounds ($P \leq 0.005$) thus according well with the clinical description. There was no significant difference between blisters and non infected post operative or decubitus wounds regarding lysozyme levels. Similarly, there were no significant differences of lysozyme levels in infected wounds from decubitus or post operative wounds.

Table 5: Lysozyme activity in wound fluid samples: There is a significant difference between lysozyme activity of infected and non-infected wounds (P≤0.005).

Clinical description of the wound	Infected	Non-infected
Blisters	0	8
Post-operative wounds	8	2
Decubitus wounds	5	3
Lysozyme activity [U/ml]	4,830 ±1,848	376 ±240
Lysozyme concentration [µg/ml]	24.15 ± 9.24	1.79 ± 1.22

Lysozyme (EC 3.2.1.17) belongs to the enzyme class of gycosidases which are able to catalyse the hydrolysis of the glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine-residues of the bacterial cell wall peptidoglycan [18]. One of the biological functions of lysozyme is believed to be self-defense against infection by bacteria [17], as it is normally released from monocytes, macrophages and polymorphonuclear leucocytes [25,32]. Elevated serum lysozyme levels are well documented for different diseases like tuberculosis

and sarcoidosis [22,23]. Therefore, the measurement of lysozyme concentration in serum or urine is useful for the diagnosis or screening of diseases. In addition, raised lysozyme levels were identified as a serum marker of active chronic inflammation [25-27]. Furthermore, sensitivity of lysozyme as a marker for sarcoidosis was shown to be high [24,33].

Wound infection is one of the most common reasons for the non - healing of a wound, leading to death in the worst case. It is widely assumed that wound fluid has the potential to provide information which can be used as a diagnostic indicator for the overall status of a wound or as prognostic indicators [28,29]. In detail, Naomi *et al.,* [29] found comparable levels of the C-reactive protein, which is a widely used marker for inflammation, in serum and wound fluid. Additionally, neutrophils and monocytes are predominant cell markers of wound infection [13,14]. Here we found clearly different lysozyme levels in wound fluid of clinically infected and non infected wounds. Thus, this observed difference between infected and non infected wounds probably reflects the number of monocytes/macrophages and neutrophils in the tissue. The significantly elevated lysozyme levels in infected wounds show the potential of this parameter for diagnosis of wound infection and prompted us to use lysozyme for the development of different enzyme responsive devices based on enzymatically controlled release for a simple detection of this enzyme.

To confirm the differences found in lysozyme levels, a zymogram based assay was performed. We and others have successfully developed and used zymogram based techniques involving renaturation of enzymes for a variety of other polysaccharide hydrolysing enzymes [34]. Figure 2 shows zymograms of polyacrylamide gels containing (4% (w/v)) *Micrococcus lysodeikticus* cell walls with commercial lysozyme and wound fluid samples.

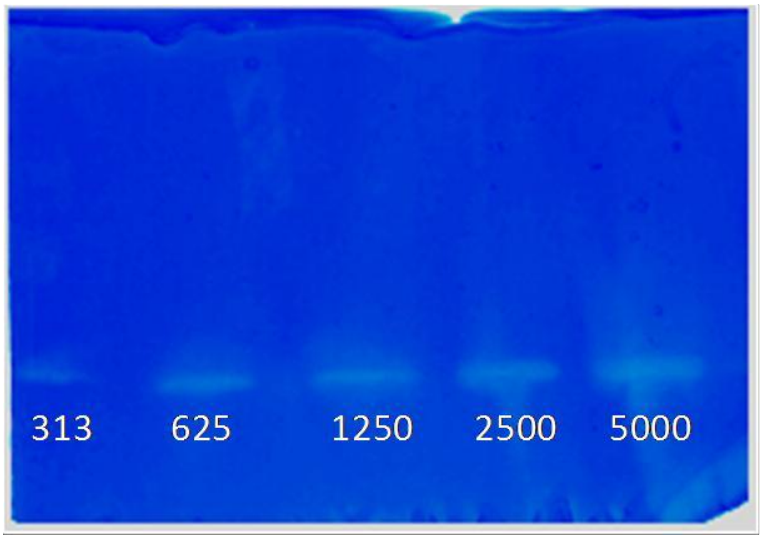

Figure 2: Zymogram of *Micrococcus lysodeikticus cells*: Zymogram gel containing 4% (w/v) *Micrococcus lysodeikticus* cell walls loaded with different lysozyme activities ranging from 313 to 5,000 U/ml

Lysozyme catalyzed hydrolysis of peptidoglycan incorporated into SDS-gels lead to observable clearing zones in a blue-stained background after staining. The intensity correlated well to the enzyme activity applied (see Figure 2). Additionally this method was successfully used for wound fluid samples thus allowing semi-quantitative measurement of lysozyme activity. Only infected wound fluid samples showed clear digestion zones, while non-infected samples did not (data not shown). As a negative control bovine serum albumin failed to yield clear spots indicating that the clearing spots are not artifacts of electrophoresis or refolding.

3.2 Diagnostic Devices Based on Enzymatically Controlled Dye Release

Device A: Transparency Increase of Agarose/Peptidoglycan Blend-Layers

For the development of a simple diagnostic tool the liquid lysozyme assay was adapted to a solid system. Therefore, in a first stage, the composition of agarose/peptidoglycan layers was optimized. An approach comprising 0.45% (w/v) peptidoglycan and casting in microtiter plates at 45°C turned out most successful. A higher content of peptidoglycan led to inhomogeneous layers with PG particles on the surface. Additionally, no correlation between increase of transparency and lysozyme activity could be observed. On the other hand, a lower content resulted in layers with already too high transparency. A layer thickness of 2.0 mm was

appropriate for visual inspection of a transparency change within 30 minutes of incubation. Using these conditions, the transparency change depending on enzyme activity of commercial lysozyme (Figure 3) and wound fluids (Figure 4) was monitored.

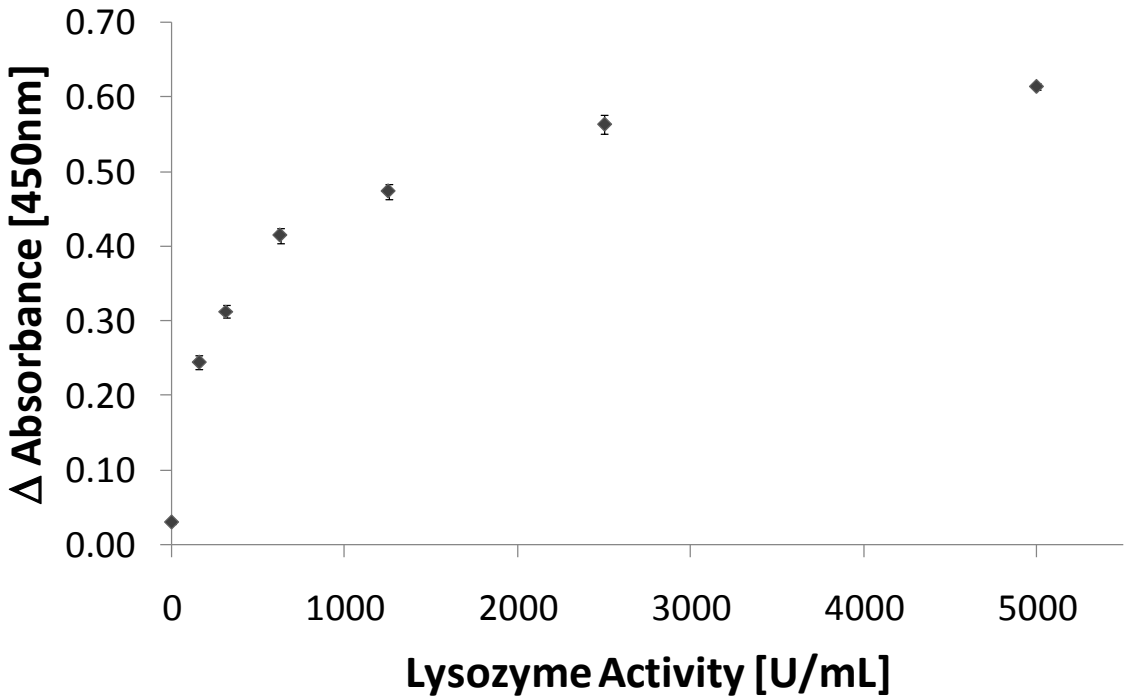

Figure 3: Correlation of transparency increase and lysozyme activity: Transparency increase of agarose/peptidoglycan blend layers (0.45% (w/w)) after incubation with various lysozyme activities at 37°C for 2 hours, measured at 450 nm.

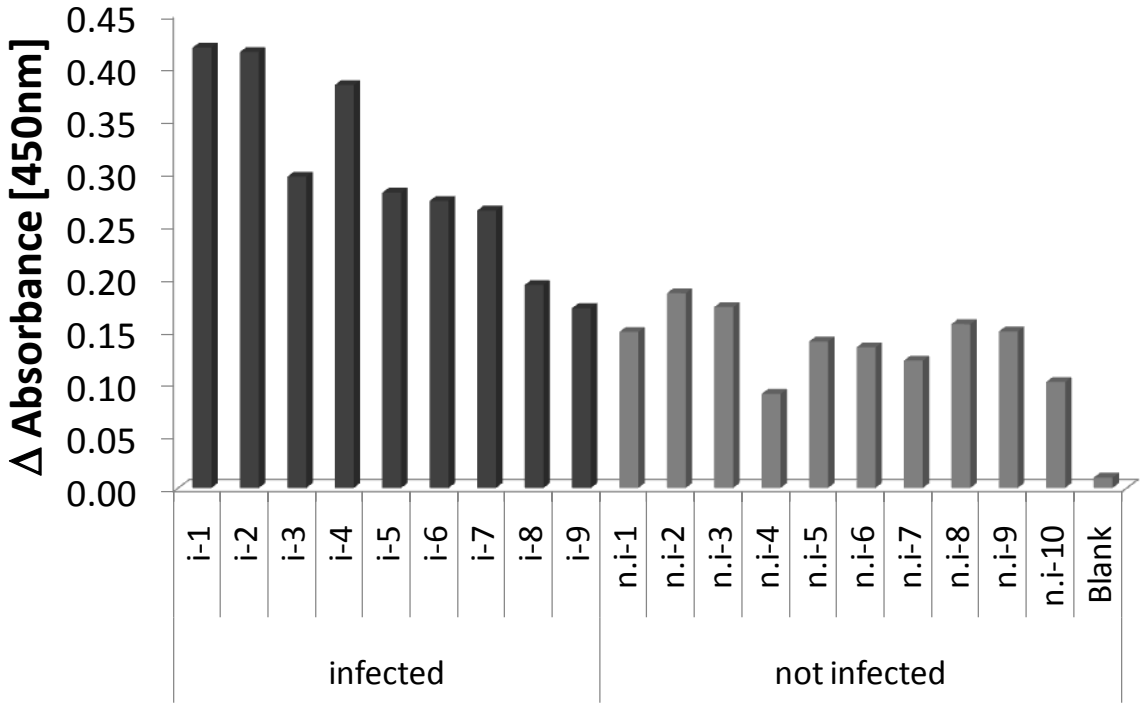

Figure 4: Distinction of infected and non infected wound fluid samples: Transparency increase (450 nm) of agarose-peptidoglycan blend layers (0.45% (w/w)) after incubation with infected (i) and non-infected (n.i) wound fluid samples at 37°C for 2 hours. Average absorbance changes of infected wound fluid samples are 2-fold higher than those of not infected wounds (P≤0.005).

Using unstained agarose/peptidoglycan blend layers, infected and non-infected wound fluid samples showed observable and significant (P≤0.005) differences in the change of absorbance after 2 hours of incubation. There is a clear tendency of high absorbance changes in case of infected wound fluids. However, the absorbance changes obtained in two out of nine infected samples are rather close to those of non-infected samples. The decrease of the shape and the short linear region (up to 300 U/ml) of the calibration curve of this system seemed to be the limiting factor as we found lysozyme levels around 4,000 U/ml in infected wounds. Since blue labelled *Micrococcus lysodeikticus* cells has previously shown higher sensitivity in turbidimetric assays or incorporated in zymograms [20,21], staining of peptidoglycan was considered to improve the detection.

Device B: Dye Release from Agarose/Peptidoglycan Mono-Layers

To improve the detection sensitivity of the above system, agarose/peptidoglycan mono-layers were constructed using peptidoglycan covalently dyed with remazol brilliant blue. It is known

that RBB binds preferentially to 6-hydroxyl groups of hexoses [21]. In our study, the RBB content was calculated to be 14% (w/v). Therefore, every 4th disaccharide and every 8th saccharide of the peptidoglycan backbone was stained with one molecule of Remazol Brilliant Blue. For both unstained PG and RBB-stained PG the major hydrolysis product apart from higher oligomers was identified by LC-MS to be the disaccharide (m/z 496.9) consisting of N-acetylgucosamine and N-acetyl muramic acid (Figure 5). This indicates that partial derivatization of peptidoglycan with RBB did not sterically affect hydrolysis by lysozyme.

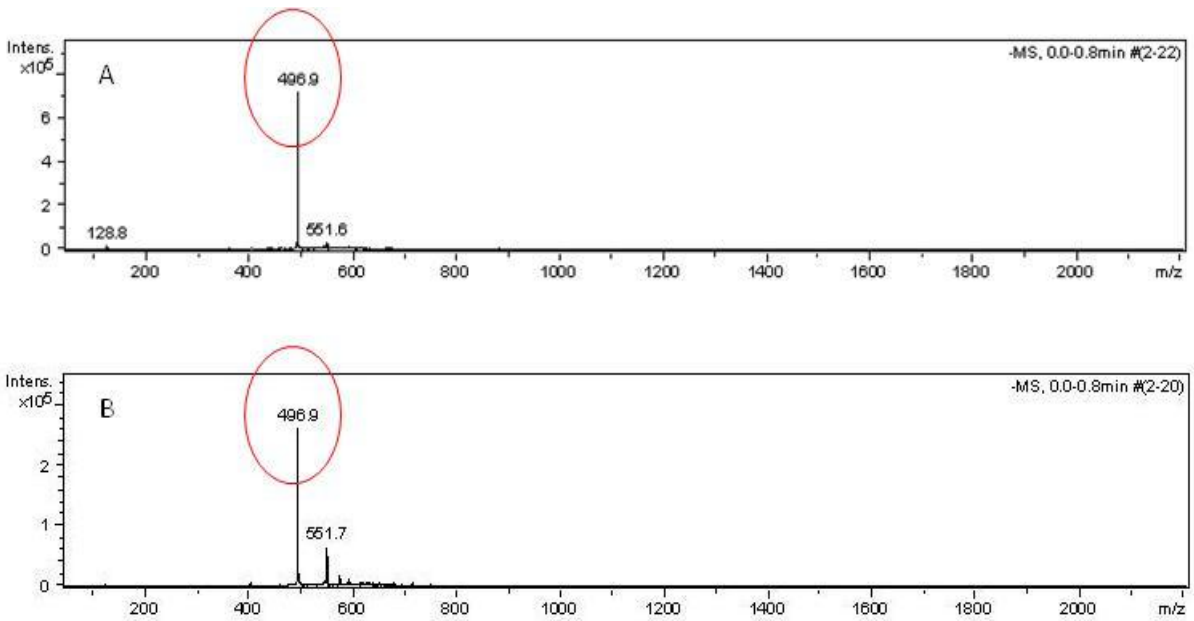

Figure 5: MS-Spectrum of digested RBB-stained (A) and unstained peptidoglycan (B): Staining procedure of peptidoglycan did not influence the degradation ability of lysozyme (6,666 U/ml)

Different amounts of stained PG (8 to 40% (w/v)) were tested for optimization of the device. The optimal concentration of stained PG turned out to be 8% (w/v) and was therefore used for all further experiments. Higher amounts of stained PG resulted in an unspecific release of dye even in the absence of enzyme. Incubation of this optimized system was carried out with different concentrations of commercial lysozyme. A linear correlation regarding the dye released up to 2,500 U/ml was observed (Figure 6). Using lysozyme activities higher than 2,500 U/ml, the absorbance increase levelled off, which could be due to steric hindrance or inaccessibility.

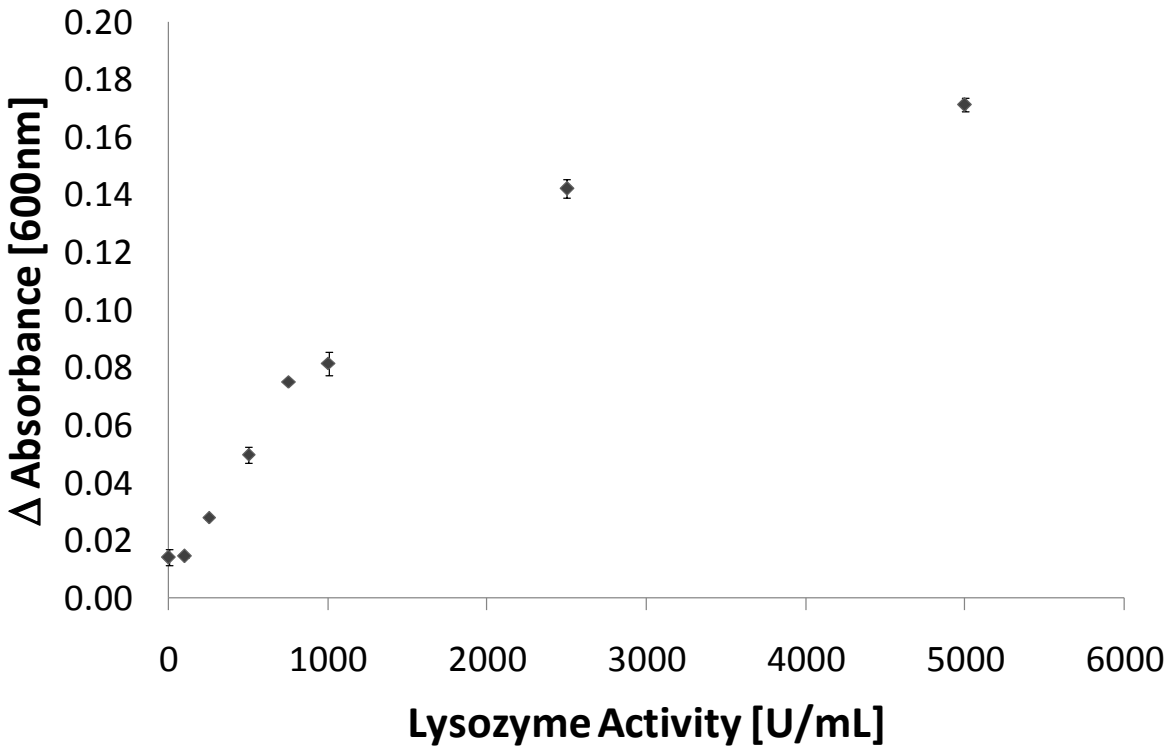

Figure 6: Correlation of colour release and lysozyme activity: Colour release from stained (8% (w/w)) agarose/peptidoglycan blend layers was measured at a wavelength of 600 nm after incubation with different lysozyme activities ranging from 313 up to 10,000 U/ml at 37°C for 120 minutes.

Additionally, diluted (1:10) wound fluid samples of infected (i) and non-infected wounds were investigated regarding its ability for controlled release of the dye (Figure 7).

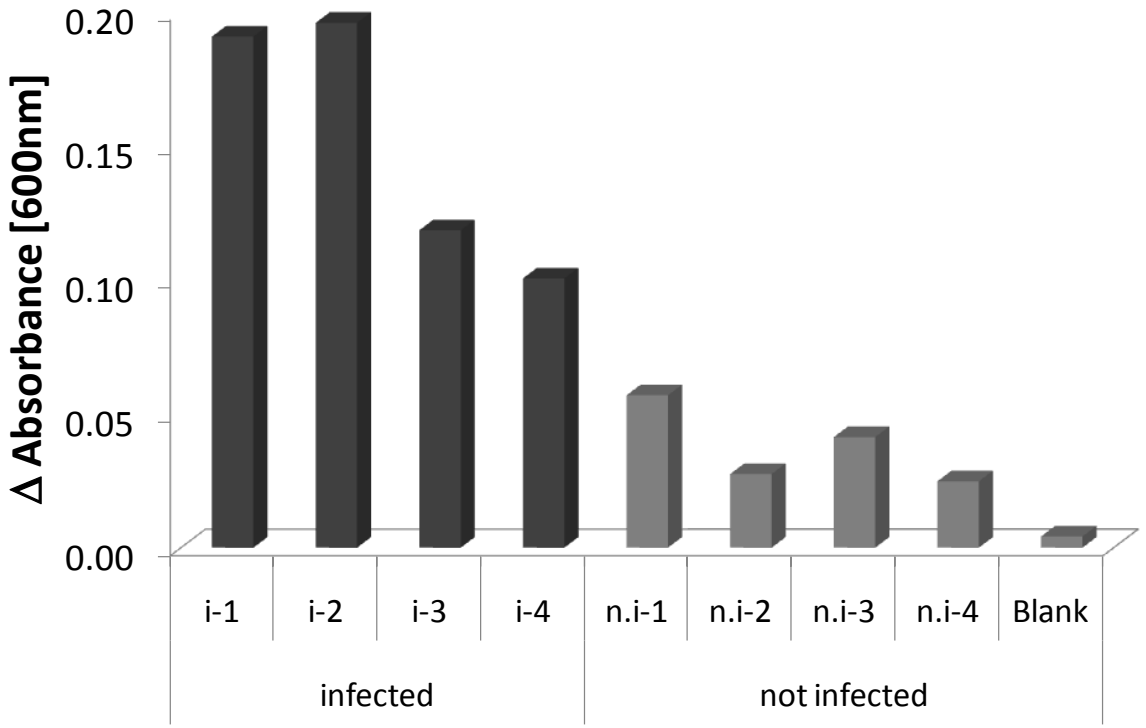

Figure 7: Distinction of infected and non infected wound fluid samples: Colour release from stained (8% (w/w)) agarose/peptidoglycan blend mono layers after incubation with infected (i-1 – i.5) and non infected (n.i-1 – n.i-4) wound fluid samples for 2 hours at 37°C. Average absorbance changes of infected wound fluid samples are 4-fold higher than those of non infected wounds (P≤0.005).

Compared to the unstained system, linearity of the calibration curve could be increased. This improvement of the system is of great importance for a precise differentiation between infected and non-infected wounds. With this system a clear difference (P≤0.005) between infected and non-infected wound fluids was detected regarding the release of dye (Figure 7). The difference with this system was indeed much more pronounced (factor 4) when compared to the system involving unstained PG (factor 2).

Device C: Dye Release from Agarose/Peptidoglycan Double-Layers

To avoid separation of the supernatant and spectrophotometric analysis of which, a system was constructed allowing simple visual judgment of wound infection. Therefore, peptidoglycan/agarose double layers were constructed. The lower layer contained stained PG and the upper layer unstained PG. Consequently, these layers appeared white to pale yellow from the top (Figure 8).

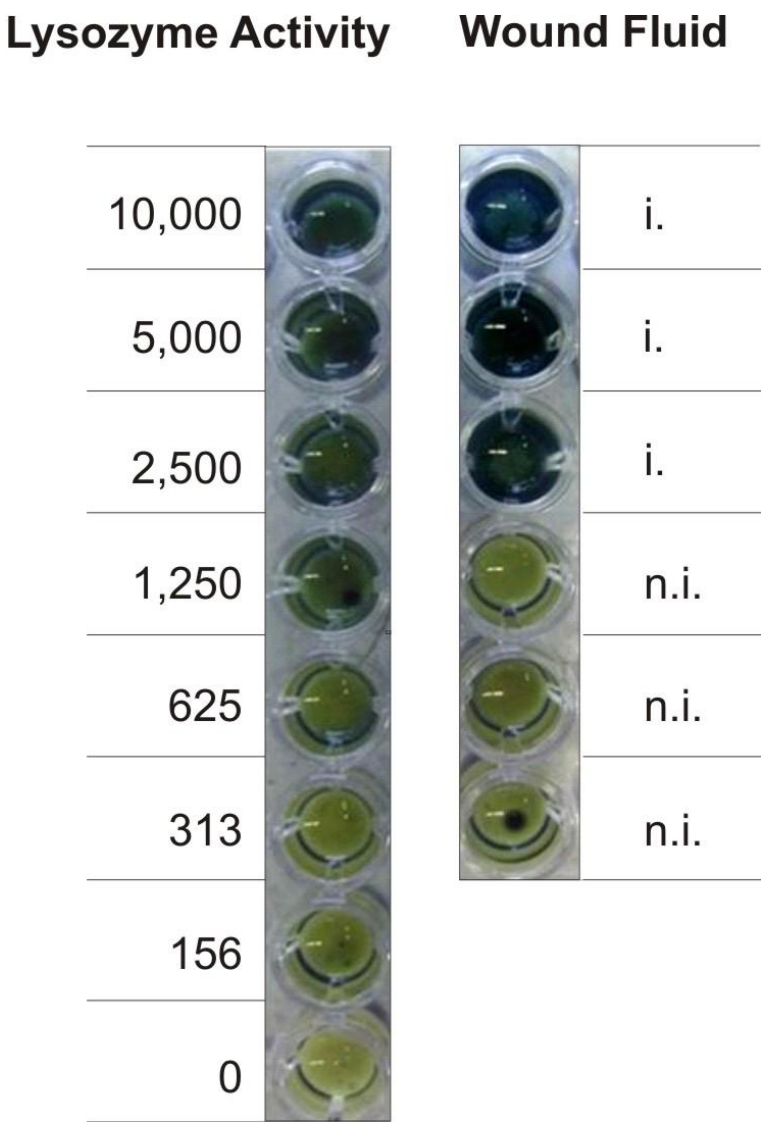

Figure 8: Visual detection approach: Layer alignment of stained and unstained peptidoglycan in microtiter plates for detection of wound infection. Double-layers were incubated with different lysozyme activities (10,000 – 156 U/ml) and with 3 infected (i) and 3 non infected (n.i.) wound fluid samples

Upon incubation with high lysozyme levels, the upper layer was hydrolysed and subsequently, the blue peptidoglycan from the lower layer appeared, yielding to a colour change from pale yellow to dark blue (Figure 8). Similarly, incubation of infected wound samples resulted in the same change from white to dark blue after 120 minutes.

As all devices described above use agarose as solid matrix, possible contribution of agarases to hydrolysis of the matrix should be considered. Agarases are capable of degrading agarose and are found in bacteria like *Pseudomonas sp*., *Bacillus sp., Alteromonas* and *Vibrio*. As

Pseudomonas spp. are known to cause infection in chronic wounds [37], their presence in wounds and secretion of agarases could enhance response of this system due to hydrolysis of agarose [38-40].

Device D: Dye Release from Alginate/Peptidoglycan Beads

In order to reduce incubation time, beads comprising dyed peptidoglycan in alginate were constructed. Due to a higher surface area, beads should lead to a faster release of dye (or dyed fragments) upon incubation with lysozyme compared to the layers described above. Again, peptidoglycan 8% (w/v) was most suitable while a higher PG content led to an inhomogeneous suspension resulting in difficulties in beads preparation.

The colour release from stained alginate/peptidoglycan beads after incubation for 60 minutes and 120 minutes with different lysozyme activities and wound fluid samples is shown in Figure 9 and Figure 10.

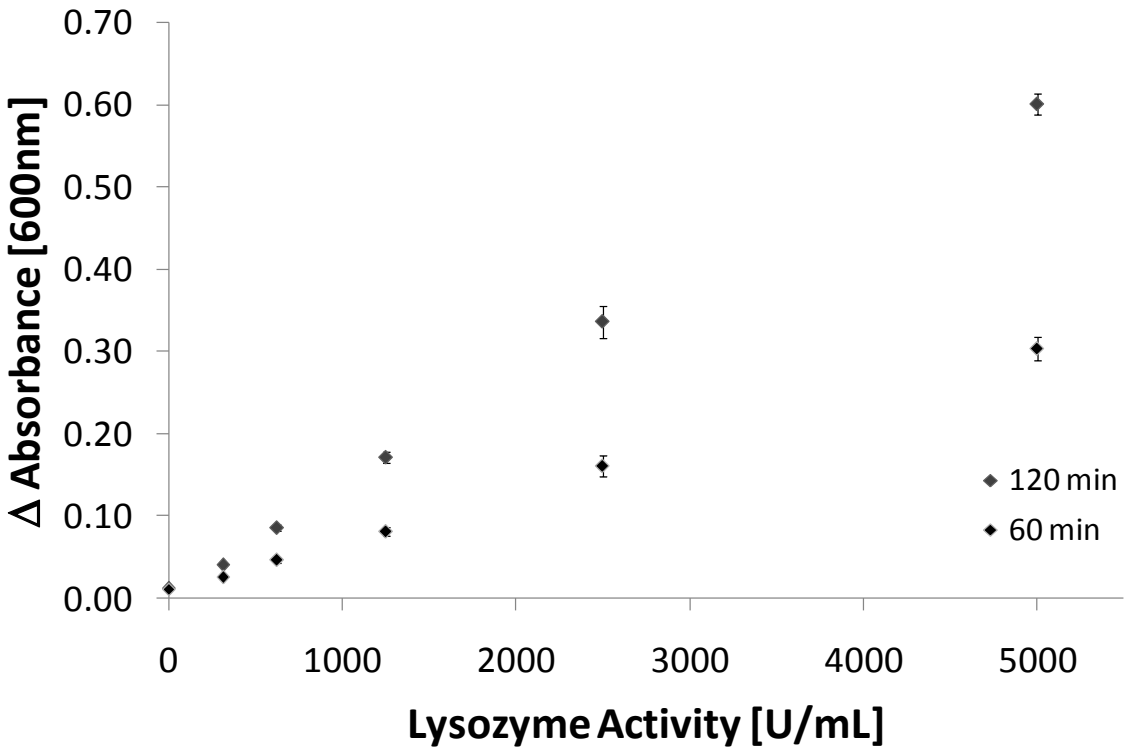

Figure 9: Correlation of colour release and lysozyme activity: Colour release from stained (8% (w/w)) agarose/peptidoglycan beads after incubation with different lysozyme activities ranging from 313 up to 5000 U/ml at 37°C for 60 and 120 minutes.

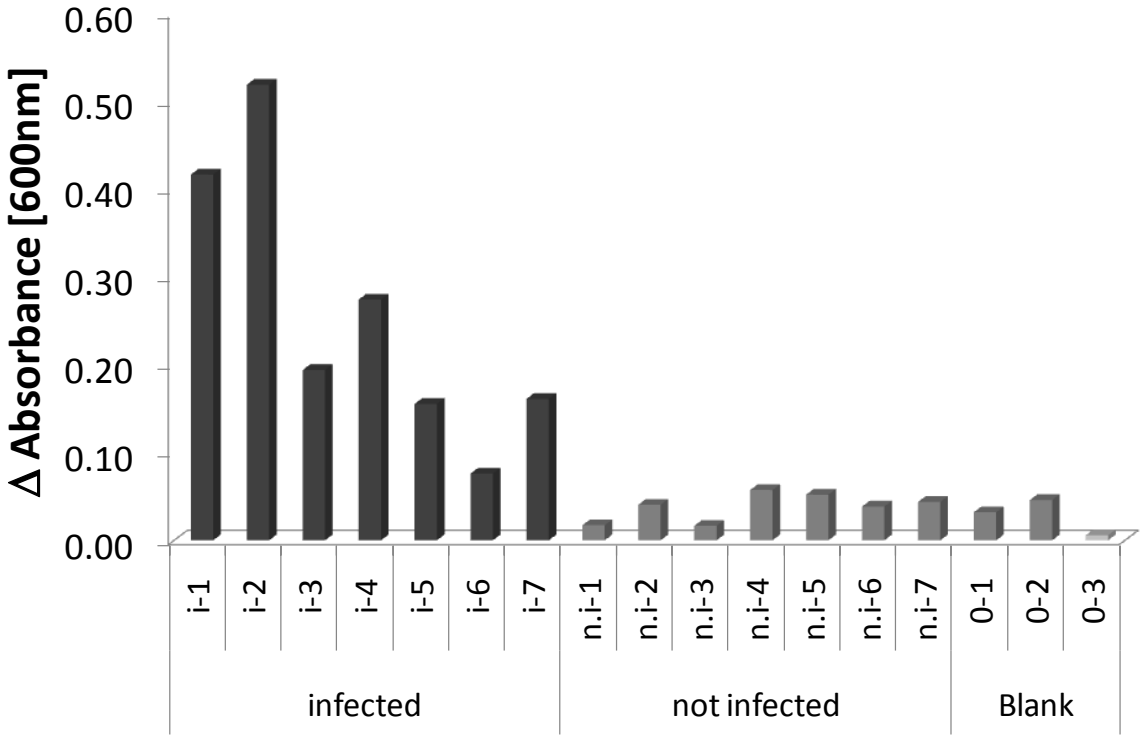

Figure 10: Distinction of infected and non infected wound fluid samples: Colour release from stained 8% (w/v) alginate/peptidoglycan beads after incubation with infected (i-1 – i.5) and non infected (n.i-1 – n.i-4) wound fluid samples for 2 hours at 37°C. Average absorbance changes of infected wound fluid samples are 7-fold higher than those of not infected wounds (P≤0.005).

Much higher absorbance changes were reached after incubation of beads for 2 hours, compared to stained peptidoglycan layers, as expected (Figure 9). Additionally, incubation for 60 minutes led to higher absorbance changes than incubation of device B for 120 minutes, which is probably due to a higher surface area. This behaviour was observed for commercial lysozyme as well as for wound fluids. Additionally, a broad linear range of the calibration curve could be achieved. With proceeding hydrolysis the absorbance further increased while a maximum for all enzyme activities was reached after 24 hours of incubation (data not shown). Concerning wound fluid samples, a clear difference between infected and non-infected wounds was reached (Figure 10). The difference between infected and not infected wound fluid samples was much more pronounced compared to stained peptidoglycan layers (factor 7 compared to factor 4). Apart from spectrophotometric quantification of released dye and dye-PG fragments, this colour change can be easily seen in the system. Therefore, 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, this behaviour was tested. Beads were stable for at least 3 months and showed no loss of reactivity.

Again the influence of alginolytic enzymes or so called alginases has to be considered. The following bacteria are known for producing alingate lyase: *Pseudomonas sp*., *Bacillus sp., Sphingomonas sp., Streptomyces sp.*, *Klebsiella sp*. [41-43]. The presence of the above organisms in wounds could enhance the response of the system due to secretion of alginases partially degrading peptidoglycan/ alginate beads.

3.3 Influence of Proteases on Digestion of Peptidoglycan

Wound fluids are a very complex matrix, comprising a great number of different biomolecules. Neutrophil-derived elastase and MMPs are the major proteases present in chronic wounds playing an important role in delayed wound healing. Elevated levels of different proteinases (elastase, cathepsin-G, gelatinases and collagenases) in infected and chronic wounds are well documented. While acute wounds show low levels of MMPs and elastase [15,30,32], chronic wounds contain excessive amounts of MMPs as shown by [33] and [30]. High levels of MMP-activity in chronic wounds decrease as soon as wounds heal.

Due to the presence of peptide bonds in cell wall peptidoglycan, the influence of elastase and collagenase potentially present in wound fluid samples was investigated. Indeed, in infected wound samples elastase activity of 1.82 U/ml and gelatinase activity of 10.5 U/ml was measured. As can be seen in Figure 11, incubation of stained peptidoglycan beads with lysozyme and gelatinase MMP-9 as well as elastase respectively, showed an improved hydrolysis of remazol-dyed peptidoglycan.

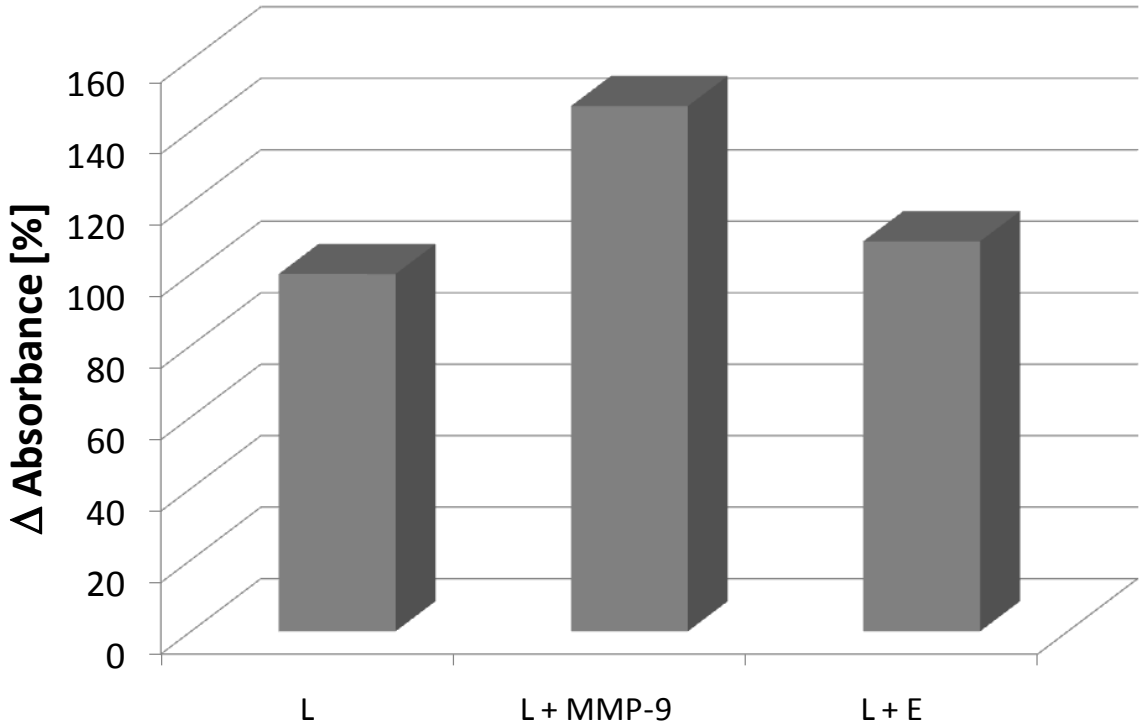

Figure 11: Influence of matrix metalloproteinase MMP-9 and elastase (E) on the release of dye fragments: Alginate/peptidoglycan beads were incubated with MMP-9 and elastase together with lysozyme (L) and compared with lysozyme L (250 U/ml) alone after an incubation-time of 28 hours at 37°C.

Interestingly, the addition of different proteases to lysozym*e* led to a synergistic increase in the release of dye and dye-PG fragments, while collagenase type IV showed no effect (data not shown). Especially the combination of MMP-9 and lysozyme enhanced the hydrolysis of peptidoglycan dramatically. In the presence of MMP-9 and lysozyme the absorbance measured at 600 nm increased 40% compared to lysozyme alone. Additional incubation with neutrophil elastase led to an increase of 10%. A possible explanation for this observed effect is the ability of proteases to hydrolyze peptide bonds of PG as is described well in literature for PG-peptidases [44]. Peptidoglycan hydrolysases are enzymes with a broad variety of enzyme specificities, including peptidases as well as glycosidases [45]. Glycosidases are able of cleaving glycosidic bonds between the sugar-units of PG whereas peptidases cleave peptide bonds of the pentapeptide.

3.4 Western Blot

To determine the nature of gelatinase activity potentially contributing to hydrolysis of PG, Western blot analysis was carried out. Enhanced levels of matrix-metalloproteinase MMP-9 has previously been reported in human blood originating from neutrophils upon addition of PG from *Staphylococcus* [46] as well as in chronic wound fluid [33]. Purified human gelatinase MMP-9 as a standard was detected between 66.2 kDa and 97.4 and was thus verified as the 92 kDa MMP-9 proenzyme (Figure 12), Additionally, MMP- 9 was detected in infected wound fluid samples. Thus, a contribution of MMP-9 to hydrolysis of PG is possible.

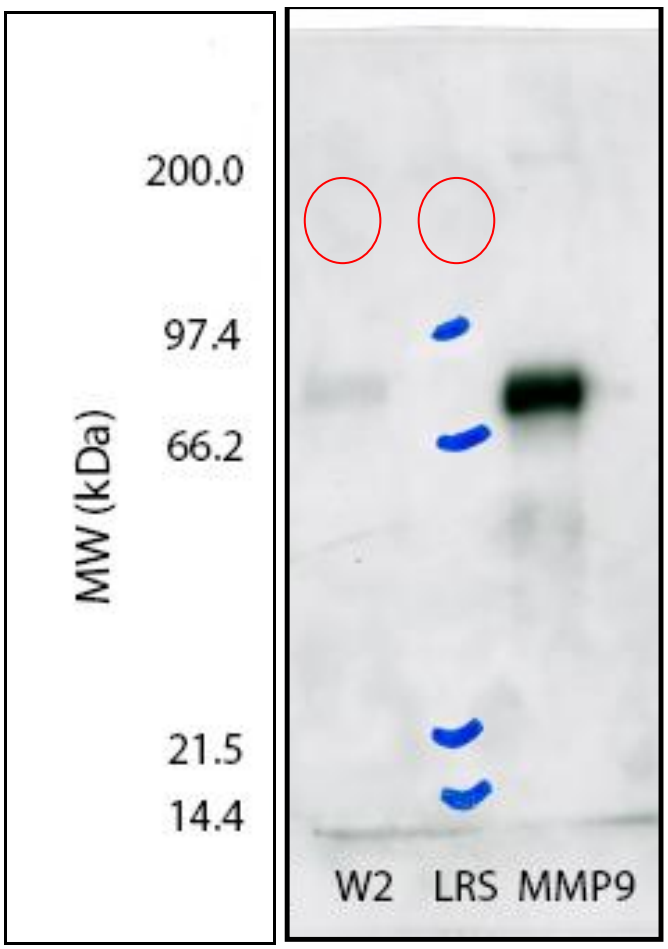

Figure 12: Detection of MMP-9 via western blot: W2: infected wound fluid sample, LRS: low range standard, MMP-9: purified MMP-9 proenzyme

4 Conclusion

Significantly elevated levels of lysozyme, elastase and gelatinase were detected in infected and non infected wound fluids. The sensitivity and specificity of lysozyme detection in wound fluids was enhanced by using RBB-labeled peptidoglycan/agarose layers or peptidoglycan/alginate beads. Using a double layer system, wound infection can be indicated by simple visual inspection. A synergistic effect in dye release has been found for matrix metalloproteinase MMP-9 and neutrophil elastase, which would enhance the response of the system in wound fluid. Such a diagnostic tool would allow early intervention with suitable treatment and could reduce clinical intervention and the use of antibiotics. The tool is therefore based on human enzymes which can be detected in fast assays in wound fluid samples and which were shown to be elevated in case of infection.

5 Acknowledgement

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6 References

1. P. Hayward, J. Hokanson, J. Heggers, J. Fiddes, C. Klingbeil, M. Goeger, and M. Robson, Fibroblast Growth-Factor Reverses the Bacterial Retardation of Wound Contraction, Am.J.Surg. 163 (1992) pp. 288-293.

2. B.D. Stenberg, L.G. Phillips, J.A. Hokanson, J.P. Heggers, and M.C. Robson, Effect of bFGF on the Inhibition of Contraction Caused by Bacteria, J.Surg.Res. 50 (1991) pp. 47-50.

3. R.F. Diegelmann and M.C. Evans, Wound Healing: An Overview of Acute, Fibrotic and Delayed Healing, Front.Biosci. 9 (2004) pp. 283-289.

4. M.J. Callam, C.V. Ruckley, D.R. Harper, and J.J. Dale, Chronic Ulceration of the Leg - Extent of the Problem and Provision of Care, Br.Med.J. 290 (1985) pp. 1855-1856.

5. O. Nelzen, D. Bergqvist, A. Lindhagen, and T. Hallbook, Chronic Leg Ulcers - An Underestimated Problem in Primary Health-Care Among Elderly Patients, J .Epidemiol.Community Health 45 (1991) pp. 184-187.

6. R.A.F. Clark, The Molecular and Cellular Biology of Wound Repair, Springer Us, 1996.

7. S.G. Jones, R. Edwards, and D.W. Thomas, Inflammation and Wound Healing: The Role of Bacteria in the Immuno-Regulation of Wound Healing, Int.J.Low.Extrem.Wounds 3 (2004) pp. 201-208.

8. M.C. Robson, Wound Infection: A Failure of Wound Healing Caused by an Imbalance of Bacteria, Surg.Clin.North.Am. 77 (1997) pp. 637-650.

9. W.K. Stadelmann, A.G. Digenis, and G.R. Tobin, Impediments to Wound Healing, Am.J.Surg. 176 (1998) pp. 39S-47S.

10. M. Collier, Recognition and Management of Wound Infections, World Wide Wounds (2004)

11. D. Krasner, Chronic Wound Care: A Clinical Source Book for Professionals, 1990.

12. J. Hart, Inflammation. 1: It`s Role in the Healing of Acute Wounds, J.Wound Care 11 (2002) pp. 205-209.

13. C.J. Sylvia, The Role of Neutrophil Apoptosis in Influencing Tissue Repair, J.Wound Care 12 (2003) pp. 13-6.

14. K. Tschaikowsky, R. Sittl, G.G. Braun, W. Hering, and E. Rugheimer, Increased Fmet-Leu-Phe Receptor Expression and Altered Superoxide Production of Neutrophil Granulocytes in Septic and Posttraumatic Patients, Clin.Investigator 72 (1993) pp. 18-25.

15. D.R. Yager and B.C. Nwomeh, The Proteolytic Environment of Chronic Wounds, Wound Repair Regen. 7 (1999) pp. 433-441.

16. E.F. Osserman, M. Klockars, J. Halper, and R.E. Fischel, Effects of Lysozyme on Normal and Transformed Mammalian-Cells, Nature 243 (1973) pp. 331-335.

17. M.R. Salton, Anatomy of Bacterial Surface, Bacteriol.Rev. 27 (1961) pp. 77-&.

18. D. Shugar, The Measurement of Lysozyme Activity and the Ultra-Violet Inactivation of Lysozyme, Biochim.Biophys.Acta 8 (1952) pp. 302-309.

19. Y. Ito, H. Yamada, and T. Imoto, Colourimetric Assay for Lysozyme using Micrococcus Luteus Labelled with a Blue Dye, Remazol brilliant Blue R, as a Substrate, Chem.Pharm.Bull. 40 (1992) pp. 1523-6.

20. M. Hardt, Y .Guo, G. Henderson, and R.A. Laine, Zymogram with Remazol Brilliant Blue-Labeled Micrococcus lysodeikticus cells for the Detection of Lysozymes: Example of a New Lysozyme Activity in Formosan Termite Defense Secretions, Anal.Biochem. 312 (2003) pp. 73-76.

21. E.F. Osserman and D.P. Lawlor, Serum and Urinary Lysozyme (Muramidase) in Monocytic and Monomyelocytic Leukemia, J.Exp.Med. 124 (1966) pp. 921-52.

22. R.S. Pascual, J.B.L.Gee, and S.C.Finch, Usefulness of Serum Lysozyme Measurement in Diagnosis and Evaluation of Sarcoidosis, New Engl. J. Med (1973) pp. 1074-1076.

23. H. Tomita, S. Sato, R. Matsuda, Y. Sugiura, H. Kawaguchi, T. Niimi, S. Yoshida, and M. Morishita, Serum Lysozyme Levels and Clinical Features of Sarcoidosis, Lung 177 (1999) pp. 161-7.

24. I. Torsteinsdottir, L. Hakansson, R. Hallgren, E. Gudbjornsson, N.G. Arvidson, and P. Venge, Serum Lysozyme: A Potential Marker of Monocyte/Macrophage Activity in Rheumatoid Arthritis, Rheumatology 38 (1999) pp. 1249-1254.

25. O. Selroos and M. Klockars, Serum Lysozyme in Sarcoidosis - Evaluation of its Usefulness in Determination of Disease Activity, Scand.J.Respir.Dis. 58 (1977) pp. 110-116.

26. C.W.G. Turton, E. Grundy, G. Firth, D. Mitchell, B.G. Rigden, and M. Turner-Warwick, Value of Measuring Serum Angiotensin I Converting Enzyme and Serum Lysozyme in the Management of Sarcoidosis, Thorax 34 (1979) pp. 57-62.

27. D.R. Yager, R.A. Kulina and L.A. Gilman, Wound Fluids: A Window into the Wound Environment?, Int.J.Low.Extrem.Wounds 6 (2007) pp. 262-72.

28. N.J. Trengove, S.R. Langton, and M.C. Stacey, Biochemical Analysis of Wound Fluid from Nonhealing and Healing Chronic Leg Ulcers, Wound Repair Regen. 4 (2002) pp. 234-239.

29. R. Mani, Science of Measurements in Wound Healing, Wound Repair Regen. 7 (1999) pp. 330-334.

30. N.J. Trengove, M.C. Stacey, S. Macauley, N. Bennett, J. Gibson, F. Burslem, G. Murphy, and G. Schultz, Analysis of the Acute and Chronic Wound Environments: The Role of Proteases and their Inhibitors, Wound Repair Regen. 7 (1999) pp. 442-452.

31. S. Pricelius, M. Murkovic, P. Souter, and G.M. Guebitz, Substrate Specificities of Glycosidases from Aspergillus Species: Pectinase Preparations on Elderberry Anthocyanins, J.Agric.Food Chem. 57 (2009) pp. 1006-1012.

32. B. Barrick, E.J. Campbell, and C.A. Owen, Leukocyte Proteinases in Wound Healing: Roles in Physiologic and Pathologic Processes, Wound Repair Regen. 7 (1999) pp. 410-422.

33. A.B. Wysocki, L. Staiano-Coico, and F. Grinnell, Wound Fluid from Chronic Leg Ulcers Contains Elevated Levels of Metalloproteinases MMP-2 and MMP-9, J.Investig. Dermatol. 101 (1993) pp. 64-68.

34. B. Haneberg, J. Glette, I. Talstad, S. Sornes, and C.O. Solberg, In vitro Release of Lysozyme from Monocytes and Granulocytes, J.Leukocyte Biol. 35 (1984) pp. 573-582.

35. C. Gronhagen-Riska and O. Selroos, Angiotensin Converting Enzyme. IV. Changes in Serum Activity and in Lysozyme Concentrations as Indicators of the Course of Untreated Sarcoidosis., Scand.J.Respir.Dis. 60 (1979) pp. 337-344.

36. J. Hart, Inflammation. 1: Its Role in the Healing of Acute Wounds, J.Wound Care 11 (2002) pp. 205-209.

37. A.L. Goodman, B. Kulasekara, A. Rietsch, D. Boyd, R.S. Smith, and S. Lory, A Signaling Network Reciprocally Regulates Genes Associated with Acute Infection and Chronic Persistence in Pseudomonas aeruginosa, Dev.Cell 7 (2004) pp. 745-754.

38. J. Carlsson and M. Malmqvist, Effects of Bacterial Agarase on Agarose Gel in Cell Culture, In Vitro Cell Dev-Pl 13 (1977) pp. 417-422.

39. M. Malmqvist, Degradation of Agarose Gels and Solutions by Bacterial Agarase, Carbohydr.Res. 62 (1978) pp. 337-348.

40. I. Hassairi, R. Ben Amar, M. Nonus, and B.B. Gupta, Production and Separation of alpha-Agarase from Altermonas agarlyticus Strain GJ1B, Bioresour.Technol. 79 (2001) pp. 47-51.

41. T. Sawabe, M. Ohtsuka, and Y. Ezura, Novel Alginate Lyases from Marine Bacterium Alteromonas sp. Strain H-4, Carbohydr.Research 304 (1997) pp. 69-76.

42. L. Cao, L. Xie, X. Xue, H. Tan, Y. Liu, and S. Zhou, Purification and Characterization of Alginate Lyase from Streptomyces Species Strain A5 Isolated from Banana Rhizosphere, J. Agr.Food Chem. 55 (2007) pp. 5113-5117.

43. T.Y. Wong, L.A. Preston, and N.L. Schiller, Alginate Lyase: Rreview of Major Sources and Enzyme Characteristics, Structure-Function Analysis, Biological Roles, and Applications, Annu.Rev.Microbiol. 54 (2000) pp. 289-340.

44. G.D. Shockman and J.V. Höltje, Chapter 7 Microbial Peptidoglycan (Murein) Hydrolases, In: New Comprehensive Biochemistry, 1994. pp. 131-166.

45. J.M. Ghuysen, D.J. Tipper, and J.L. Strominger, Enzymes that Degrade Bacterial Cell Walls, Method.Enzymol. 8 (1966) pp. 685-699.

46. Y.Y. Wang, A.E. Myhre, S.J. Pettersen, M.K. DaHNEw, S.J. Foster, C. Thiemermann, K. Björnland, A.O. Aasen, and J.E. Wang, Peptidoglycan of Staphyloccus Aureus Induces Enhanced Levels of Matrix Metalloproteinase-9 in Human Blood Originating From Neutrophils, Shock 24 (2005)

4

ANALYSIS OF MYELOPEROXIDASE IN WOUND FLUIDS AS MARKER FOR INFECTION

Neutrophilic polymorphonuclear leukocytes (PMNs) play a crucial role in the host defense against bacterial and fungal infections. They participate in the inflammatory response through the liberation of peptides and enzymes like myeloperoxidase (MPO). Therefore myeloperoxidase was chosen as marker enzyme for diagnosis of infection. Novel assay systems for myeloperoxidase activity in wound fluid for detection of infection were investigated. Substrate specificities and reaction pathways of MPO were investigated for crystal violet, leuko crystal violet, fast blue RR (4-benzoylamino-2,5-dimethoxybenzenediazonium chloride) and various systematically substituted model substrates based on 2,7-dihydroxy-1-(4-hydroxyphenylazo)naphtalene-3,6-disulfonic acid. In addition, fast blue RR was covalently bound to siloxanes allowing immobilisation of the substrate, while cellobiosedehydrogenase was integrated for generation of hydrogen peroxide required by MPO. Elevated levels of myeloperoxidase were found in infected wounds compared to non infected wounds (92.2 $\pm$ 45.0 U/ml) versus 1.7$\pm$ 4.4 U/ml). Incubation of the novel MPO substrates with infected wound fluid samples for 10 minutes resulted in a clear colour change in case of elevated myeloperoxidase levels thus allowing early diagnosis of infection.

Annals of Clinical Biochemistry, submitted

1 Introduction

Myeloperoxidase (MPO) is a heme-containing protein, consisting of two dimers connected by a disulfide bond. Each dimer comprises a β-heavy subunit (59 kDa) and a light α–subunit (14 kDa) [1,2]. MPO is stored in the azurophilic granules of neutrophils and in the lysosomes of monocytes [3] and is not released until activation of neutrophils and degranulation [4]. MPO catalyzes the production of hypochlorous acid by converting hydrogen peroxide and chloride-ions [5]. Hypochlorous acid is the most powerful bactericidal oxidant produced by neutrophils [6].

Neutrophils are normally the first cells at site of inflammation or injury [7,8]. The infiltration of neutrophils to injured tissue is a characteristic tool of the host defense and inflammation [9], as neutrophilic polymorphonuclear leukocytes (PMNs) play a crucial role in the host defense against bacterial and fungal infections. They participate in the inflammatory response through the liberation of peptides and enzymes like MPO [10]. Leukocyte activation prompts the release of MPO and the formation of oxidants important in host defence [11]. Therefore elevated levels of MPO are a potentially useful marker for quantification of tissue PMN accumulation and is correlated with the MPO release by neutrophils due to inflammatory processes as already shown [12,13].

MPO measured in blood is now considered as a marker of neutrophil activation and degranulation [14,15] and are measured by enzyme immunoassays, which quantify the concentration of the enzyme without measuring its true enzymatic activity. Different studies have shown the importance of myeloperoxidase for cardiovascular diseases like coronary artery disease (CAD), acute coronary syndrome (ACS) and acute myocardial infarction [10,16-17]. As patients with coronary artery disease have elevated Myeloperoxidase levels, MPO-levels can be used for predicting future cardiovascular events after risk factors and C-reactive protein are controlled for [11;14;18;19].

Additionally, levels of myeloperoxidase are significantly higher in the tissues of pressure ulcers compared with those of acute healing wounds [20]. It is widely assumed that wound fluid has the potential to provide important biochemical information which can be used as a diagnostic indicator providing information for determining the overall status of a wound and for monitoring the progression of wound healing. Furthermore, wound fluid can be used to

monitor specific parameters that have been identified as prognostic indicators or are targets of a particular therapy [21-23].

As there is a strong need for a fast prognostic aid which would assist in predicting clinical infection of a wound prior to obvious clinical symptoms, we have determined myeloperoxidase levels in different types of chronic wounds. Based on these data we have investigated enzyme responsive devices using novel substrates for simple detection of this enzyme and, as a consequence, the development of a rapid and simple diagnostic system for detection of wound infection.

2 Materials and Methods

2.1 Sample Collection and Preparation

Fluid from 8 post-operative wounds, three decubitus ulcer wounds, three malum perforens wounds and three ulcus cruris wounds were collected and analyzed by biochemical techniques. Exudates were collected directly from exudating wound surface by using needles or a spoon before the daily treatment. Four samples were collected during V.A.C therapy in special canisters without starch. Additionally, fluid from ten blisters was collected. The samples were centrifuged three times at 420 g for 20 minutes to remove cells and tissue material and stored at -20°C until analyzed. 8 wounds out of the 17 wounds were described as clinically infected by the attending doctors. The patients studied so far were grouped and labeled with the following abbreviations (followed by a number): i = infected wound; n. i = non infected wound; b = blisters). Permission to collect wound fluid was obtained from the Ethics Committee of the Medical University Graz.

2.2 Determination of Hemoglobin Concentration

The hemoglobin concentration in the samples was determined spectrophotometrically at 405 nm using hemoglobin as a standard (0.5 µg/ml to 2.5mg/ml, Hemoglobin human, Sigma-Aldrich). In this concentration range eventual oxidation of MPO substrates by hemoglobin was recorded and substracted from MPO activity data given below.

2.3 Determination of Myeloperoxidase Activity

Myeloperoxidase activity was determined according to the method described by Desser *et al*., [24]. Briefly, 10 µl wound fluid or enzyme solution was mixed with 290 µl substrate solution, containing 100 mM Guaiacol (Sigma Aldrich) and 0.017% (w/w) H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0). Enzyme standards containing 0.16 U/ml up to 10.0 U/ml myeloperoxidase (Sigma Aldrich) were prepared for calibration. Formation of tetra-guaiacol was continuously measured for 15 minutes (every 20 seconds) at 470 nm. The change of optical density per minute was calculated from the initial rate. Additionally, the increase in absorbance after 100 seconds was used for calculation of MPO activity.

2.4 Myeloperoxidase Activity on Different Substrates

TMB and ABTS

As previously described by Bozeman *et al.* [25] and Venezie *et al.* [26], TMB and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonicacid) were used for assaying MPO activity. Therefore, 3346 µl of succinate buffer (pH 5.4) comprising 0.3 M sucrose were used, 105 µl 1% (v/v) H_2O_2 and 49 µl of the substrates (10 mM ABTS or 25 mM TMB) were added. TMB was firstly dissolved in N,N-methylformamide, while ABTS was dissolved in water. 5 µl of infected and non-infected wound fluid samples were incubated with 100 µl of the substrate solutions for detection myeloperoxidase activity. Visual inspection of the samples after 30 minutes of incubation at room temperature was carried out. Additionally, absorbance was measured at 410 nm (ABTS) and 650 nm (TMB).

Fast Blue RR (4-Benzoylamino-2,5-dimethoxybenzenediazonium chloride)

Fast Blue RR was tested for its ability to be used as MPO substrate. The reaction mixture contained 5 ml sodium acetate buffer (200 mM, pH 4.0) and 500 µl Fast Blue RR (0.5 mM in 0.06 M HCl). 8 µl H_2O_2 were added to 2 ml of reaction solution. For Initiation of the color-reaction, 5 µl of sample (MPO or wound fluid samples) was mixed with 295 µl of the reaction mix. Absorbance was measured at 305 nm.

Leuco Crystal Violet (LCV)

The leuco crystal violet method [27] is based on the oxidation of 4,4´,4´´-methylidynetris (N,N-dimethylaniline, LCV) in the presence of H_2O_2 and peroxidase, forming a crystal violet ion, CV^+, which has an absorbance maximum at a wavelength of 590 nm. CV^+ is stable for several days [28]. Oxidation of leuco-crystal violet to crystal violet was carried out using an assay modified from the previously published method from *Pricelius et al.* [29]. The reaction mixture contained 5 ml sodium acetate buffer (200 mM, pH 4.0) and 500 µl leuco-crystal violet (1 mM in 0.06 M HCl). To 2 ml of reaction solution, 8 µl H_2O_2 were added. To initiate the color-reaction, 25 µl MPO or 5 µl wound fluid samples were mixed with 275 µl or 295 µl of the reaction mix. Absorbance was measured at 590 nm.

Crystal Violet (Gentian Violet)

An aqueous dye solution (1% mg/ml) was prepared. To assure a total dissolving of the dye, the solution was incubated for 15 minutes with ultrasonic radiation. Then, 70 µl of this solution were diluted in 10 ml potassium phosphate buffer (100 mM, pH 7.0). To 2 ml of this reaction

mix, 8 µl H_2O_2 (1% v/v) were added. To measure the enzyme conversion, 100 µl dye-solution, 25 µl MPO (150 U/ml) were used. Additionally, enzyme conversion was carried out using infected and non-infected wound fluid samples. Absorbance was measured from 300-800 nm with the plate reader.

Analysis of Crystal Violet Degradation Products with HPLC-MS

The conversion of crystal violet by myeloperoxidase was measured with HPLC/MS. The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with an electrospray ionisation operated in positive and negative ion mode and the electrospray voltage was set to 3500 V. Dry gas flow was set to 10 l min^{-1} with a temperature of 350°C, nebulizer set to 50 psi. As eluent a mixture of acetonitrile/formic acid/water (80/8/12 %v/v) was used with a flow rate of 0.8 ml/min for 30 minutes. Scans were carried out from m/z 100-1000.

For analysis of the degradation products, 70 µl of an aqueous crystal violet solution (1% (w/v)) was diluted in 10 ml ammonium acetate buffer (pH 6.8). Buffer, myeloperoxidase (150 U/ml) and H_2O_2 (1% (w/v)) were incubated together with the dye-solution. Incubation was carried out at 37°C with a relative humidity of 70% for 24 hours. After 0, 1, 6 and 24 hours, reactions were stopped by cooling down the solutions to -72°C. After cooling, the solutions were transferred into glass vials which were placed in the auto-sampler of the LC system for automation of the MS injection.

Systematically Substituted Azo - Dyes for Detection of MPO-Activity

18 structurally related azo-dyes with 2,7-dihydroxy-1-phenylazonaphtalene-3,6-disulfonic acid as basic structure, were synthesized as previously described [30] (Figure 13). These dyes have been systematically substituted with a representative set of functional groups at the benzene ring in ortho-, meta- or para-position to the chromophoric centre to test the steric influence of different substition patterns.

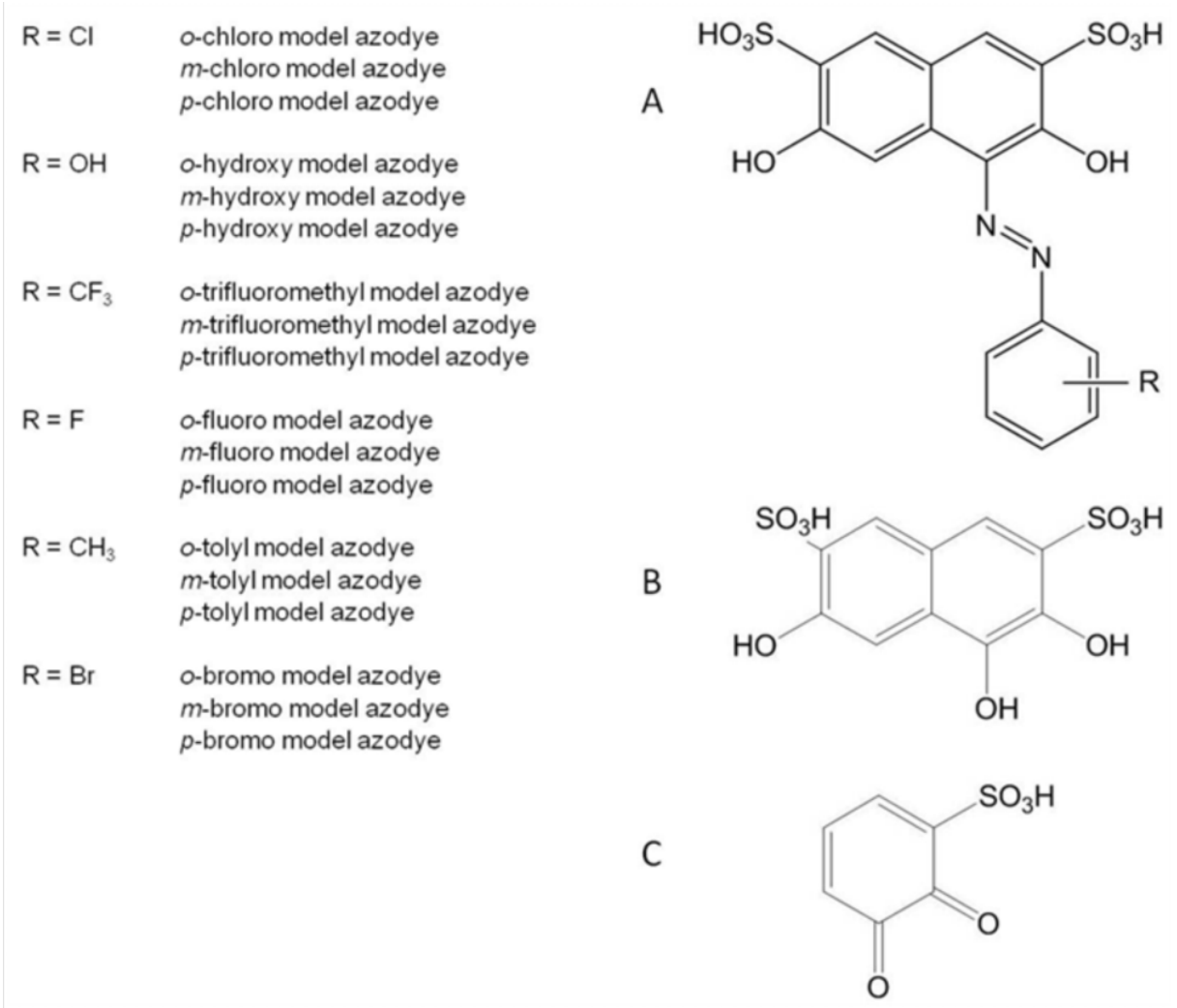


Figure 13: Molecule framework (2,7-dihydroxy-1-phenylazonaphtalene-3,6-disulfonic acid) of synthesized azodyes (A) and possible oxidation products (B, C).

The reaction mixture contained 5 ml sodium acetate buffer (200 mM, pH 4.0) and 500 µl model dye (5.0, 1.0 and 0.5 mM in 0.06 M HCl). To 2 ml of reaction solution, 8 µl H_2O_2 were added. To initiate the color-reaction, 5 µl of MPO or wound fluid samples were mixed with 295 µl of the reaction mix. Absorbance was measured between 400 and 800 nm.

Analysis of Model Dye Degradation Products of with HPLC-MS

For HPLC separation of the oxidation products of these substrates, a waters C8 column (3.5 µm) of dimensions 2.1 mm i. d. x 50 mm was used with an eluent consisting of a mixture of 90 parts acetic acid (0.1 %) and 10 parts methanol (95 %) containing 0.1 % acetic acid. The MS

spectra were recorded with an Agilent Ion Trap SL (Palo Alto, CA, USA) electrospray voltage was set to 4000 V. Dry gas flow was set to 8 L min^{-1} with a temperature of 350°C, nebulizer set to 40 psi. Scans were carried out from m/z 50-500.

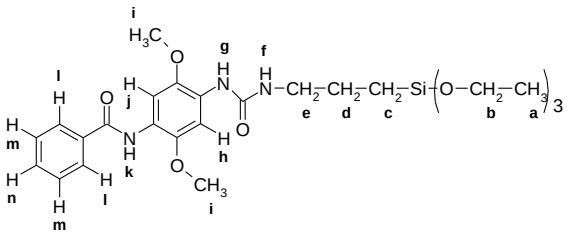
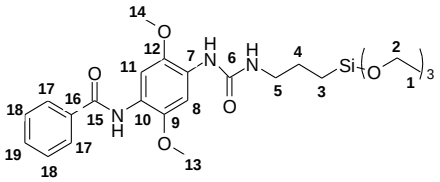
2.5 Immobilisation of Myeloperoxidase Substrates

Coupling of Myeloperoxidase Substrates to Siloxanes

Crystal violet, 2,7-dihydroxy-1-(4-hydroxyphenylazo)naphtalene-3,6-disulfonic acid and Fast Blue RR (N-(4-amino-2,5-dimethoxyphenyl) benzamide) were assessed for coupling to alkoxysilanes for immobilization. 2.67 g of Fast Blue RR were dissolved in 75 ml of abs. THF and were placed in a 100 ml three-necks round bottom flask. With a syringe, 2.51 g of 3-(triethoxysilyl)propyl isocyanate (Sigma-Aldrich) were slowly added. The reaction mixture was then heated to reflux and maintained at this temperature for 29 hours. According to thin layer chromatography (TLC) control, the solvent was removed on a rotary evaporator and the residue was suspended in ethyl acetate. The organic phase was extracted three times with 0.01 M hydrochloric acid and the combined aqueous phases were solvent extracted with ethyl acetate. The crude product (5.10 g) was re-crystallized from petrol ether : ethyl acetate (1:1), resulting in 3.15 g (65%) brown acicular crystals.

The molecule structure of the synthesized substrate was proven by ^{1}H and ^{13}C NMR as is shown for Fast Blue in Table 6. In addition, successful coupling was verified with FT-IR measurements (Bruker Tensor 35; ATR, single reflection diamond cell; 32 Scans), by observing significant changes within the FT-IR spectra of Fast Blue RR and the purified coupling product. The amine group signal of Fast Blue RR (ν_s 3434 cm^{-1}, ν_{as} 3355 cm^{-1}) disappeared in coupling product and a broad amide signal appeared at 3303 cm^{-1}. In addition, a new IR band was measured at 1075 cm^{-1}, representing the signals of the introduced alkoxysilane substituent (ν (SiO)+ ν (SiC) of R-$(CH_2)_2$-$\underline{CH_2}$-Si-O-CH2-R´).

Table 6: Chemical structure and observed ^{1}H and ^{13}C-NMR signals of the alkoxysilane Fast Blue RR coupling product.

Structure	NMR-Spectra
	^{1}H-NMR (300 MHz, in d_6-DMSO, 100mg/ml): δ= 0.54 (t, J= 5.58 Hz, 2 H_c), 1.05 (t, J= 6.97 Hz, 3 H_a), 1.48 (qin, J= 7.74 Hz, 2 H_d), 3.05 (quart, J= 6.97 Hz, 2 H_e), 3.44 (quart., J= 6.97 Hz, 2 H_b), 3.73 (s, 6 H_i) 6.91 (d, J= n.a., 1H_f), 7.44 (s, 1H_g) 7.51(m, 2 H_m+1 H_n), 7.95(d, J= 6.66 Hz, 2H_l), 7.96 (s, 1H_h), 8.05(s, 1H_j), 9.34(s, 1H_k)
	^{13}C-NMR (75.5 MHz, in d_6-DMSO, 100mg/ml): δ= 7.6 (C_3), 18.8 (C_1), 23.8 (C_4), 42 (C_5), 56.3 (C_{13}), 57.9 (C_2), 102.8 (C_8), 108.6 (C_{11}), 119.3 (C_{10}), 119.4 (C_7), 127.6 (C_{17}), 128.6 (C_{18}), 131.7 (C_{19}), 134.8 (C_{16}), 140.6 (C_{12}), 145.7 (C_9), 155.4 (C_6), 165 (C_{15})

Coating of TLC Plates with Functionalised Siloxanes

The coating of silica thin layer chromatography plates (TLC plates) was carried out as follows. Squares were cut out with a size of 2 cm x 2 cm, sprinkled with 100 µl of the substrate, dried for one hour at room temperature, and were then placed for 24 hours at ~ 105 ° C in the oven.

Biotransformation of the Immobilised Substrates using Myeloperoxidase

The TLC plates with immobilized substrate were incubated in 100 µl potassium phosphate buffer (100 mM; pH 7.1), 1.5 µl hydrogen peroxide solution (1% (v/v)) and 50 µl MPO (150 U/ml) or 150 µl buffer and 1.5 µl of hydrogen peroxide (1% (v/v)) for the blank. Additionally, oxidation of immobilized dyes was carried out with infected and non-infected wound fluid samples, using 100 µl potassium phosphate buffer (100 mM; pH 7.1) and 50 µl wound fluid. The conversion of immobilised substrates was quantified with a spectrophotometer (ColorLite sph850) based on the colour difference between the reference and the sample giving ΔE values according to the CIELab concept.

2.6 In-Situ Generation of Hydrogen Peroxide with Cellobiose Dehydrogenase (CDH)

Cellobiose dehydrogenase (EC 1.1.99.15) was used for generation of hydrogen peroxide required by myeloperoxidase. H_2O_2 production was based on a modified method published by Pricelius *et al.,* [29]. Essentially, the reaction mixtures as described above contained

cellobiose (5 mM), desferrioxamine mesylate (1 mM) and 1.5 U/ml of CDH instead of hydrogen peroxide.

3 Results

3.1 Myeloperoxidase Activity in Wound Fluid

In this study, significantly higher myeloperoxidase activity was detected in infected wounds when compared to non infected wounds based on the oxidation of guaiacol [24] and using commercial MPO as standard. 9 wounds (4 post operative and 4 chronic wounds) were classified as not infected by the attending medical doctors. 4 post operative and 4 chronic wounds were described as clinically infected (Table 7). Additionally, fluids from 10 non-infected blisters served as negative controls. MPO activity in infected wound fluids was significantly ($P \leq 0.005$) higher (92.2 ± 49.0 U/ml) than in non- infected wounds and blisters (1.7 $\pm$ 3.4 U/ml), which was in good agreement with the clinical description. There was no significant difference between blisters and non infected post operative or chronic wounds regarding MPO levels. Similarly, there were no significant differences of MPO levels between infected chronic wounds and infected post operative wounds.

Table 7: MPO activity in wound fluid samples: There is a significant difference between MPO activity of infected and non-infected wounds (P<0.005).

Clinical description of the wound	Infected	Non-infected
Blisters	0	10
Post-operative wounds	4	4
Decubitus wounds	4	5
MPO activity [U/ml]	92.2 ± 49.0	1.7 ±3.4

3.2 Novel MPO Substrates for Detection of Infection

Guaiacol is widely used for spectrophotometric quantification of MPO activity in general and was successfully applied for detection of MPO activity in wound fluids. In this study, commercial MPO as well as wound fluid from infected and non infected wounds were used for developing a reliable and yet fast diagnostic system for wound infection. ABTS (10 mM,

measured at 410 nm), TMB (25 mM, measured at 650 nm), LCV (1 mM, measured at 590 nm) and FBRR (0.5 mM, measured at 305 nm) were incubated with infected and non infected wound fluid samples. Additionally, fluids of five blisters served as negative controls (Figure 13). Additionally, hemoglobin content of the wound fluid samples was determined and MPO activity corrected for the contribution of hemoglobin to substrate oxidation.

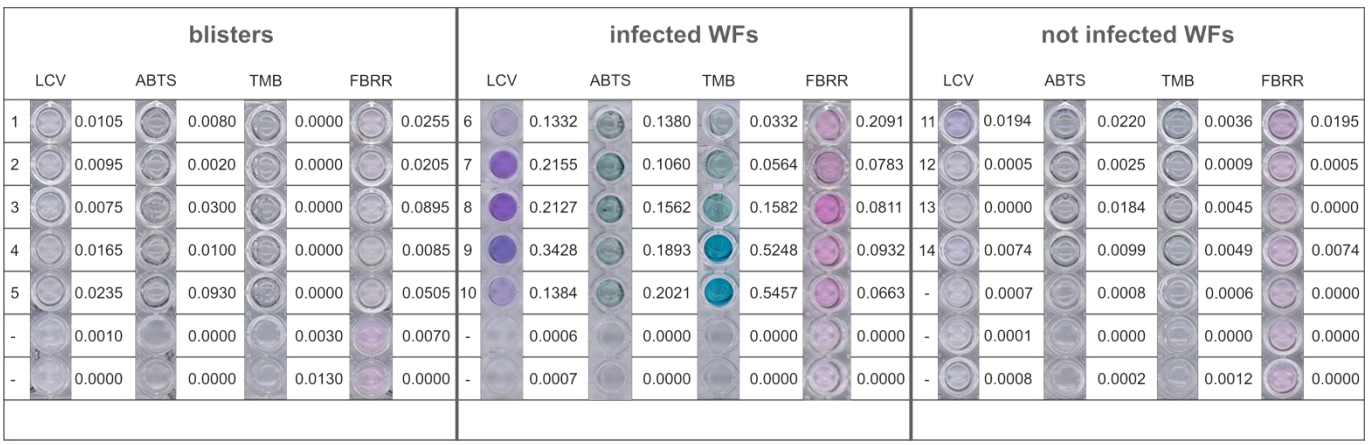


Figure 14: MPO activity in wound fluids using ABTS (10 mM, measured at 410 nm), TMB (25 mM, measured at 650 nm), LCV (1 mM, measured at 590 nm) and FBRR (0.5 mM, measured at 305 nm). Samples were grouped as follows: blisters (1-5), infected (6-10) wound fluid samples, not infected wound fluid samples (11-15) and controls (-) containing substrate only. Contribution of hemoglobin to substrate conversion was subtracted. There is a significant difference between infected wound fluids and blisters (P≤0.005).

Using TMB and ABTS as MPO substrates, only in case of infection a clear color change can be observed after an incubation time of 30 minutes. These results are according very well with the clinical description and with the MPO activity determined with guaiacol as a substrate. Concerning the different substrates, Thereby TMB was more sensitive than ABTS while the substrate solution containing hydrogen peroxide was stable for at least 12 weeks at 4°C.

Incubation of infected wound fluid samples with Fast Blue RR resulted in a clear pink coloration of the sample after 30 minutes of incubation, while there was only a very light coloration in the two other groups. It is thus shown for the first time that Fast Blue RR can serve as a suitable and sensitive substrate for determining MPO activity in wound fluid.

Incubation of infected wound fluid samples with LCV resulted in formation of crystal violet with a dark violet color after 10 minutes of incubation. Non infected wound fluid samples and blisters showed only a very light coloration. Also with this substrates, infected wound fluid samples can clearly be distinguished from blisters and non-infected wound fluids based on elevated MPO activity. Further oxidation or of crystal violet with MPO resulted in an absorbance shift from 580 nm to 560 nm after one hour of incubation (150 U/ml) at 25°C (Figure 15). This wavelength-shift resulted in discoloration of the sample. H_2O_2 and buffer did not show any bleaching of the violet color of crystal violet.

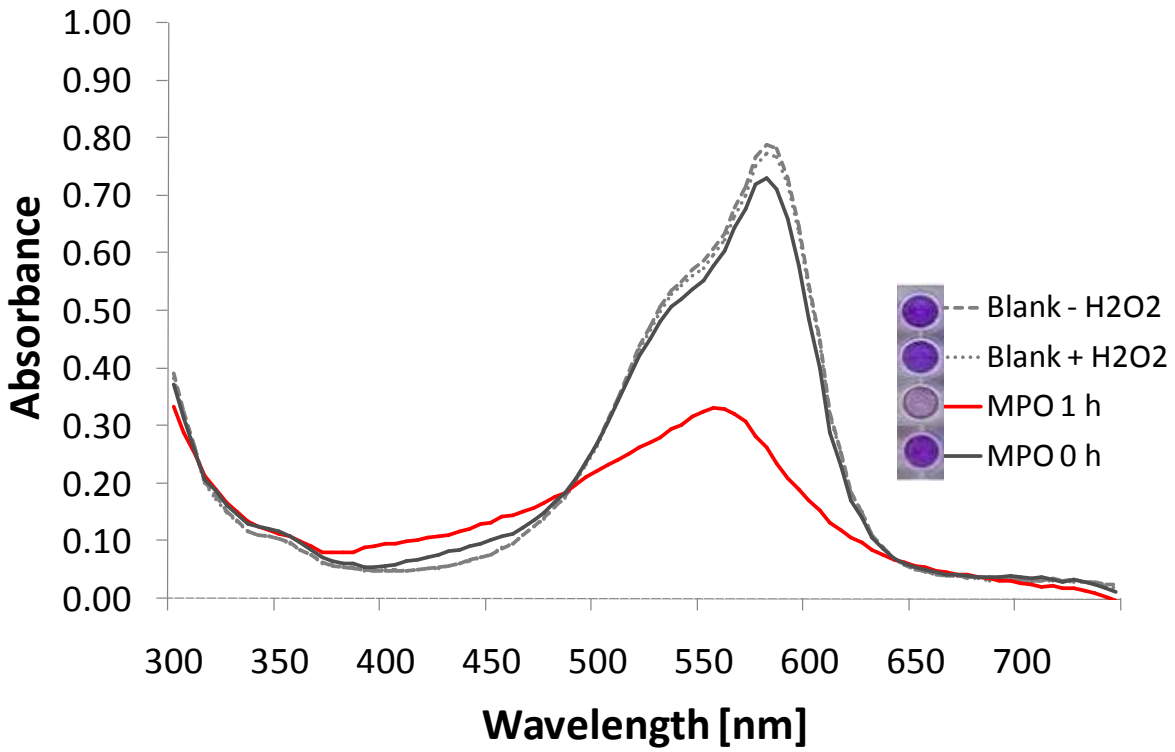


Figure 15: Measurement of MPO activity using crystal violet: Absorbance scan and colour change (inlet picture) of crystal violet incubated with MPO for 0 hours (MPO 0 h) and 1 hour (MPO 1 h)

The MPO catalyzed oxidation of crystal violet by myeloperoxidase was investigated by using HPLC-MS. The characteristic peak for crystal violet 372.53 (m/z) completely disappeared after incubation with MPO for 24 hours. After incubation for 6 hours, a small peak at mass/charge ratio of 332.4 appeared. This indicates a demethylated and deaminated molecule. After 24 hours of incubation, this was no longer evident suggesting a further degradation. The complete MPO initiated breakdown of the molecule thus involved

successive cleavage of all methyl groups followed by deamination (Figure 16). Finally, the backbone was cleaved. However, due to the relatively high MPO activity of (150 U/ml) required for this reaction, no significant color change was seen after incubation with wound samples for 30 minutes (like with the other substrates).

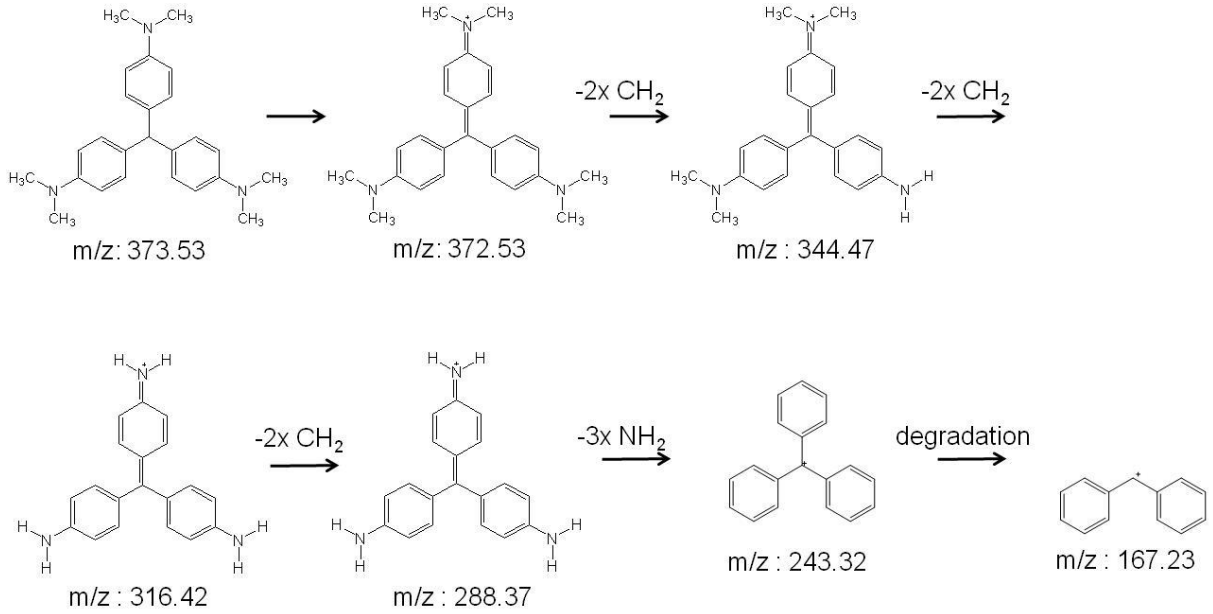


Figure 16: Hypothetic reaction pathway of MPO catalyzed oxidation of crystal violet and its leuco form as determined with mass spectrometry

To further study the reaction mechanism of MPO, oxidation of systematically substitutes model substrates based on 2,7-dihydroxy-1-(4-hydroxyphenylazo)naphtalene-3,6-disulfonic acid. Among them those derivatives carrying hydroxyl groups in ortho and para positions to the azo bond showed highest conversion after incubation with MPO (i.e. decolorization) (Figure 17). On the other hand, the meta-substituted analog, the methyl derivatives and the un-substituted derivative only showed marginal conversion.

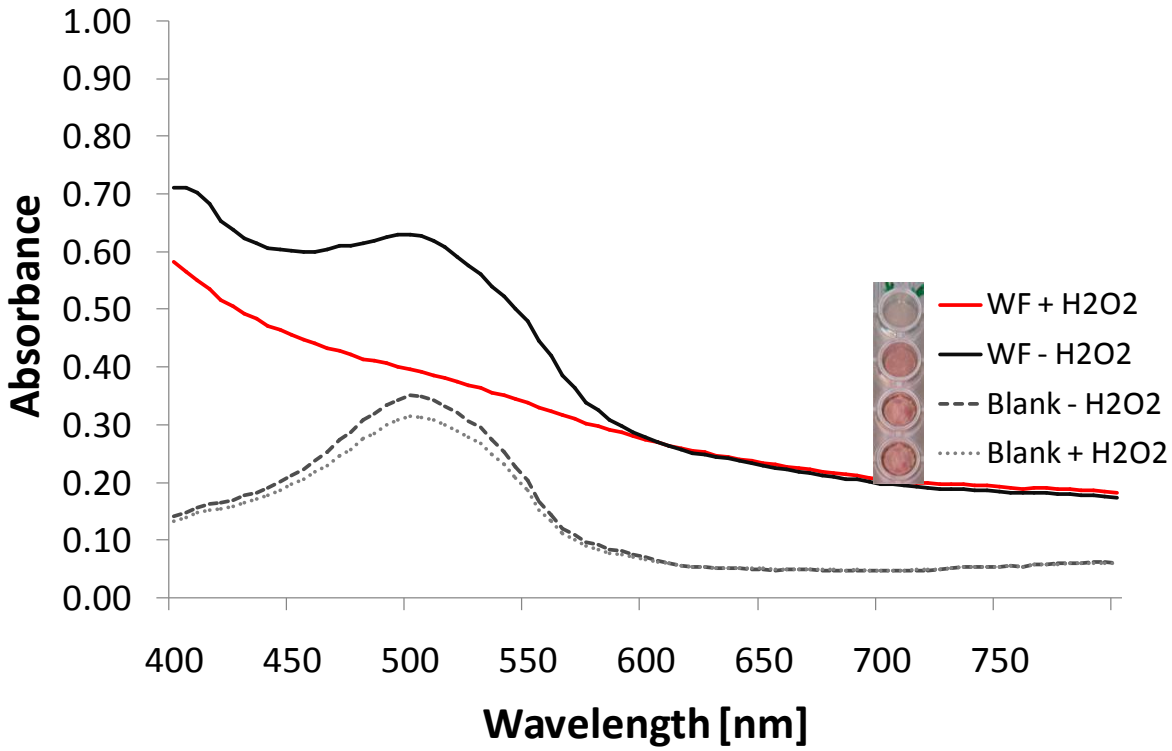


Figure 17: Measurement of MPO activitiy: Absorbance scan and colour change (inlet picture) of 2,7-dihydroxy-1-(4-hydroxyphenylazo)naphtalene-3,6-disulfonic acid incubated with wound fluid (WF). MPO activity is shown as a decrease of absorbance at a wavelength of 490 nm.

Upon chromatographic and mass spectral analysis, all reactive substrates showed similar fragmentation patterns indicating lower molecular weight compounds after cleavage of the chromophoric bond. In all these cases as a reaction product the 3,4,6-trihydroxynaphtalin-2,7-disulfonic acid (m/z 335; Figure 13B) was found at a retention time of 8.5 minutes. This compound, as the common structural feature among others gave two major mass fragments with m/z 318 (dehydroxylated fragment) and m/z 188. Further fragmentation of this latter compound in a secondary mass spectrum lead to a predominantly present fragment identified as 2, 3-dioxobenzene sulfonic acid (Figure 13C).

Comparing the studied MPO substrates regarding their potential in the assessment of wound fluid enzyme levels, Fast Blue RR, 2,7-dihydroxy-1-(4-hydroxyphenylazo)-naphtalene-3,6-disulfonic acid and Leuko crystal violet seemed to be most suitable (Table 8).

Table 8: Summary of results obtained with different MPO substrates and commercial MPO and wound fluid

Substrates	Activity MPO	Activity Wound Fluids
Guaiacol	+++	Colour not stable
ABTS	++	+
TMB	++	++
Leuko crystal violet	+++	+++
Crystal violet	+	slow reaction
Fast Blue RR	+++	+++
2,7-dihydroxy-1-(4-hydroxyphenylazo)naphtalene-3,6-disulfonic acid	+++	+++
2,7-dihydroxy-1-(2-hydroxyphenylazo)naphtalene-3,6-disulfonic acid	+	+

3.3 Covalent Immobilisation of Myeloperoxidase Substrates

Since immobilization of MPO substrates on solid materials would facilitate application of the diagnostic system (e.g. test strips, integration in bandages), coupling of the tested MPO substrates to carriers (i.e. siloxanes) was assessed. However, coupling of all investigated substrates except fast blue RR failed, since no stable products were obtained and/or they did not react with MPO (data not shown). Fast Blue RR was successfully coupled to alkoxysilane isocyanates resulting in a stable urea compound as indicated by ^{1}H and ^{13}C NMR analysis (Figure 18). The resulting functionalized siloxanes were polymerized on silica gel plates and glass slides as carriers. MPO was indeed able to oxidize the immobilized substrate after one day of incubation, resulting in a color change towards dark red. This color change corresponded to a ΔE of 17.95. Additionally, incubation with infected and non infected wound fluid samples was performed. Again, a color change was determined for infected wound fluid samples, while there was no color change in non infected wound samples (data not shown).

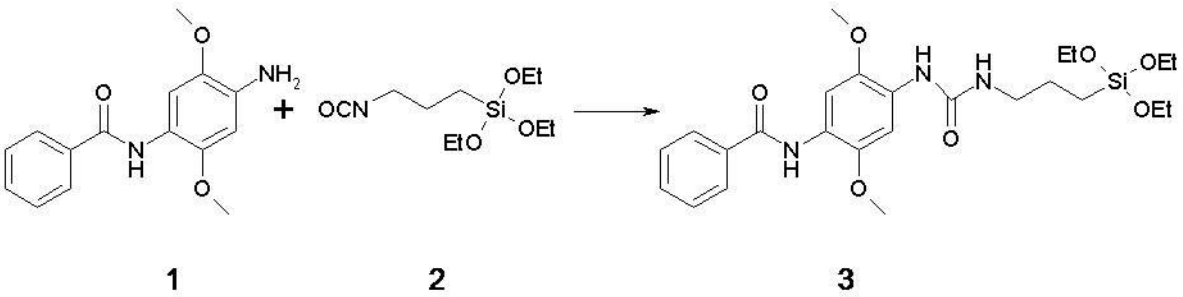


Figure 18: Fast Blue RR (1) coupled to 3-(triethoxysilyl)propyl isocyanate (2) resulting in the stable urea product (3).

3.4 Enzymatic In-Situ Production of Hydrogen Peroxide

Leuko Crystal Violett was incubated either with commercial MPO or wound fluids in the presence of cellobiosedehydrogenase and cellobiose instead of hydrogen peroxide. Using hydrogen peroxide, an absorbance of 0.56 was measured at 590 nm after 60 minutes of incubation with MPO. Replacement of hydrogen peroxide by the CDH gave a similar value of 0.708. Likewise, incubation of wound fluids with MPO in the presence of CDH/cellobiose and hydrogen peroxides gave comparable absorbance changes.

4 Discussion

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. It is widely assumed that wound fluid has the potential to provide information which can be used as a diagnostic indicator for the overall status of a wound or as prognostic indicators [21-23].

A significant difference in myeloperoxidase (MPO) levels between infected wounds and non-infected wounds or blisters was found in this study. This was according well with the clinical description of the attending medical doctors. There was no significant difference between blisters and non infected post operative or chronic wounds regarding myeloperoxidase levels. Thus, this observed difference between infected and non infected wounds probably reflects the number of monocytes/macrophages and neutrophils in the tissue. As neutrophils are normally the first cells at site of inflammation or injury [7,31], the infiltration of neutrophils to injured tissue is a characteristic tool of the host defence and inflammation [9]. In fact, neutrophils and monocytes are predominant cell markers of wound infection [32,33].

Therefore, elevated levels of MPO released in case of activation of neutrophils can be correlated with the MPO release of neutrophils due to inflammatory processes [4]. Increased levels of MPO are thus a potentially marker for inflammatory processes due to infiltration into the tissues [10,34,35].

In order to develop a fast diagnostic tool indicating the presence of neutrophils before manifestation of infection, a screening for new suitable substrates for MPO detection in wound fluids was carried out.

Guiacol is indeed a suitable substrate for spectrophotmetric MPO activity measurement in wound fluids. However, apart from its toxicity, we and others [36] have experienced that the formed colour is not stable in some cases. It is well known that peroxidase catalysed oxidation of guaiacol proceeds via coloured oligomers like tetraguaiacol [36-38] and polymers which can precipitate [39].

ABTS and TMB are well known substrates for peroxidases. Benzidine and its derivates are widely used for both detection and quantification of peroxidase activity. However, their carcinogenic and mutagenic properties present serious drawbacks to their use in the laboratory [40]. Therefore, one of their derivates, TMB was chosen, since it is known to be neither carcinogenic nor mutagenic [41]. In addition, a high sensitivity compared to benzidine-

based assays has been reported [42]. Additionally, the chlorination- and peroxidase-activity of MPO can be measured with TMB [43]. Comparing the sensitivity of ABTS and TMB, the latter seems to be more suitable for a diagnostic system.

Crystal violet and its leuco form were for the first time shown to be converted by MPO. In agreement with previous results for horse radish peroxidase [44], N-demethylation seems to be the first step in crystal violet degradation. This is supported by the fact that the absorption maximum of the dye underwent a hypsochromic shift since N-demethylation products are known to have absorption maxima at wavelengths lower than that of crystal violet [45]. Thereafter, step-wise de-amination finally leads to the breakdown of the backbone as indicated by LC-MS data. Oxidation of the leuco form was found to be a considerable faster process which is in agreement with data reported for other peroxidases [46]. Consequently, regarding assessment of wound infection, leuco crystal violet was found more suitable giving clearly visible color changes when incubated with infected wound fluids for only 10 minutes. Although the wound samples contained varying amounts of hemoglobin potentially contributing to oxidation of MPO substrates, yet a clear distinction of infected and non-infected diluted wound samples was possible

In an attempt to obtain more information on the structural requirements for substrates to be oxidized efficiently by MPO, model substrates were synthesized. Based on 2,7-dihydroxy-1-phenylazonaphtalene-3,6-disulfonic acid, systematically substituted derivatives were constructed in order to gain mechanistic information on MPO catalyzed oxidation. In agreement with our previous investigations involving laccases [30] and [47] the nature and position of the substituent of the molecule had a dramatic influence on MPO-catalyzed oxidation. Hydroxy groups and, to a lower extent, methyl substituted derivatives were readily oxidized while there was no detectable reaction with derivatives carrying chlorine or nitro groups. This is in agreement with results previously found for laccases [30]. Most likely electron donating substituents can stabilize the aromatic cation formed after two subsequent electron abstractions [48].

Hydrogen peroxide is a required co-substrate in MPO catalyzed oxidation of all above substrates. To improve storage stability of a diagnostic kit, enzymatic in- situ production of hydrogen peroxide was investigated. A recently reported cellobiose dehydrogenase (EC 1.1.99.15) from *Myriococcum thermophilum* which accepts a wide range of sugars and even polysaccharides was used to produce hydrogen peroxide [29]. Indeed, it was possible to

replace hydrogen peroxide in the assay solution by addition of this enzyme together with cellobiose.

Immobilization of MPO substrates would have advantages both in terms of simpler handling (e.g. test strips) as well as when the diagnostic device is to be integrated in bandages. In latter case it will be essential that there is no dye release into wounds. Here we showed for the first time that Fast blue RR can be covalently coupled to alkoxysilanes still remaining a substrate for MPO. This functionalized alkoxysilane can easily be polymerized onto a large variety of materials ranging from polyesters and cotton as materials of bandages to glass or silica gel as potential carriers in test strips.

Summarizing our results we have demonstrated the potential of novel, partly immobilized MPO substrates for detection of MPO activity in wound fluids. Using these substrates, visible colour changes indicate elevated enzyme levels in infected wounds.

5 Acknowledgements

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6 References

1. S.J. Klebanoff, Myeloperoxidase, Proc.Assoc.Am.Physicians 111 (1999) pp. 383-389.

2. R. Fenna, J. Zeng, and C. Davey, Structure of the Green Heme in Myeloperoxidase, Arch.Biochem.Biophys. 316 (1995) pp. 653-656.

3. F. Lanza, Clinical Manifestation of Myeloperoxidase Deficiency, J.Mol.Med. 76 (1998) pp. 676-681.

4. W.M. Nauseef, Myeloperoxidase Deficiency, Hematol.Oncol,Clin.North.Am. 2 (1988) pp. 135-158.

5. D.C. Dale, L. Boxer, and W.C. Liles, The Phagocytes: Neutrophils and Monocytes, Blood 112 (2008) pp. 935-945.

6. M.B. Hampton, A.J. Kettle, and C.C. Winterbourn, Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing, Blood 92 (1998) pp. 3007-3017.

7. O. Soehnlein, An Elegant Defense: How Neutrophils Shape the Immune Response, Trends Immunol. 30 (2009) pp. 511-512.

8. W.K. Stadelmann, A.G. Digenis, and G.R. Tobin, Physiology and Healing Dynamics of Chronic Cutaneous Wounds Am.J.Surg. 176 (1998) pp. 26S-38S.

9. J.A. Smith, Neutrophils, Host Defense, and Inflammation: A Double-Edged Sword, J.Leukocyte Biol. 56 (1994) pp. 672-686.

10. J. Alegre, J. Jufresa, C. Aleman, R. Segura, L. Armadans, R.N. Marti, C. Cervera, and T. Fernandez de Sevilla, Pleural Fluid Myeloperoxidase as a Marker of Infectious Pleural Effusions, Eur.J. Intern.Med. 12 (2001) pp. 357-362.

11. M.L. Brennan, M.S. Penn, F. Van Lente, V. Nambi, M.H. Shishehbor, R.J. Aviles, M. Goormastic, M.L. Pepoy, E.S. McErlean, E.J. Topol, S.E. Nissen, and S.L. Hazen, Prognostic Value of Myeloperoxidase in Patients with Chest Pain, N.Engl.J.Med 349 (2003) pp. 1595-1604.

12. D.W. Laight, B. Woodward, and J.F. Waterfall, Assessment of Myeloperoxidase Activity in Renal Tissue after Ischemia/Reperfusion, Eur.J.Pharmacol. 292 (1994) pp. 81-88.

13. T. Schneider and A.C.I ssekutz, Quantitation of Eosinophil and Neutrophil Infiltration Into Rat Lung by Specific Assays for Eosinophil Peroxidase and Myeloperoxidase Application in a Brown NorwayRat Model of Allergic Pulmonary Inflammation, J.Immunol.Methods 198 (1996) pp. 1-14.

14. M.L. Brennan and S.L. Hazen, Emerging Role of Myeloperoxidase and Oxidant Stress Markers in Cardiovascular Risk Assessment, Curr.Opin.Lipidol. 14 (2003) pp. 353-359.

15. C.C. Wu, J.S. Chen, W.M. Wu, T.N. Liao, P.L. Chu, S.H. Lin, C.H. Chuang, and Y.F. Lin, Myeloperoxidase Serves as a Marker of Oxidative Stress During Single Haemodialysis Session Using two Different Biocompatible Dialysis Membranes, Nephrol. Dial.Transpl. 20 (2005) pp. 1134-1139.

16. S.V. Parikh and J.A. De Lemos, Biomarkers in Cardiovascular Disease: Integrating Pathophysiology into Clinical Practice, Am.J.Med.Sci. 332 (2006) pp. 186-197.

17. R.M. Segura, Useful Cinical Biological Markers in Diagnosis of Pleural Effusions in Children, Paediatr.Respir.Rev 5 (2004) pp. S205-S212.

18. Z.L. Zhang, M.L. Brennan, X.M. Fu, R.J. Aviles, G.L. Pearce, M.S. Penn, E.J. Topol, D.L. Sprecher, and S.L. Hazen, Association between Myeloperoxidase Levels and Risk of Coronary Artery Disease, J.Am.Med.Assoc 286 (2001) pp. 2136-2142.

19. S. Baldus, C. Heeschen, T. Meinertz, A.M. Zeiher, J.P. Eiserich, T. Munzel, M.L. Simoons, and C.W. Hamm, Myeloperoxidase Serum Levels Predict Risk in Patients with Acute Coronary Syndromes, Circulation 108 (2003) pp. 1440-1445.

20. T.A. Dechert, A.E. Ducale, S.I. Ward, and D.R. Yager, Hyaluronan in Human Acute and Chronic Dermal Wounds, Wound Repair Regen.14 (2006) pp. 252-258.

21. N.J. Trengove, S.R. Langton, and M.C. Stacey, Biochemical Analysis of Wound Fluid from Nonhealing and Healing Chronic Leg Ulcers, Wound Repair Regen. 4 (2002) pp. 234-239.

22. D.R. Yager, R. A. Kulina, and L.A. Gilman, Wound Fluids: A Window into the Wound Wnvironment?, Int.J.Low.Extrem.Wounds 6 (2007) pp. 262-72.

23. R. Mani, Science of Measurements in Wound Healing, Wound Repair Regen. 7 (1999) pp. 330-334.

24. R.K. Desser, S.R. Himmelhoch, W.H. Evans, M. Januska, M. Mage, and E .Shelton, Guinea Pig Heterophil and Eosinophil Peroxidase, Arch.Biochem.Biophys. 148 (1972) pp. 452-465.

25. P.M. Bozeman, D.B.Learn, and E.L. Thomas, Assay of the Human Leukocyte Enzymes Myeloperoxidase and Eosinophil Peroxidase, J.Immunol.Methods 126 (1990) pp. 125-133.

26. R.D. Venezie, J.W. Jenzano, and R.L. Lundblad, Differentiation of Myeloperoxidase and Glandular Peroxidase in Biological Fluids: Application to Human Saliva, J.Clin.Lab.Anal. 5 (1991) pp. 57-59.

27. H.A. Mottola, B.E. Simpson, and G. Gorin, Absorptiometric Determination of Hydrogen Peroxide in Submicrogram Amounts with Leuco Crystal Violet and Peroxidase as Catalyst, Anal.Biochem. 42 (1970) pp. 410-411.

28. C.A. Cohen, A. Pak, D. Strongin, and M. Schoonen, Quantifying Hydrogen Peroxide in Iron-Containing Solutions using Leuco Crystal Violet, Geochem.Trans. 6 (2005) pp. 47-51

29. S. Pricelius, R. Ludwig, N. Lant, D. Haltrich, and G. Guebitz, Substrate Specificity of Myriococcum Thermophilum Cellobiose Dehydrogenase on Mono-, Oligo-, and Polysaccharides Related to In Situ Production of H2O2, Appl.Microbiol.Biot. 85 (2009) pp. 75-83.

30. A. Kandelbauer, O. Maute, R.W. Kessler, A. Erlacher, and G. Guebitz, Study of Dye Decolourization in an Immobilised Laccase Enzyme-Reactor Using Online Spectroscopy, Biotechnol.Bioeng. 87 (2004) pp. 552-563.

31. W.K. Stadelmann, A.G. Digenis, and G.R. Tobin, Physiology and Healing Dynamics of Chronic Cutaneous Wounds, Am.J.Surg. 176 (1998) pp. 26S-38S.

32. C.J. Sylvia, The Role of Neutrophil Apoptosis in Influencing Tissue Repair, J.Wound Care 12 (2003) pp. 13-6.

33. K. Tschaikowsky, R. Sittl, G.G. Braun, W. Hering, and E. Rugheimer, Increased Fmet-Leu-Phe Receptor Expression and Altered Superoxide Production of Neutrophil Granulocytes in Septic and Posttraumatic Patients, Clin.Investigator 72 (1993) pp. 18-25.

34. D. Hurst and P. Venge, Levels of Eosinophil Cationic Protein and Myeloperoxidase from Chronic Middle Ear Effusion in Patients with Allergy and/or Acute Infection, Arch.Otolaryngol.Head Neck Surg. 114 (1996) pp. 531-544.

35. G.A.B. Edelstam, Ö. Lundkvist, P. Venge, and T.C. Laurent, Hyaluronan and Myeloperoxidase in Human Peritoneal Fluid during Genital Inflammation, Inflammation 18 (1994) pp. 13-21.

36. R.S. Koduri and M. Tien, Oxidation of Guaiacol by Lignin Peroxidase. Role of Veratryl Alcohol, J.Biol.Chem. 270 (1995) pp. 22254-8.

37. A.E. Zeraik, F. Souza, O. Fatibello-Filho, and O.D. Leite, Development of a Spot Test for Peroxidase Activity Monitoring during a Purification Procedure, Quím.Nova. 31 (2008) pp. 731-734.

38. T. Hiroyuki, U. Hiroshi, N. Ritsuko, and K. Shiro, Guaiacol Oxidation Products in the Enzyme-Activity Assay Reaction by Horseradish Peroxidase Catalysis, Chem.Lett. 33 (2004) pp. 796-

39. K. Iwahara, Y. Honda, T. Watanabe, and M. Kuwahara, Polymerization of Guaiacol by Lignin-Degrading Manganese Peroxidase from Bjerkandera adusta in Aqueous Organic Solvents, Appl.Microbiol.Biotechnol. 54 (2000) pp. 104-111.

40. G. Choudhary, Human Health Perspectives on Environmental Exposure to Benzidine: A Review, Chemosphere 32 (1996) pp. 267-291.

41. L.A. Marquez and H.B. Dunford, Mechanism of the Oxidation of 3,5,3',5'-Tetramethylbenzidine by Myeloperoxidase Determined by Transient- and Steady-State Kinetics, Biochemistry 36 (1997) pp. 9349-9355.

42. M.M. Mesulam, Tetramethyl Benzidine for Horseradish Peroxidase Neurohistochemistry: a Non-Carcinogenic Blue Reaction Product with Superior Sensitivity for Visualizing Neural Afferents and Efferents, J.Histochem.Cytochem. 26 (1978) pp. 106-117.

43. M. Berger and M.P. Schneider, Regioselectivity of Lipases in Organic Solvents, Biotechnol.Lett. 13 (1991) pp. 333-338

44. F.R. Gadelha, F.M. Hanna, R.P. Mason, and R. Docampo, Evidence for Free Radical Formation during Horseradish Peroxidase-Catalyzed N-demethylation of Crystal Violet, Chem.Biol.Interact. 85 (1992) pp. 35-48.

45. R. Bangert, W.A ichele, E. Schollmeyer, B. Weinmann, and H. Herlinger, Photooxidation von Malachitgrün und Kristallviolett, Melliand Textilber 5 (1977) pp. 404-

46. C.A. Cohn, A. Pak, D. Strongin, and M.A. Schoonen, Quantifying Hydrogen Peroxide in Iron-Containing Solutions using Leuco Crystal Violet, Geochem.Trans. 6 (2005) pp. 47-

47. J. Maier, A. Kandelbauer, A. Erlacher, A. Cavaco-Paulo, and G.M. Guebitz, A New Alkali-Thermostable Azoreductase from Bacillus sp. Strain SF, Appl.Environ.Microbiol. 70 (2004) pp. 837-844.

48. M. Chivukula and V. Renganathan, Phenolic Azo Dye Oxidation by Laccase from Pyricularia oryzae, Appl.Environ.Microbiol. 61 (1995) pp. 4374-4377.

5

NEW SENSOR MATERIALS FOR THE DETECTION OF HUMAN NEUTROPHIL ELASTASE- AND CATHEPSIN G-ACTIVITY IN WOUND FLUID

Human neutrophil elastase (HNE) and cathepsin G (CatG) are involved in the pathogenesis of a number of inflammatory disorders. These serine proteinases are released by neutrophils and monocytes in case of infection. Wound infection is a severe complication regarding wound healing causing diagnostic and therapeutic problems. In this study we have shown the potential of HNE and CatG to be used as markers for early detection of infection. Significant differences in HNE and CatG levels in infected and non infected wound fluids were observed. Peptide substrates for these two enzymes were successfully immobilized on different surfaces, including collagen, modified collagen, polyamide polyesters and silica gel. HNE and CatG activities were monitored directly in wound fluid via hydrolysis of the chromogenic substrates. Infected wound fluids led to significant higher substrate hydrolysis compared to non-infected ones. These different approaches could be used for the development of devices which are able to detect elevated enzyme activities before manifestation of infection directly on bandages. This would allow a timely intervention by medical doctors thus preventing severe infections.

Experimental Dermatology, in press

1 Introduction

Wound healing is a complex process regulated by a multiplicity of biomolecules, including cytokines, growth factors and enzymes. In a chronic wound, the normal process of healing is disrupted at one or more points, mostly in the inflammatory or proliferative phase [1]. Whereas healing acute wounds have low levels of protein-degrading enzymes, exudates from non-healing chronic wounds contain elevated levels of proteases, like matrix metalloproteinases (MMPs) and human neutrophil elastase (HNE) produced by neutrophilic granulocytes [2-4].

The neutrophilic granulocyte is the first nucleated cell to infiltrate the wound bed after the integrity of the skin has been disrupted. This innate immune cell mediates the first line of defence and marks the start of the inflammatory response of wound healing. The main function of neutrophils at the site of infection is to decontaminate the open wound by the destruction of invading microbes and therefore minimizing the chance for infectious complications [5]. Under physiological conditions, the number of PMN granulocytes decreases after a few days. However, in chronic wounds, several authors have shown a massive and constant infiltration of PMN granulocytes [3,6]. Neutrophils express a large number of proteases like CatG and HNE.

Human neutrophil elastase (HNE: EC 3.4.21.37) and cathepsin G (CatG: EC 3.4.21.20) are two enzymes of the chymotrypsin family that are stored in the primary (azurophil) granules of polymorphonuclear neutrophils (PMNs). Their activities depend upon a catalytic triad composed of aspartate, histidine and serine residues which are widely separated in the primary sequence, but are brought together at the active site of the enzymes in their tertiary structures [7-9].

HNE is able to degrade a variety of structural and functional proteins deposited in wounds such as collagen and fibronectin, as well as key growth factors such as TNF-α [10-12]. CatG has a broad substrate specificity too, including substrates like collagen, fibronectin, proteglycans and elastin and is therefore involved in the degradation of extacellular matrix components [13,14]. The excessive production of HNE and other proteolytic enzymes by PMN granulocytes leads to extensive pathological tissue destruction in a number of disorders, including delayed and chronic wound healing [15,16].

HNE is known to be involved in the pathogenesis of a number of inflammatory disorders [17-19]. Cystic fibrosis, which is characterized by chronic airway infection and inflammation

invariably dominated by neutrophils [20], chorioamnionitis [21], intrauterine infection [22] and urethral infections in men show elevated HNE levels [23].

A number of studies have found significantly elevated mean levels of HNE activity in pressure ulcers and in leg ulcers [24-26]. In contrast to HNE, the knowledge about CatG levels in inflammatory disorders and chronic and infected wounds is marginal. It is well established that CatG has different important functions during inflammatory processes, including platelet activation [27], microbiocidal activity [28], conversion of angiotensin I to angiotensin II [29] and cleavage of clotting factors [30]. Additionally, CatG is able to inactivate cytokines like interleukin-1 (IL-1), interleukin-8 (IL-8) and tumour necrosis factor α (TNF-α) [13,14]. Furthermore, CatG is involved in the pathogenesis of chronic diseases like emphysema and cystic fibrosis [31]. The role of CatG during inflammation is underscoren by the fact that CatG deficient mice have an impaired wound healing response and increased susceptibility to bacterial infections [14].

Wound infection is a severe complication during wound healing causing diagnostic and therapeutic problems [32,33]. Infection is characterized by an excessive stimulation of neutrophils, resulting in the release of proteolytic enzymes like HNE and CatG into the plasma [34]. Due to the fact that the presence of neutrophils and monocytes is a well documented marker for infection [35], HNE is frequently used as both a predictor and an indicator of inflammatory disease severity [34,36-38], Since elevated HNE levels are described at the very beginning of infection by Hofer *et al*. [39,40] HNE was therefore selected as well as CatG as diagnostic parameter for detection of wound infection directly in wound fluid. In this study, levels of both enzymes in infected and non infected wound fluid samples were determined, thus assessing their potential monitoring wound status. In a further step, immobilisation of specific chromogenic enzyme substrates on different supporting materials was performed. Materials were selected which are used as bandages or dressings in the field of wound treatment. This would allow a possible development of a so called “online wound monitoring” system incorporated directly into bandages and wound dressings.

2 Materials and Methods

2.1 Sample Collection and Preparation

Fluid from six post-operative wounds, two decubitus ulcer wounds, six malum perforens wounds and four ulcus cruris wounds were collected and analyzed by biochemical techniques. Exudates were collected directly from exudating wound surface by using needles or a spoon before the daily treatment. Two samples were collected during V.A.C therapy in special canisters without starch. Additionally, fluid from nine blisters was collected. The samples were centrifuged three times at 420 g for 20 minutes to remove cells and tissue material and stored at -20°C until analyzed. 9 wounds out of those 18 wounds were described as clinically infected by the attending doctors. The patients studied so far were grouped and labeled with the following abbreviations (followed by a number): i = infected wound; n.i = non infected wound; b = blisters. Permission to collect wound fluid was obtained from the Ethics Committee of the Medical University Graz.

2.2 Elastase and Cathepsin G Activity Measured in Wound Fluid

HNE activity was determined by measuring the cleavage of N-methoxysuccinyl-ala-ala-proval-p-nitroanilide (MeOSuc-AAPV-pNA) as the chromogenic substrate, while for the determination of CatG activity *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide (Suc-AAPF-pNA) was used [1]. A solution of 1 mM MeOSuc-AAPV-pNA and 3 mM Suc-AAPF-pNA (diluted in DMSO) in 0.1 M HEPES buffer (pH 7.4, containing 0.5 M NaCl) was used. To 5 µL sample (wound fluid) or commercial enzyme (HNE: 0.1 U/ml - 1 U/ml; CatG: 1.25 U/ml - 5 U/ml), 100 µL of the substrate solution is added. Substrate degradation was continuously monitored by measuring the increasing absorbance at 405 nm every 60 seconds at a temperature of 30°C for HNE and at 37°C for CatG. The initial speed of the reaction (linear range) was used for calculations.

2.3 Immobilisation of Elastase and Cathpesin G Substrates on APTS modified Silica Gel

Modification of Silica Gel using 3-Aminopropyl-triethoxysilane (APTS)

Silica gel was modified using 3-Aminopropyl)triethoxysilane for creating functional groups (amino groups) which can be used for further immobilisation of enzyme substrates. The

modification of silica gel with APTS is based on the work of *Le Berre et al.* with some alterations [2]. The reaction time was 15 hours at and temperature of 40°C. For the modification step, 10 g silica gel were immersed in 30 ml of a 20% (w/v) APTS solution (in EtOH 95% (v/v) + 200 µl HCl diluted) and stirred for 4 hours at 40°C. The mixture was washed for three times with EtOH 70% by centrifuging and pouring away the supernatant. Then the wet residue was dried in the desiccator over night. The dried powder was stored at room temperature in the dark.

Immobilisation of Enzyme Substrates

MeOSuc-AAPV-pNA and Suc-AAPF-pNAas HNE and CatG substrates were linked to APTS modified silica gel by using N-(3-dimethylaminopropyl)-N-ethylcarbodiimid-hydrochlorid (EDAC) and hydroxy-benzotriazole-hydrate (HOBT). For the immobilisation procedure a solution of EDAC (50 mg), HOBT (5 mg) and CatG substrate (50 mg) was prepared in ethanol (20 ml, absolute). Then, 5 g silica powder were added to the solution and stirred for 30 minutes at room temperature. After centrifugation at 2000 rpm for 10 minutes, the cross-linked substrates were washed several times with 70% (v/v) ethanol. As last step the gel was dried in the desiccator until constant weight was achieved. The dried powder was stored at room temperature in the dark for further application.

Enzymatic Conversion of Immobilised Suc-AAPF-pNA and MeOSuc-AAPV-pNA

Immobilised HNE and CatG substrates were incubated with commercial enzyme as well as with wound fluid. 170 mg of silica gel were re-suspended in 1000 µl of 0.1 M HEPES buffer (pH 7.4, containing 10% (v/v) DMSO, 0.5 M NaCl). 100 µl of this solution were incubated with 5 µl enzyme (HNE: 10 U/ml; CatG: 50 U/ml) or wound fluid samples (undiluted) at 37°C over night. Absorbance was measured at a wavelength of 405 nm.

2.4 Immobilisation of Cysteamid-Suc-AAPV-pNA on Various Surfaces

To facilitate coupling of the HNE substrate, a cysteinamid was synthesized at the N-terminus of Suc-AAPV-pNA. The modified peptide (Cysteamid-Suc-AAPV-pNA) was linked via thiol (SH) groups to the amino-groups (NH_2) of different supports. Briefly, sulfo-GMBS was dissolved in water (10 mM), diluted with the conjugation buffer (pH 6.5) and was then added to different surfaces with a final concentration of 1 mM. After incubation for 30 minutes at room temperature, the excess cross-linker was removed by several washing steps using

reaction buffer. The modified peptide was dissolved in DMSO (10 mM) and diluted with the conjugation buffer (1.5 mM). After incubation for 30 minutes at room temperature, 5 washing steps were performed. Reduced cysteine at a concentration of 20 mM was added and incubated for 30 minutes at room temperature. After removal of the cysteine solution, 5 washing steps were performed. As surfaces, collageneous matrices as well as polyamide (PA) and polyethyleneterephthalate (PES) were selected.

Polyamide and Polyethyleneterephthalate

To improve the efficiency of the coupling, activated PA and PES fibers were used**.** Therefore, the number of functional groups on the surface of PA and PES, was increased as previously described by Heumann *et al.* [3], [4] and Eberl *et al.* [5]. Essentially, PA and PES were treated with polyamidase and cutinase to insert novel amino- and hydroxyl groups, respectively, as anchors for binding the protease substrate.

Fabrication of Collagen (Coll), Hyluronic Acid (HA)-Coll and Thiolated Hyaluronic Acid (THA)-Coll Matrices

Different collagenous matrices were fabricated as previously described by Park *et al.* [6].

Collagen: Type I collagen from bovine (Lohmann & Rauscher) was prepared according to the instruction of the manufacturer. Briefly, frozen collagen (1.68% (w/v)) in acetic acid, pH 3.0) was defrosted at room temperature overnight. The concentration was brought close to 1.1% (w/v) with distilled water and this mixture was homogenized usingan external stirrer (IKA T25 digital ultratorax). Then the pH was adjusted to pH 6.0 using 1 M NaOH.

Hyaluronic acid (HA) preparation: Hyaluronic acid sodium salt was hydrated in distilled H_2O to obtain 0.4% (w/v) for 30 minutes.

Thiolated hyaluronic acid (THA): Hyaluronic acid (0.1 g) was dissolved in 50 ml distilled water to obtain 0.2% (w/v) solution. 1-ethyl-(dimethylaminopropyl) carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) were added (10 mM). The pH was set to pH 5.5 with 0.1 M HCl and solution was left for 15 min under stirring to complete activation of carboxyl moieties in HA. Thereafter L-cysteine ethyl ester HCl (Sigma) was added in final concentration of 50 mM. The pH of the solution was set to pH 6.0 and stirred for 3 hours at room temperature in a closed vial. The product was dialyzed 3 times against 5 liters of 1% (w/v) NaCl and finally once against distilled water. The product was lyophilized thereafter.

Determination of free thiol groups immobilised on THA, was carried out spectrophotometrically using Ellman's reagent, following the method described by *Hornof et al.* [7]. Brievly, 1 mg of THA and control was hydrated in 0.50 ml of 0.5 M phosphate buffer (PB, pH 8.0). Then 0.50 ml of Ellman's reagent (3 mg of 5,5'- dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in 10 ml of PB –buffer (0.5 M, pH 8.0) were added. The samples were incubated for 3 hours at room temperature. Thereafter, samples were centrifuged (5000 rpm, 5 min) and 300 µl of solution was transferred into the microplate reader where the absorbency was measured at a wavelength of 450 nm. L-cysteine ethyl ester HCl standards were used to calculate the amount of thiol groups immobilised on the polymer. The control sample did not contain thiols.

Films: Films were prepared as follows. Pure collagen or mixtures of collagen and thiolated and non-thiolated hyaluronic acid (ratio 1:1) were cast on a petri dish and dried on air for 24 h at room temperature. Obtained films were neutralized with 0.1 M aqueous NaOH solution and washed thoroughly with distilled water before crosslinked with EDAC/NHS/MES (2-morpholinoethane sulfonic acid) solution for 4 hours. The final concentrations of the compounds in the crosslinking solution were: EDAC 10 mg/ml, NHS 2.5 mg/ml, MES 50 mM. The samples were again thoroughly washed with distilled water and air-dried. Films were stored at +4ºC until further use.

Enzymatic Conversion of Immobilised Cys-Suc-AAPV-pNA on Various Surfaces

Incubation of different support materials was carried out to measure the conversion of crosslinked HNE substrate (Cys-Suc-AAPV-pNA). Therefore, these surfaces were incubated for 1 day with 150 µl HEPES buffer comprising 10 µl HNE (5 U/ml), or wound fluid sample. Conversion of substrate by enzyme was measured photometrically at 405 nm.

3 Results and Discussion

3.1 Enzyme Activity in Wound Fluid

Activities of human neutrophil elastase (HNE: EC 3.4.21.37) and Cathepsin G (CatG: EC 3.4.21.20) were determined directly in wound fluid (Table 9). Significantly higher HNE and CatG activity was detected in infected wounds when compared to non infected wounds ($P \leq 0.05$). These results are in good accordance with the clinical description of the wounds. Additionally, fluid from 9 blisters was used as a negative control. There was a significant difference between blisters and non infected post operative or chronic wounds regarding enzyme levels. However, there were no significant differences of enzyme levels in infected chronic wounds or post operative wounds.

Table 9: Elastase and cathepsin g activity in wound fluid samples: There is a significant difference between elastase and cathepsin G activity in infected and non-infected wounds (P<0.005).

Clinical Description of the wound	Infected	Non-infected
Blisters	0	9
Post-operative wounds	3	3
Decubitus wounds	2	0
Malum perforens	2	4
Ulcus cruris	2	2
Elastase activity [U/ml]	22.97 $\pm$ 13.27	2.89 $\pm$ 1.27
Cathepsin G activity [U/ml]	50.85 $\pm$ 36.76	7.05 $\pm$ 4.93

Human neutrophil elastase and cathepsin G play a crucial role in physiological and pathological processes. HNE is known to be involved in the pathogenesis of a number of inflammatory disorders [18]. Elevated activities of HNE are described for diseases like rheumatoid arthritis [48], [49], hereditary emphysema, chronic obstructive pulmonary disease

[50], adult respiratory distress syndrome [51], ischemia/reperfusion injury, emphysema, cystic fibrosis and tumor progression [52]. Additionally, elevated HNE levels in chronic wounds and burn wounds have been demonstrated [53,54]. As HNE and CatG are responsible for degradation of growth factors [53,54] and proteinase inhibitors [3], an over expression of these enzymes is a possible reason for the non-healing of a wound.

Furthermore, HNE was found to be elevated at the very beginning of infection. Therefore it was suggested as diagnostic marker for detection of wound infection by Hofer et *al.* [39,40]. Wound infection is one of the most common reasons for impaired wound healing, leading to death in the worst case. Infection is characterized by an excessive stimulation of neutrophils, resulting in the release of proteolytic enzymes like HNE and CatG [34]. Due to the fact that the presence of neutrophils and monocytes is a well documented marker for infection [35,55,56], HNE measured in serum, plasma and urine is frequently used as both a predictor and an indicator of inflammatory disease severity [36- 38]

However, it is well documented that neutrophilic enzymes can also be measured in wound fluid. Naomi *et al.,* [41] found comparable levels of the C-reactive protein, which is a widely used marker for inflammation, in serum and wound fluid, assuming the potential of wound fluid to provide useful information regarding the overall status of a wound [41,57,58]. Different studies have shown high levels of HNE in wound fluid of chronic, non-healing wounds [43-45,53,59]. However, no or less comparable data is available for CatG levels in acute, chronic and especially infected wounds.

As we found significantly elevated enzyme levels of HNE and CatG in infected wounds, detection of these two enzymes in wound fluid could give early stage warning of wound infection. Analysis of wound fluid is a non-invasive method that possesses a powerful tool for characterization of proteinases in the wound environment. As these enzymes are known to be elevated in early stages of infection, determination of these markers is suggested to be done routinely in traumatology and in case of chronic wounds thus allowing early treatment of the wound before obvious clinical signs of infection are present [39,40]. To allow integration of the detection system into bandages for online monitoring, we investigated immobilisation of different enzyme substrates onto materials used as/in bandages and wound dressings.

3.2 Immobilisation of Human Neutrophil Elastase and Cathepsin G Substrates

Natural and synthetic enzyme substrates can be immobilised on various surfaces for detecting enzyme activity. In contrast to natural enzyme substrates, a single specific single cleavage site can be introduced into the desired surface in case of synthetic substrates, thus resulting in highly sensitive and specific detection systems. Therefore, we decided to use the chromogenic peptides MeOSuc-AAPV-pNA and Suc-AAPF-pNA which are hydrolysed by HNE and CatG, respectively were for immobilisation on different surfaces.

APTS Modified Silica Gel

Silica gel has been widely used as a food supplement and is now available as wound dressing (Bayer Innovation, Düsseldorf, Germany). Here, silica gel was preliminary modified using APTS to introduce functional groups, which are capable of covalently binding peptide substrates. For this approach, highly specific substrates for HNE (MeOSuc-AAPV-pNA) and CatG (Suc-AAPF-pNA) were chosen. These two substrates were then covalently linked to APTS modified silica gel using EDAC as coupling agent. Amination of the silica gel is essential for the successful binding of enzyme substrates. Without amination no hydrolysis of peptide substrate and thus release of p-nitroanilide could be detected. Incubation of these immobilized substrates with wound fluid samples resulted in release of p-nitroanilide and thus colouration of the supernatant in case of infected (i) samples. Incubation with non infected wound fluid samples led to a negligible release in both cases. As a control, commercial HNE and CatG were used. Absorbance changes measured at 405 nm are shown in Figure 19.

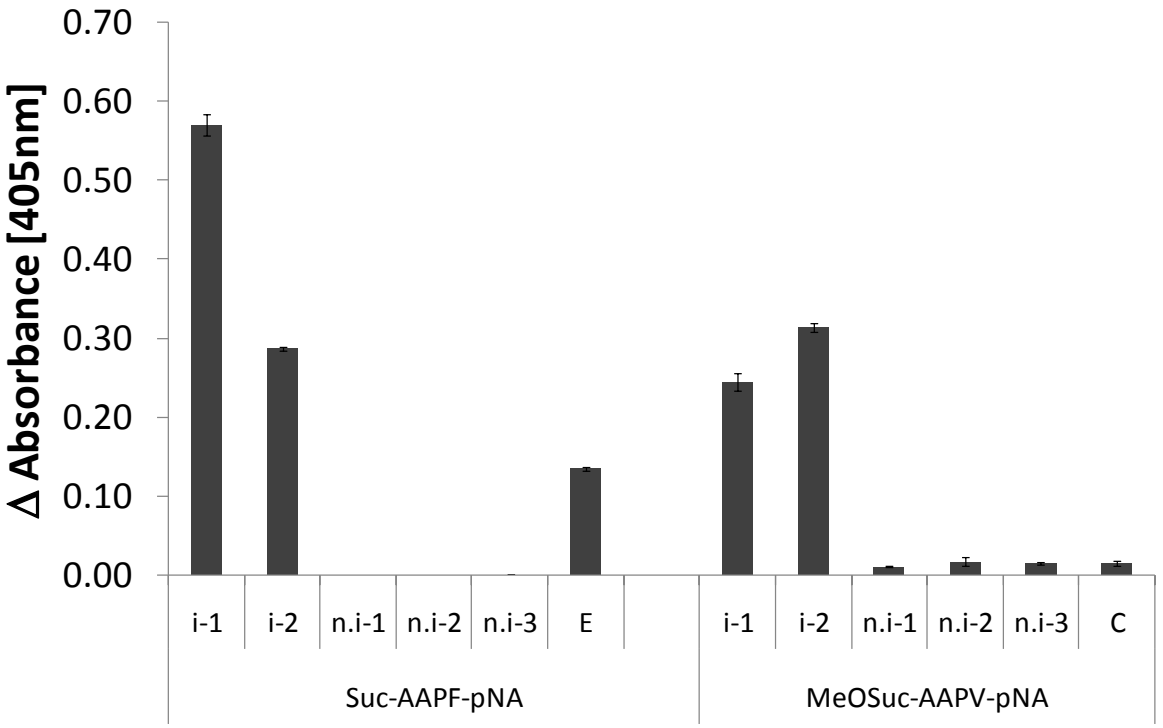

Figure 19: Distinction of infected and non infected wound fluid samples: Hydrolysis of Suc-AAPF-pNA and MeOSuc-AAPV-pNA immobilized on APTS-modified silica gel with non infected (n.i) and infected (i) wound fluid samples, HNE (E) and cathepsin (C) for 2 days. Substrate hydrolysis was monitored at 405 nm.

Specificity is a very important fact for the development of diagnostic devices based on enzyme activity. As was shown by Castillo *et al*. and Nakajima *et al.*, MeOSuc-AAPV-pNA is selectively hydrolyzed by HNE, but is not a substrate for human leukocyte CatG [60,61]. Furthermore it was demonstrated that only activity from HNE released from neutrophils but not from macrophages is measured with this peptide [62]. On the other hand, *N*-Suc-AAPF-pNA, (CatG), cannot be hydrolysed by human leukocyte HNE [61].

The use of immobilised enzyme substrates has gained increasing interest during the last years. A lot of research was focused on the immobilization of substrates suitable for human leukocyte HNE. Kamarun *et al.* described the immobilization of the peptide sequence AAPVAAK on polyacrylamide hydrogel films synthesized on piezoelectric quartz crystals [63]. Another approach was illustrated by Edwards *et al.*, who attached N-succinyl-ala-ala-pro-val-p-nitroanilide (Suc-AAPV- pNA) to glycine-cross-linked ethoxylate acrylate resins (Gly-CLEAR) by a carbodiimide reaction [64]. Furthermore, the chromogenic peptide substrate Succinyl-Ala-Ala-Pro-Ala-pNA and its analogue Succinyl-Ala-Ala-Pro-Val-pNA were attached

to derivatized cellulose [65]. In addition, immobilization of HNE and CatG substrates would allow their integration into a bandage or wound dressing for online monitoring of the wound status. Due to the more pronounced turnover rate of the HNE substrate, this peptide was chosen for further investigations with other carrier materials.

Collagenous Matrices

Pure collagen (Coll) as well as collagen crosslinked to thiolated and non-thiolated hyaluronic acid (THA and HA) were used for immobilization of peptide comprising the specific HNE cleavage site AAPV. Thiolation was carried out to introduce more reactive anchor-groups for covalent binding of the substrate. Cys-Suc-AAPV-pNA was linked to collagen and functionalised (HA-Coll, THA-Coll and Coll), mediated by a carbodiimide (EDAC). The presence of reactive groups like sulfhydryl groups on the surface of different materials is a requirement for the covalent linking of different peptide substrates. Enzymatic activity was measured by monitoring the release of p-nitroanilide. Infected (i) and non infected (n.i) wound fluids samples, as well as HNE, were used for incubation. Successful hydrolysis of the peptid substrate was seen in case of infected wound fluids and HNE. Only slight hydrolysis was observed in case of non infected wound fluids (Figure 20).

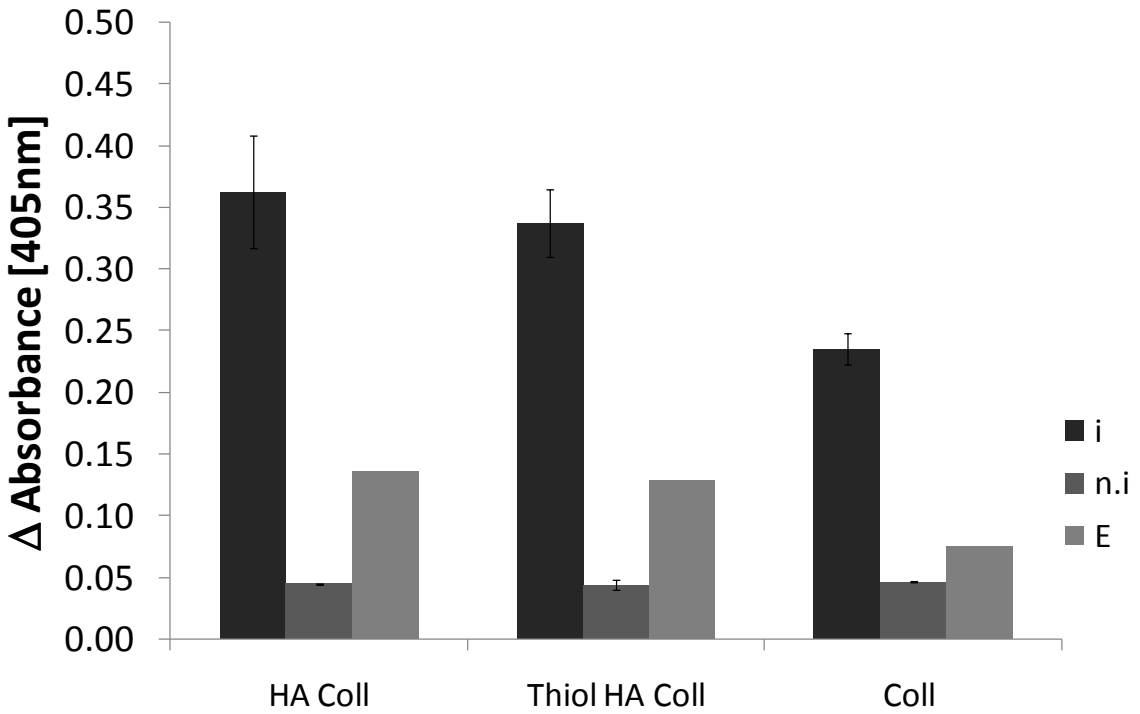

Figure 20: Distinction of infected and non infected wound fluid samples: Hydrolysis of Cys-Suc-AAPF-pNA immobilized on collagenous matrices (HA Coll; Thiol HA Coll and Coll) with infected and non infected (i; n.i) wound fluids and HNE (E). Release of p-NA was measured at 405 nm. There is a significant difference in increase of absorbance in case of infected wound fluids compared to non infected ones ($P \leq 0.005$).

HA-Coll and ThiolHA-Coll films show similar absorbance changes. Again, a significant difference in increase of absorbance in case of infected wound fluids compared to non infected ones ($P \leq 0.005$) can be seen. Even though the successful introduction of reactive thiol groups could be affirmed using Ellman´s reagent (the amount of SH in the thiolated HA was calculated to be 78.3 ± 4.2 µmol/g of conjugate) the thiolation of hyaluronic acid has no effect on the binding behaviour of the HNE substrate. A possible explanation could be the fact that thiol groups can be easily oxidized in presence of atmospheric oxygen [66].

Although a significantly higher increase of absorbance in case of infected wound fluids compared to non infected ones ($P \leq 0.005$) can be seen in case of the matrix comprising pure collagen (Coll), absorbance change is lower compared to the modified collagen films. This can be explained by the molecular composition of collagen, as its amino acid sequence is characterized by very high amounts of glycine, proline and alanine and the presence of

hydroxyl proline and hydroxylysin, following the pattern: Gly-Pro-X or Gly-X-Hyp. In detail, every third amino acid of collagen is glycine while proline or hydroxyproline constitute about 1/6 of the total sequence. Due to the large amounts of hydroxlysine and hydroxyproline, the collagen-polypeptide chains are able to build intra- and intermolecular cross-links. Thus, there are only a few functional groups available for additional interaction with other molecules [67].

Nevertheless, collagen is a very important material for wound dressings. Collagen dressings are able to keep the wound moist [68,69] and absorb large amounts of fluid [70,78]. Furthermore, overabundance of matrix-metalloproteinases (MMPs) in chronic wounds is reduced in case of collagen based dressings. This allows fibroblasts to proliferate, resulting in the formation of a new extracellular matrix (ECM) and leading to the granulation of the tissue [71]. Additionally, pure collagen has efficient HNE binding ability [67]. As increased levels of HNE lead to reduced amounts of growth factors and proteinase inhibitiors, binding of HNE is a positive side-effect using collagen as support matrix.

Modified collagen as well has a very positive impact on wound healing. Beside the very good structural properties of HA-Coll conjugates, both macromolecules separately, collagen and hyaluronic acid, have a strong benefit on the healing behaviour of infected and chronic wounds. Specific interaction of cells with their surrounding extracellular matrix accounts for regulation of the wound healing process as shown by many studies [46,72,73]. Especially the benefits of HA and Coll on the enhancement of cell migration and division is well documented [70,74-76]. An additional chemical modification of collagen-HA, for example cross linking with EDAC would provide membranes with improved mechanical property as well as a reduced biodegradation [72].

PA and PES as Surfaces for Immobilisation of Enzyme Substrates

Polyamide and polyesters are widely used materials integrated in wound dressings to increase the number of available reactive groups for immobilisation of the HNE substrate, enzymatic modification was carried out as described by Eberl *et al.* and Heumann *et al*. [43-45] (Figure 21).

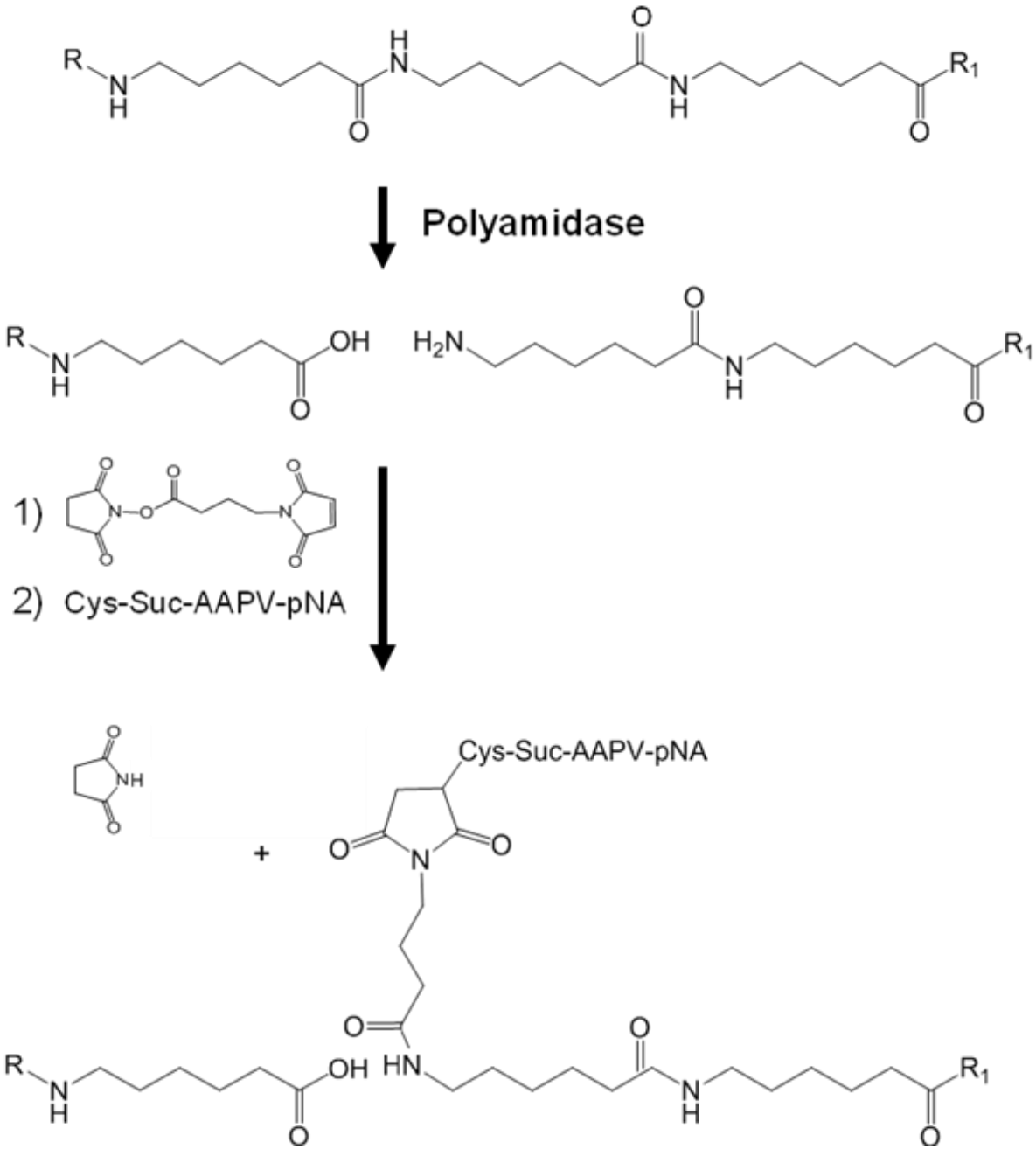

Figure 21: Covalent immobilisation of HNE substrate onto polyamide after enzymatic surface hydrolysis

After incubation with infected and non infected wound fluid samples, a clear difference in substrate hydrolysis and colour formation as a consequence, was observed. In case of the enzymatically modified materials (PA^+, PES^+) a significant higher hydrolysis of the bound HNE substrate compared to surfaces without modification (PA, PES) was achieved (Figure 22).

This can be explained by the fact that higher amounts of substrate are bound due to introduction of novel amino- and hydroxyl groups to the surface (data shown).

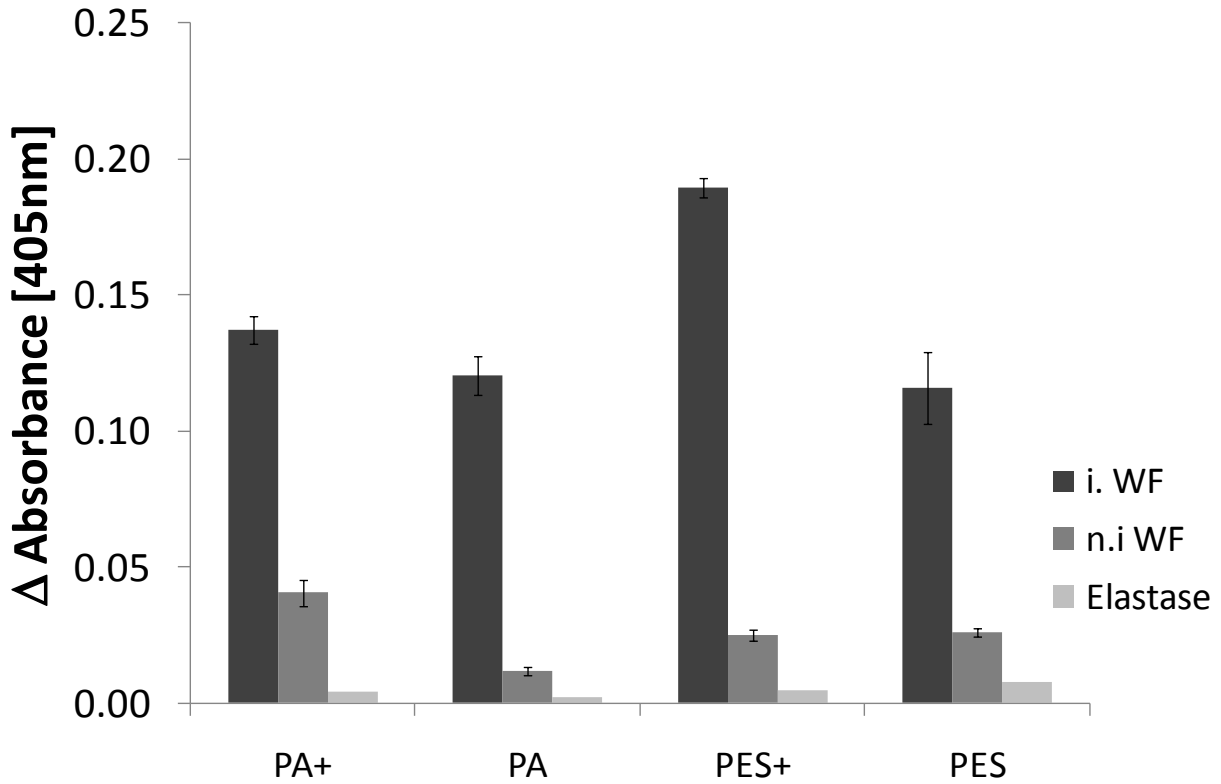

Figure 22: Distinction of infected and non infected wound fluid samples: Hydrolysis of Cys-Suc-AAPF-pNA immobilized on different fibers (PA; PA+; PES and PES+) with infected (i.) and non infected (n.i) wound fluids, as well as HNE (E). Release of p-NA was measured at 405 nm. There is a significant higher absorbance increase in case of infected wound fluids compared to non infected ones ($P \leq 0.05$). PA+ and PES^{+}

Enzymatic surface modification is a very useful tool in polymer functionalisation. In contrast to chemical hydrolysis, the endo-acting enzymes do not degrade the material but lead to large oligomers on the surface which remain tightly bound. This was recently revealed by XPS and MALDI-TOF analysis [45,77]. In our case, this modification was used for increasing the number of binding sites for the HNE substrate while consequently a higher amount of substrate bound on the surface should increase the response of the system. Furthermore, a drastic increase of hydrophilicity was demonstrated both for PET and PA upon enzymatic surface hydrolysis, probably enhancing biotransformation of the immobilised HNE substrate

due to modified enzyme sorption characteristics [44,45]. As covalent immobilisation of enzyme substrates brings a lot of advantages, an enhancement of the bonding capacity of materials is desirable. Covalent immobilisation of enzyme substrates avoids desorption from the support surface, it provides the highest irreversible surface loading of enzyme substrate and additionally can improve resistance to altering environmental conditions. [77,78]. The use of enzymatic surface activation prior to immobilisation is, in contrast to chemical activation, a surface specific environmentally friendly method without impact on the material bulk properties.

In case of wound dressings, immobilisation of the peptide substrates for HNE and CatG for integration directly into bandages would have strong benefits. In contrast to in-vitro enzyme activity assays, on-line monitoring of wound infection would avoid handling with wound fluids and could be used in home-care. Covalent immobilisation of the enzymes substrates would avoid any leaching and stabilize the substrate during storage. Wound infection is indeed one of the most common reasons for impaired wound healing. Here we demonstrated that infection of wounds can be assessed based on biotransformation of HNE and CatG substrates immobilised on typical bandage or wound dressing materials.

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5 References

1. G.S. Lazarus, D.M. Cooper, D.R. Knighton, D.J. Margolis, R.E. Pecoraro, G. Rodeheaver, and M.C. Robson, Definitions and Guidelines for Assessment of Wounds and Evaluation of Healing, Arch.Dermatol. 130 (1994) pp. 489-493

2. N. J. Trengove, M.C. Stacey, S. Macauley, N. Bennett, J. Gibson, F. Burslem, G. Murphy, and G. Schultz, Analysis of the Acute and Chronic Wound Environments: The Role of Proteases and their Inhibitors, Wound Repair.Regen. 7 (1999) pp. 442-452

3. D. R. Yager and B.C. Nwomeh, The Proteolytic Environment of Chronic Wounds, Wound Repair.Regen. 7 (1999) pp. 433-441

4. B. Barrick, E.J. Campbell, and C.A. Owen, Leukocyte Proteinases in Wound Healing: Roles in Physiologic and Pathologic Processes, Wound Repair. Regen. 7 (1999) pp. 410-422

5. S. J. Leibovich and R. Ross, Role of Macrophage in Wound Repair - Study with Hydrocortisone and Antimacrophage Serum, Am.J.Pathol. 78 (1975) pp. 71-99

6. R. F. Diegelmann, Excessive Neutrophils Characterize Chronic pressure Ulcers, Wound Repair.Regen 11 (2003) pp. 490-495

7. A. Janoff and J. Scherer, Mediators of Inflammation in Leukocyte Lysosomes: Elastinolytic Activity in Granules of Human Polymorphonuclear Leukocytes, J.Exp.Med. 128 (1968) pp. 1137-&

8. R. C. Kao, N.G. Wehner, K.M. Skubitz, B.H. Gray, and J.R. Hoidal, Proteinase-3 - A Distinct Human Polymorphonuclear Leukocyte Proteinase That Produces Emphysema in Hamsters, J.Clin.Invest. 82 (1988) pp. 1963-1973

9. M. Baggiolini, B. Schnyder, U. Bretz, B. Dewald, and W. Ruch, Cellular mechanisms of proteinase release from inflammatory cells and the degradation of extracellular proteins, Ciba Foundation Symposium 75 (1980) pp. 105-121

10. J. A. Mcdonald and D.G. Kelley, Degradation of Fibronectin by Human-Leukocyte Elastase - Release of Biologically-Active Fragments, J.Biol.Chem. 255 (1980) pp. 8848-8858

11. P. Scuderi, P.A. Nez, M.L. Duerr, B.J. Wong, and C.M. Valdez, Cathepsin-G and Leukocyte Elastase Inactivate Human Tumor-Necrosis-Factor and Lymphotoxin, Cell.Immunol. 135 (1991) pp. 299-313

12. W. Kafienah, D.J. Buttle, D. Burnett, and A.P. Hollander, Cleavage of Native Type I Collagen by Human Neutrophil Elastase, Biochem.J. 330 (1998) pp. 897-902

13. R. E. Abbott, C.J. Corral, D.M. MacIvor, X. Lin, T.J. Ley, and T.A. Mustoe, Augmented Inflammatory Responses and Altered Wound Healing in Cathepsin G-Deficient Mice, Arch.Surg. 133 (1998) pp. 1002-1006

14. D. M. MacIvor, S.D. Shapiro, C.T.N. Pham, A. Belaaouaj, S.N. Abraham, and T.J. Ley, Normal Neutrophil Function in Cathepsin G-Deficient Mice, Blood 94 (1999) pp. 4282-4293

15. S. J. Weiss, J.T. Curnutte, and S. Regiani, Neutrophil-Mediated Solubilization of the Subendothelial Matrix - Oxidative and Nonoxidative Mechanisms of Proteolysis Used by Normal and Chronic Granulomatous-Disease Phagocytes, J.Immunol. 136 (1986) pp. 636-641

16. S. Herrick, G. Ashcroft, G. Ireland, M. Horan, C. McCollum, and M. Ferguson, Up-Regulation of Elastase in Acute Wounds of Healthy Aged Humans and Chronic Venous Leg Ulcers are Associated With Matrix Degradation, Lab.Invest. 77 (1997) pp. 281-288

17. S. J. Weiss, Tissue Destruction by Neutrophils, N.Engl.J.Med 320 (9-2-1989) pp. 365-376

18. S. J. Weiss, Tissue Destruction by Neutrophils, N.Engl.J.Med. 320 (1989) pp. 365-376

19. C. T. Lee, A.M. Fein, M. Lippmann, H. Holtzman, P. Kimbel, and G. Weinbaum, Elastolytic Activity in Pulmonary Lavage Fluid from Patients with Adult Respiratory-Distress Syndrome, N.Engl.J.Med 304 (1981) pp. 192-196

20. T.F. Boat, M.J. Welsh, and A.L. Beaudet, Cystic Fibrosis, In: C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle (Eds.), The Metabolic Basis of Inherited Diseases, 1989 pp. 2649-2680.

21. T. Chimura, T. Hirayama, and M. Takase, Lactoferrin in Cervical Mucus of Patients with Chorioamnionitis, Jap.J.Antibiot 46 (1993) pp. 318-22

22. R. P. Nugent, M.A. Krohn, and S.L. Hillier, Reliability of Diagnosing Bacterial Vaginosis Is Improved by A Standardized Method of Gram Stain Interpretation, J.Clin.Microbiol. 29 (1991) pp. 297-301

23. P. A. Fraser, J. Teasdale, K.S. Gan, R. Eglin, S.C. Scott, and C.J.N. Lacey, Neutrophil Enzymes in Urine for the Detection of Urethral Infection in Men, Genitourin.Med 71 (1995) pp. 176-179

24. F. Grinnell and M.F. Zhu, Fibronectin Degradation in Chronic Wounds Depends on the Relative Levels of Elastase, alpha 1-Proteinase Inhibitor, and alpha 2-Macroglobulin, J.Invest.Dermatol. 106 (1996) pp. 335-341

25. D. R. Yager, S.I. Ward, O.O. Olutoye, R.F. Diegelman, and I.K. Cohen, Ability of Chronic Wound Fluids to Degrade Peptide Growth Factors is Associated with Increased Levels of Elastase Activity and Diminished Levels of Proteinase Inhibitors, Wound Repair.Regen. 5 (1997) pp. 23-32

26. C. N. Rao, D.A. Ladin, Y.Y. Liu, K. Chilukuri, Z.Z. Hou, and D.T. Woodley, Alpha 1-Antitrypsin is Degraded and Non-Functional in Chronic Wounds but Intact and

Functional in Acute Wounds: The Inhibitor Protects Fibronectin from Degradation by Chronic Wound Fluid Enzymes, J.Invest.Dermatol. 105 (1995) pp. 572-8

27. M. A. Selak, M. Chignard, and J.B. Smith, Cathepsin G is a Strong Platelet Agonist Released by Neutrophils, Biochem.J. 251 (1988) pp. 293-299

28. W. M. Shafer, J. Pohl, V.C. Onunka, N. Bangalore, and J. Travis, Human Lysosomal Cathepsin G and Granzyme B Share a Functionally Conserved Broad Spectrum Antibacterial Peptide, J.Biochem. 266 (1991) pp. 112-116

29. C. F. Reilly, D.A. Tewksbury, N.M. Schechter, and J. Travis, Rapid Conversion of Angiotensin I to Angiotensin II by Neutrophil and Mast Cell Proteinases, J.Biol.Chem 257 (1982) pp. 8619-8622

30. P. T. Turkington, Cathepsin G, a Regulator of Human Vitamin K, Dependent Clotting Factors and Inhibitors, Throm.Res. 67 (1992) pp. 147-155

31. P. I. Bird, Regulation of Pro-Apoptotic Leucocyte Granule Serine Proteinases by Intracellular Serpins, Immunol.Cell.Biol. 77 (1999) pp. 47-57

32. H. Hofer, E. Kukovetz, G. Egger, G. Khoschsorur, R. Wildburger, W. Petek, and R. Schaur, PMN-Related Parameters for the Monitoring of Wound healing in Traumatology, Eur.J.Orthop.Surg.Traumatol. 5 (1995) pp. 21-26

33. T. Tsaka and K.R. Herkner, Polymorphonuclear Elastase in Neonatal Sepsis, Clin.Chim.Acta 193 (14-12-1990) pp. 103-111

34. K. M. Peters, K. Koberg, T. Rosendahl, and H.D. Haubeck, PMN Elastase in Bone and Joint Infections, Int.Orthop. 18 (1994) pp. 352-355

35. T.J. Williams, Factors that Affect Vessel Reactivity a Leukocyte Emigration, In: R.A.F. Clark and P.M. Henson(Eds.), Molecular and Cellular Biology of Wound Repair, 1988. pp. 115-183.

36. A. B. Groeneveld, P.G. Raijmakers, C.E. Hack, and L.G. Thijs, Interleukin 8-Related Neutrophil Elastase and the Severity of the Adult-Respiratory-Distress-Syndrome, Cytokine 7 (1995) pp. 746-752

37. A. Orem, O. Deger, G. Cimsit, and S. Bahadir, Plasma Polymorphonuclear Leukocyte Elastase Levels and its Relation to Disease Activity in Psoriasis, Clin.Chim.Acta. 264 (8-8-1997) pp. 49-56

38. F. B. Smith, F.G. Fowkes, A. Rumley, A.J. Lee, G.D. Lowe, and C.M. Hau, Tissue Plasminogen Activator and Leucocyte Elastase as Predictors of Cardiovascular Events in Subjects with Angina Pectoris: Edinburgh Artery Study, Eur.Heart J. 21 (2000) pp. 1607-13

39. H. P. Hofer, E. Kukovetz, W. Petek, F. Schweighofer, R. Wildburger, and R.J. Schaur, Released PMN Elastase: An Indicator of Postsurgical Uneventful Wound Healing and Early Inflammatory Complications. A Contribution to the Search for Objective Criteria in Wound Healing Monitoring. Injury (1995) pp. 103-106

40. H. P. Hofer, E. Kukovetz, W. Petek, G. Khoschsorur, R. Wildburger, F. Schweighofer, F. Quehenberger, and R.J. Schaur, Biochemical Wound Monitoring. PMN Elastase in Different Healing Stages after Trauma Surgery-Orthopedic Interventions, Unfallchirurg 96 (1993) pp. 292-298

41. N. J. Trengove, S.R. Langton, and M.C. Stacey, Biochemical Analysis of Wound Fluid from Nonhealing and Healing Chronic Leg Ulcers, Wound Repair.Regen. 4 (2002) pp. 234-239

42. V. Le Berre, E. Trevisiol, A. Dagkessamanskaia, S. Sokol, A.M. Caminade, J.P. Majoral, B. Meunier, and J. Franois, Dendrimeric Coating of Glass Slides for Sensitive DNA Microarrays Analysis, Nucleic.Acids Res. 31 (2003) pp. 88

43. S. Heumann, A. Eberl, H. Pobeheim, S. Liebminger, G. Fischer-Colbrie, E. Almansa, A. Cavaco-Paulo, and G.M. Gubitz, New Model Substrates for Enzymes Hydrolysing Polyethyleneterephthalate and Polyamide Fibres, J.Biochem.Biophys.Methods 69 (2006) pp. 89-99

44. S. Heumann, A. Eberl, G. Fischer-Colbrie, H. Pobeheim, F. Kaufmann, D. Ribitsch, A. Cavaco-Paulo, and G.M. Guebitz, A Novel Aryl Acylamidase from Nocardia Farcinica Hydrolyses Polyamide, Biotechnol.Bioeng. 102 (2009) pp. 1003-1011

45. A. Eberl, S. Heumann, T. Brueckner, R. Araujo, A. Cavaco-Paulo, F. Kaufmann, W. Kroutil, and G.M. Guebitz, Enzymatic Surface Hydrolysis of Poly(ethylene terephthalate) and Bis(benzoyloxyethyl) terephthalate by Lipase and Cutinase in the Presence of Surface Active Molecules,J.Biotech. 143 (2009) pp. 207-212

46. S. N. Park, J.C. Park, H.O. Kim, M.J. Song, and H. Suh, Characterization of Porous Collagen/Hyaluronic Acid Scaffold Modified by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide Cross-linking, Biomaterials 23 (2002) pp. 1205-1212

47. M. D. Hornof, C.E. Kast, and A. Bernkop-Schnürc, In-vitro Evaluation of the Viscoelastic Properties of Chitosan-Thioglycolic Acid Conjugates, Eur.J.Pharm.Biopharm. 55 (2003) pp. 185-190

48. K. A. Elsaid, G.D. Jay, and C.O. Chichester, Detection of Collagen Type II and Proteoglycans in the Synovial Fluids of Patients Diagnosed with Non-Infectious Knee Joint Synovitis Indicates Early Damage to the Articular Cartilage Matrix, Osteoarthr.Cartilage 11 (2003) pp. 673-680

49. O. Ekerot and K. Ohlsson, Interactions of granulocyte proteases with inhibitors in rheumatoid arthritis, Adv.Exp.Med.Biol. (1984) pp. 335-344

50. S. D. Shapiro, Proteinases in Chronic Obstructive Pulmonary Disease, Biochem.Soc.Trans.30 (2002) pp. 98-102

51. C. Jaffray, J. Yang, G. Carter, C. Mendez, and J. Norman, Pancreatic Elastase Activates Pulmonary Nuclear Factor Kappa B and Inhibitory Kappa B, Mimicking Pancreatitis-ssociated Adult Respiratory Distress Syndrome., Surgery 128 (2000) pp. 225-231

52. G. M. Tremblay, M.F. Janelle, and Y. Bourbonnais, Anti-inflammatory Activity of Neutrophil Elastase Inhibitors, Curr.Opin.Investig.Drugs 4 (2003) pp. 556-565

53. D. R. Yager, S.I. Ward, O.O. Olutoye, R.F. Diegelmann, and I.K. Cohen, Ability of Chronic Wound Fluids to Degrade Peptide Growth Factors is Associated with Increased Levels of Elastase Activity and Diminished Levels of Proteinase Inhibitors, Wound.Repair.Regen. 5 (1997) pp. 23-32

54. F. Grinell and M.F. Zhu, Identification of Neutrophil Elastase as the Proteinase in Burn Wound Fluid Responsible for Degradation of Fibronectin, J.Invest.Dermatol. 103 (1994) pp. 155-164

55. J. Hart, Inflammation. 1: Its Role in the Healing of Acute Wounds, J.Wound Care 11 (2002) pp. 205-209

56. C. J. Sylvia, The Role of Neutrophil Apoptosis in Influencing Tissue Repair, J.Wound Care 12 (2003) pp. 13-6

57. M. Frohm, H. Gunne, A.C. Bergman, B. Agerberth, T. Bergman, A. Boman, S. Liden, H. Jörnvall, and H.G. Boman, Biochemical and Antibacterial Analysis of Human Wound and Bister Fuid, Eur.J.Biochem. 237 (1996) pp. 86-92

58. D. R. Yager, Kulina R.A., and L.A. Gilman, Wound Fluids: a Window into the Wound Environment?, Int.J.Low.Extrem.Wounds 6 (2007) pp. 262-72

59. F. Grinnell and M.F. Zhu, Identification of Neutrophil Elastase as the Proteinase in Burn Wound Fluid Responsible for Degradation of Fibronectin, J.Invest.Dermatol. 103 (1994) pp. 155-161

60. M. J. Castillo, K. Nakajima, M. Zimmerman, and J.C. Powers, Sensitive Substrates for Human Leukocyte and Porcine Pancreatic Elastase: A Study of the Merits of Various Chromophoric and Fluorogenic Leaving Groups in Assays for Serine Proteases, Anal.Biochem. 99 (1979) pp. 53-64

61. K. Nakajima, J.C. Powers, B.M. Ashe, and M. Zimmerman, Mapping the Extended Substrate Binding Site of Cathepsin G and Human Leukocyte Elastase. Studies with Peptide Substrates related to the Alpha 1-Protease Inhibitor Reactive Site, J.Biol.Chem. 254 (1979) pp. 4027-4032

62. R. Hoffman, S. Starkey, and J. Coad, Wound Fluid from Venous Leg Ulcers Degrades Plasminogen and Reduces Plasmin Generation by Keratinocytes, J.Invest.Dermatol. 6 (1998) pp. 1140-1144

63. D. Kamarun, X. Zheng, L. Milanesi, C.A. Hunter, and S. Krause, A Peptide Cross-linked Polyacrylamide Hydrogel for the Detection of Human Neutrophil Elastase, Electrochim.Acta 54 (1-9-2009) pp. 4985-4990

64. J. V. Edwards, S. Caston-Pierre, A.F. Bopp, and W. Goynes, Detection of Human Neutrophil Elastase with Peptide-bound Cross-linked Ethoxylate Acrylate Resin Analogs, J.Pept.Res. 66 (2005) pp. 160-168

65. J. V. Edwards, S. Caston-Piere, P. Howley, B. Condon, and J. Arnold, A Bio-Sensor for Human Neutrophil Elastase Employs Peptide-p-Nitroanilide Cellulose Conjugates, Sensor Lett. 6 (2008) pp. 518-523(6)

66. I. O. Egwim and H.J. Gruber, Spectrophotometric Measurement of Mercaptans with 4,4'-dithiodipyridine., Anal.Biochem. 288 (2001) pp. 188-194

67. U. Schonfelder, M. Abel, C. Wiegand, D. Klemm, P. Elsner, and U.C. Hipler, Influence of Selected Wound Dressings on PMN Elastase in Chronic Wound Fluid and their Antioxidative Potential In-vitro, Biomaterials 26 (2005) pp. 6664-6673

68. C. J. Doillon, C.F. Whyne, S. Brandwein, and F.H. Silver, Collagen-based Wound Dressings: Control of the Pore Structure and Morphology, J.Biomed.Mater.Res. 20 (1986) pp. 1219-1228

69. C. J. Doillon and F.H. Silver, Collagen-based Wound Dressing: Effects of Hyaluronic Acid and Fibronectin on Wound Healing, Biomaterials 7 (1986) pp. 3-8

70. W. Friess, Collagen-biomaterial for Drug Delivery, Eur.J.Pharmaceut.Biopharmaceut. 45 (1998) pp. 113-136

71. D. Brett, A Review of Collagen and Collagen-based Wound Dressings: Collagen-based Wound Dressings, Wounds (2008)

72. T. Shunqing, M.V. Scott, H. Hu-Ping, and S. Myron, Fabrication and Characterization of Porous Hyaluronic Acid-collagen Composite Scaffolds, J.Biomed.Mater.Res. 82 (2007) pp. 323-335

73. K. Sai and M. Babu, Collagen Based Dressings-a Review, Burns 26 (2000) pp. 54-62

74. M. E. Nimni, D. Cheung, B. Strates, M. Dokama, and K. Sheikh, Chemically Modified Collagen: A Natural Biomaterial for Tssue Replacement, J.Biomed.Mater.Res. (1987) pp. 741-771

75. J. M. Pachence, Collagen-based Devices for Soft Tissue Repair, J.Biomed.Mater Res. 33 (1996) pp. 35-40

76. C. H. Lee, A. Singla, and Y. Lee, Biomedical Applications of Collagen, Int.J.Pharm. 221 (2001) pp. 1-22

77. T. Brückner, A. Eberl, S. Heumann, M. Rabe, and G. M. Guebitz, Enzymatic and Chemical Hydrolysis of Poly(ethylene terephthalate)Fabrics. J.Polym.Sci.Pol.Chem. (2008) pp. 6435 - 6443

78. R. Ulbrich, R. Golbik, and A. Schellenberger, Protein Adsorption and Leakage in Carrier-enzyme System, Biotechnol.Bioeng. 37 (1991) pp. 280-287

79. D. Leckband and R. Langer, An Approach for the Stable Immobilization of Proteins, Biotechnol.Bioeng. 37 (1991) pp. 227-237

6

GENERAL CONCLUSION AND POTENTIAL APPLICATION

1 General Conclusion

In this work, activities of enzymes which are associated with the human immune system were determined directly in wound fluid. Four different enzymes namely lysozyme, myeloperoxidase, elastase and cathepsin G were chosen for this work. Levels of all four enzymes were found to be significantly increased in case of wound fluid originating from infected wounds, compared to wound fluids from non-infected wounds and blisters. These results were according well with the clinical description of the attending medical doctors. Thus, the observed difference between infected and non infected wounds probably reflects the number of monocytes/macrophages and neutrophils in the tissue. For all enzymes, there was no significant difference between blisters and non infected post operative or chronic wounds. We have shown that all four enzymes can be used as marker for wound infection. Based on these results different systems for detection of wound infection were developed

Lysozyme

Lysoyzme activity was determined directly in wound fluid samples of post-operative and decubitus ulcer wounds. Lysozyme activity in infected wound fluids was significantly higher (4830 ± 1848 U/ml) compared to non infected wounds (376 ± 240 U/ml). Based on these results different concepts for a fast diagnostic tool for wound infection was investigated. In a

first approach *Micrococcus lysodeikticus* cells were incorporated into agarose-layers. The sensitivity and specificity of lysozyme detection in wound fluids was drastically enhanced by using stained peptidoglycan covalently linked to remazol brilliant blue (RBB) stained. Dyed peptidoglycan was then incorporated into agarose layers or alginate beads. Consequently, incubation of 8% (w/v) labelled agarose/peptidoglycan blend layers with infected wound fluid samples for 2 hours at 37°C resulted in a 4 fold higher amount of dye released than measured for non-infected wounds. For alginate/peptidoglycan beads a 7 fold higher amount of dye was released in case of infected wound fluid samples compared to not-infected ones. Using a double layer system, wound infection can be indicated by simple visual inspection. Incubation of a double layer system consisting of stained and non-stained peptidoglycan with infected wound fluids resulted in a colour change from yellow to blue thus allowing simple visual detection of wound infection. Additionally the impact of matrix-metalloproteinase MMP-9 and elastase was investigated. When dosed in ratios typical for wounds, a slight synergistic effect was measured for peptidoglycan hydrolysis (i.e. dye release) between lysozyme and these proteases.

Myeloperoxidase

For measuring myeloperoxidase activity in wound fluid, a screening for suitable substrates was carried out. Since the common substrate guaiacol is very toxic and not stable, ABTS and TMB were tested. These substrates were classified as very suitable for the application in a diagnostic tool. Additionally, crystal violet and its leuco form were shown to be converted by MPO. N-demethylation seems to be the first step in crystal violet degradation, followed by step-wise deamination, finally leading to the breakdown of the backbone. The conversion of leuco-crystal violet by myeloeroxidase gives a clear colour change within very short incubation times. Problematic in this case is the fact, that crystal violet can also be oxidized by heme from blood.

In an attempt to obtain more information on the structural requirements for substrates to be oxidized efficiently by MPO, model substrates were synthesized. Based on 2,7-dihydroxy-1-phenylazonaphtalene-3,6-disulfonicacid, systematically substituted derivatives were constructed in order to gain mechanistic information on MPO catalysed oxidation. Here, hydroxyl groups and to a lower extent, methyl substituted derivatives were readily oxidized

Since hydrogen peroxide is required for MPO catalysed oxidation of all above substrates, in situ production of hydrogen peroxide was investigated. It was possible to replace hydrogen

peroxide by addition of cellobiose dehydrogenase (EC 1.1.99.15) from *Myriococcum thermophilum* together with cellobiose.

The immobilisation of MPO substrates has advantages especially in case of handling. We were able to show that Fast blue RR can be covalently coupled to siloxanes still remaining a substrate for MPO.

Summarizing our results we have demonstrated the potential of novel MPO substrates for the measurement of activity in wound fluids. Using these substrates, visible colour changes indicate elevated myeloperoxidase levels, thus allowing early diagnosis of wound infection.

Elastase

In this study we have shown the potential of human neutrophil elastase and cathepsin G to be used as marker for infection. These enzymes were determined directly in wound fluid. Significantly higher elastase and cathepsin G activity was detected in infected wounds when compared to non infected wounds ($P \leq 0.05$), measured with commercial substrates. In a further step the immobilisation of specific chromogenic elastase and cathepsin G substrates on different supporting materials was carried out. Materials were selected which are used as bandages or dressings in the field of wound treatment. Hence, substrates for these two enzymes were successfully bound to different surfaces like collagen, HA-Coll, THA-Coll, PA, PES and silica gel. Using this approach, HNE and CatG can be monitored directly in wound fluid via hydrolysis of chromogenic substrates. The degradation of these substrates resulted in a reproducible and selective manner upon incubation with enzymes or wound fluid. Infected wound fluids led to higher substrate hydrolysis compared to non-infected ones. Therefore this system could be used for diagnosis wound infection directly in wound fluid.

2 Potential Application

Wound infection is a common clinical problem which will increase in the next few years due to the aging population and an increasing number of immunodepressive patients [8]. Bacterial infection of wounds is the main course of impaired wound healing and a severe complication regarding the normal wound healing process. Bacterial infection can be a long lasting, in the worst case life-long complication of the normal wound healing process [9]. Diagnosis of infection is very difficult. Normally, infection is diagnosed by evaluating the typical signs of infection like *rubor*, *colour*, *tumor* and *dolor*. For diagnosing wound infection long experience of the attending doctor is necessary. But it has been shown, that these signs of inflammation are often missing. Timely diagnosis of an incipient infection is the critical factor of a successful medical treatment. Since common methods for diagnosing infection are all together time-consuming, physicians are waiting for a method that promises to detect infection in a fast way and, what is more important, prior to obvious clinical symptoms. In conclusion, in the field of wound management there is a b a big need for a diagnostic tool which would allow the early detection of infection before the onset.

During this work, a device for rapid and early diagnosis of wound infection was developed and patented (EP 09160557.6). This so called “rapid diagnostic tool” is based on enzymes present in wound fluid which are associated with an inflammatory response of the human body. The application of appropriate substrates for enzymes, which are known to be elevated in case of infection leads to a colour reaction within minutes and allows a very fast estimation of the wound status. This diagnostic device for detection of wound infection would allow an early and suitable treatment and could reduce clinical intervention and the use of antibiotics. Also, such device can be used for fast on-site assessment such as in home care without requiring trained personal and special equipment.

The diagnostic device is based on a fast colour based system exploiting a combination of elevated levels of elastase, cathepsin G, myeloperoxidase and lysozyme levels in infected wound fluid. A colour reaction associated with a certain level of enzyme present in wound fluid allows the likelihood or the presence of clinical infection to be assessed. Finally, since the ratio of activities of the four individual enzyme activities can vary considerably, it is essential to base the judgement on a combination of four enzyme activities to increase.

With this diagnostic device, wounds can be diagnosed for infection according to the methods described. The device is envisaged as being most useful in diagnosing clinical infection of chronic wounds like venous ulcers, pressure sores, diabetic ulcers and post-operative wounds.

7

APPENDIX

1 Publications

1.1 Papers

A. Hasmann, E. Wehrschütz-Sigl, G. Kanzler, U. Gewessler, E. Hulla, K. P. Schneider, B. Binder, M. Schintler, and G. M. Guebitz, Novel Peptidoglycan Based Diagnostic Devices for Detection of Wound Infection, Diag.Micr.Infec.Dis., in press

A. Hasmann, E. Wehrschütz-Sigl, A. Marold, H. Wiesbauer, R. Schoeftner, U. Gewessler, A. Kandelbauer, Doris Schiffer, K. P. Schneider, B. Binder, M. Schintler, and G. M. Guebitz, Analysis of Myeloperoxidase in Wound Fluids as Marker for Infection, Ann.Clin.Biochem., submitted

A. Hasmann, U. Gewessler, E. Hulla, K. P. Schneider, A. Francesco, Wehrschütz-Sigl, M. Schintler, J. Van Palen, G. M. Guebitz, and E. Wehrschuetz-Sigl, New Sensor Materials for the Detection of Human Neutrophil Elastase- and Cathepsin G-Activity in Wound Fluid, Exp.Dermatol., in press

K. P. Schneider, A. Rollett, E. Wehrschütz-Sigl, **A. Hasmann**, A. Zankel, A. Muehlebach, F. Kaufman and G. M. Guebitz, Bioresponsive Systems based on Polygalacturonate containing Hydrogels, Enzyme.Microb.Tech., in press

I. Perelshtein, G. Applerot, N. Perkas, E. Wehrschütz-Sigl, **A. Hasmann,** G. M. Guebitz and A. Gedanken, CuO-Textile Nanocomposite: Morphology and Antibacterial Activity, Surf.Coat.Technol. 204 (2009) pp. 254-257

I. Perelshtein, G. Applerot, N. Perkas, E. Wehrschütz-Sigl, **A. Hasmann,** G. M. Guebitz and A. Gedanken, Antibacterial Properties of an In Situ Generated and Simultaneously Deposited Nanocrystalline ZnO on Fabrics, ACS Appl.Mater.Interfaces 1 (2009) pp. 361-366

I. Perelshtein, N. Perkas, G. Applerot, E. Wehrschütz-Sigl, **A. Hasmann,** E. Hulla, G. M. Guebitz and A. Gedanken, Ultrasound Radiation as a "Throwing Stones" Technique for the Production of Antibacterial Nanocomposite Textiles, ACS Appl.Mater.Interfaces 2(7) (2010) pp.1999-2004

1.2 Posters

A. Hasmann, K. Schneider, E. Wehrschütz-Sigl, and G.M. Gübitz, Bioresponsive polymers for wound treatment, ÖGBT Symposium, (2007)

A. Hasmann, K. Schneider, E. Wehrschütz-Sigl, A. Vasconcelos, M. Fernande, B. Barros, A. Cavaco-Paulo, and G. M. Gübitz, New strategies for improved wound treatment involving bioresponsive polymers, 8th advanced summer course in cell materials interactions, Porto, Portugal (2007)

A. Vasconcelos, M. Fernandes, B. Barro, **A. Hasmann**, E. Wehrschütz-Sigl, K. Schneider, G.M. Gübitz, and A. Cavaco-Paulo, Basic characterization of keratin material as matrices for drug delivery systems, 8th advanced summer course in cell materials interactions, Porto, Portugal (2007)

A. Hasmann, E. Wehrschütz-Sigl, K. Schneider, and G. M. Gübitz, Bioresponsive Polymers for Wound Treatment, COST 868 Working Group Meeting, Graz, Austria (2007)

A. Hasmann, E. Wehrschütz-Sigl, K. Schneider, A. Eberl, S. Heumann, and G. M. Gübitz, Bioresponsive Polymers: A new Concept for controlled Drug Release Device, The 5th International Conference on Textile Biotechnology, Wuxi, China (2007)

K. Schneider, M. Schröder, A. Rollett, **A. Hasmann**, E. Wehrschütz-Sigl, and G. M. Gübitz, Trigger enzymes for pectin based bioresponsive polymers, 16th International Conference on Bioencapsulation, Dublin, Ireland (2008)

A. Hasmann, E. Wehrschütz-Sigl, G. Kanzler, K. Schneider, M. Schröder, and G. M. Gübitz, A new concept for controlled drug release devices based on enzyme activity in wound fluids. 16th International Conference on Bioencapsulation, Dublin, Ireland, (2008)

A. Hasmann, E. Wehrschütz-Sigl, G. Kanzler, K. Schneider, M. Schröder, and G. M. Gübitz, A new model system for enzymatic drug release devices due to enzyme activity in wound fluids, COST 868 Working Group Meeting, Varna, Bulgaria, (2008)

A. Hasmann, E. Wehrschütz-Sigl, G. Kanzler, K. Schneider, M. Schröder, and G. M. Gübitz, A new model system for enzymatic drug release, ÖGBT Symposium, Graz, Austria, (2008)

K. Schneider, M. Schröder, A. Rollett, **A. Hasmann,** E. Wehschütz-Sigl, and G. M. Gübitz, Pectin based bioresponsive polymers, ÖGBT Symposium, Graz, Austria, (2008)

E. Wehrschütz-Sigl, A. **Hasmann**, G. Kanzler, U. Gewessler, and G. M. Gübitz, Novel diagnostic devices for detection of infection in decubitus ulcer wounds. RCPE Symposium. Graz, Austria, (2009)

1.3 Oral Presentations

A. Hasmann, New strategies for wound treatment involving bioresponsive polymers, Cost 868 Working Group Meeting, Sitges, Spain, (2007)

E. Wehrschütz-Sigl, A. **Hasmann**, G. Kanzler, U. Mandl-Schweiger, U. Geweßler, and G. M. Gübitz, Lysozyme as a trigger enzyme for diagnostic systems, Cost 868 Working Group Meeting, Procchio, Italy, 18.05.2009

A. Francesko, **A. Hasmann**, K. Schneider, E. Wehrschütz-Sigl, G. M. Gübitz and T. Tzanov, Enzymatic acivity in wound flluids, COST 868 Working Group Meeting, Graz, Austria, (2007)

K. Schneider, **A. Hasmann,** A. Rollett, A. Marold, T. Flock and G.M. Gübitz, Polysaccharides based bioresponsive polymer composites. RCPE Symposium, Graz, Austria, (2009)

1.4 Patents

A. Hasmann, E. Wehrschütz-Sigl, and G. M. Gübitz, Infact: Diagnose von Wundinfektion, EP 09160557

M. Schröder, K. Schneider, A. Rollett, F. Kaufmann, A. Hafner, **A. Hasmann**, E. Wehrschütz-Sigl, and G. M. Gübitz, Enhanzyme: Bioresponsives System mit enzymatischer Verstärkungsreaktion zur kontrollierten Freisetzung und Sensorik

A. Hasmann, E. Almansa, A. Cavaco-Paulo, H. Chrobazcek, A. Eberl, A. Glieder, G. M. Gübitz, S. Heumann, R. Niederstadt, and K. Schöning, Biocatalytic hydrophilization of polyolefines

1.5 Book Chapters

E. Wehrschütz-Sigl, **A. Hasmann** and G. M. Gübitz, Smart Materials containing Enzymes or Enzyme Substrates In: V. Nierstrasz and A. Cavaco-Paulo (Eds), Advances in Textil-Biotechnology, Woodhead Publishing, India, 2010

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3 Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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