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Enzymes in wound fluids as triggers for diagnostic tools

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Abstract

This work was embedded in the European Lidwine project dealing with the treatment of Decubitus Ulcer. A release system triggered by enzymes present in chronic infected wounds was developed. Two different kinds of diagnostic tools were investigated for a qualitative analysis of wounds concerning microbial environment to make a statement about the condition of a wound. Therefore wound fluids were screened for enzymes. Lysozyme and collagenase turned out to be present in infected wounds in enhanced levels but only in low levels in recovering wounds and blister fluids. These enzymes were thus chosen as trigger enzymes for the release system. The system consisted of a polymer matrix blended with peptidoglycan as substrate for lysozyme. The matrix consisted of agarose in the first approach and was realised in transparent well plates. Linear correlation between transparency increase and lysozyme activity applied was found below 100 U/ml. Differentiation with the naked eye between blank and activities of 1,250 – 500 U/ml was possible. In the second approach an alginate matrix was polymerised in $CaCl_2$ solution forming stable beads while incorporated peptidoglycan was stained with Remazol Brilliant Blue. In parallel experiments the alginate matrix was blended with gelatin in different ratios. All beads were incubated with 250 U/ml of lysozyme as well as co-incubated with each 250 U/ml of lysozyme and collagenase. Enzyme incubation solutions of pure alginate beads turned deep blue after 200 minutes compared to incubation solutions of buffer incubation experiments. The differentiation between a sample of wound fluid containing 2,000 U/ml lysozyme and a sample of blister fluid containing 200 U/ml lysozyme was clearly possible via photometric measurement as well as with the naked eye. Incubation of beads blended with gelatin yielded the same but higher absorbance distribution as pure alginate beads. Indeed, the diagnostic tool was able to indicate infection in wound fluids while there was no signal in blister fluids used as controls.

1 Lidwine project

The Lidwine project is an EU-funded integrated project dedicated to SMEs (IP-SME). It focuses on the development of multifunctional medical textiles to prevent wounds such as Decubitus on the one hand and to stimulate wound healing on the other hand. The project has a socio-economic impact on the European citizen since it aims to reduce additional costs for treatments resulting from the life span extension of the human population and as well enhances quality of life. Prevention of Decubitus is realized by stimulation of the blood circulation (massage and electrotherapy), by reducing the friction between skin and textile (textile treatments at nanoscale), by keeping the skin dry (surface treatment and textile design) and by preventing bacterial infections (functionalized nanoparticles).

The work of the TUGraz is concerning the development of new drug release systems with switchable capabilities. The switch function is meant to be activated upon body fluid from the wound in case of infection to enable microbial characterisation of a wound. The work thus includes the identification of major microorganisms in wounds and their extracellular enzymes.

2 Introduction

In this chapter the processes of wound healing are explained as well as factors that are supposed to delay normal wound healing leading to the formation of pressure sores. Key enzymes of the healing process being able to trigger an enzymatic realease in diagnostic application and the matrix biopolymers alginate, agarose and gelatin are described more in detail.

2.1 Decubitus ulcer

The terminus “Decubitus” derives from the Latin word “decubare” and means to lie down (Bienstein 1997).

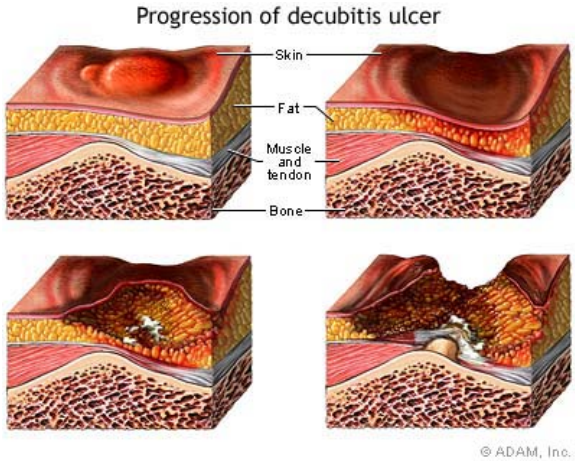
Decubitus ulcer (also known as pressure ulcer, pressure sore and bed sore) is defined as “an injury to the skin as a result of constant pressure due to staying in one position without moving” (DHS 2007, p 1).

Long term exposure to pressure leads to insufficient blood supply and causes necrosis of tissue which in terms leads to a delayed wound healing and generally to chronic and open wounds respectively (Asmussen and Söllner 2005).

In addition to external factors such as pressure, shear and friction forces internal factors such as old age, immobility, incontinence, catabolic illnesses and malnutrition support the development of pressure ulcers. Old age commonly accompanies malnutrition leading to a lack of nutrients (proteins, energy, vitamins B, C, D, folic acid) needed for wound healing (proteins, energy, arginine, zink, vitamins A, B, C) (Mathus-Vliegen 2004).

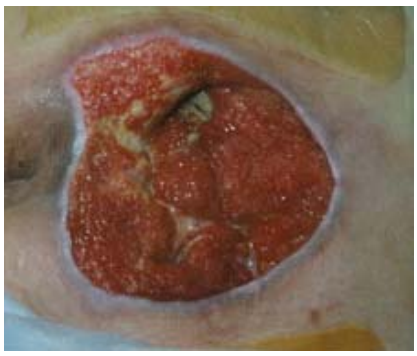
Pressure ulcers usually arise at vulnerable areas such as heels, sacral area and shoulders and are classified in four stages depending on the distribution of pressure and the architecture of vessels. The progression of decubitus ulcer is shown in Picture 1.

The first stage is a persistent reddening of the skin. It’s reversible when relieving the skin of pressure. In the second stage the epithelium, the surface of the skin, is damaged and blisters arise because of long term pressure. Wounds in this stage heal without leaving scars when medically treated. Stage three is an open wound, the corium becomes necrotic and in the fourth stage the necrosis exposes even the bone. Wounds of stages three and four heal under the development of scars, as far as they are healing (Bienstein 1997).



Picture 1: Progression of Decubitus Ulcer: stage 1 (above left), stage 2 (above right), stage 3 (below left) and stage 4 (below right) (© A.D.A.M., Inc)

Pressure ulcers generally result in open, non healing wounds. A chronic wound of stage four is shown in Picture 2.



Picture 2: Pressure ulcer stage 4 (© European Pressure Ulcer Advisory Panel)

Chronic wounds are defined as wounds which are not showing any tendency of healing in the first twelve weeks or which do not heal within twelve months despite of adequate causal and local medical care (Welt et al. 2005).

All open wounds are contaminated (presence of non-proliferating bacteria) or colonized (presence of bacteria which do proliferate or initiate a host reaction but do not affect the healing process) by bacteria but chronic wounds are likely to be infected. Classic signs of infected wounds are tumour, dolour, foul odour, pain, necrotic tissue. In contrast to colonization, infected wounds are associated with a host immune reaction and normal wound healing is impaired (Collier 2004; Bjarnsholt et al. 2008).

Infected open wounds, especially stages III and IV, have to be relieved of devitalised tissue by surgical debridement as far as antibiotic treatment fails (Schiffman et al. 2009).

Until now, there's no understanding about why chronic wounds fail to heal. An imbalance in biochemical processes and key enzymes is investigated and furthermore there's strong evidence that the formation of biofilms in chronic wounds influence the healing process (see 2.2.4).

2.2 Healing of wounds

The functioning of the immune system is essential for normal wound healing. A short introduction is therefore given here and its influence on the wound healing process. The background of the non-healing of chronic wounds isn't fully understood so far. Molecular dysfunctions and further factors of influence are described in this chapter.

2.2.1 Immune system

The immune system is the defence system against infections. It consists of the innate immunity and the specific acquired immunity. The first to defend against invading microbes is the innate immunity and most of the potential pathogens can be rendered harmless. The specific acquired immunity has developed evolutionary and specifically reacts to pathogens having passed the innate immunity (Roitt, Brostoff and Male 1991).
The immune system consists of a variety of molecules and cells. The most important cells are the leukocytes. They are divided into phagocytes which are part of the innate immunity and the lymphocytes mediating the specific acquired immunity (Roitt, Brostoff and Male 1991).

I Innate immunity

The skin as external barrier prevents potential pathogens from penetrating the body. If they manage to pass through, e.g. in case of injury, they are defended by two main operations. Soluble chemical factors on the one hand and the mechanism of phagocytosis on the other hand have a destructive effect on microbes (Delves 2006).

Phagocytosis is the engulfment and digestion of microorganisms and is carried out by the two leukocyte cell types' polymorphonuclear neutrophils and macrophages. Neutrophils are nondividing short-lived cells with granules containing antimicrobial effectors. Primary azurophil granules contain myeloperoxidase, defensins, cathepsin G, lysozyme, elastase and

the bactericidal permeability increasing protein (BPI). Secondary specific granules are peroxidase-negative and contain lactoferrin, much of lysozyme, OH^--phosphatase and cytochrome b_{558}. Neutrophils share the same stem cell precursor as macrophages (Delves 2006). Macrophages are mononuclear phagocytes and emerge from blood monocytes entering the tissue (Asmussen and Söllner 2005). They are long-lived cells and defend against bacteria, viruses and protozoa (Delves 2006).

Phagocytosis is supported by mediators and receptors. In case of penetration of microorganisms acute phase proteins and the complement system is activated. The complement system is a cascade of around 20 proteins and peptides respectively which mediate an inflammation. Peptides with different effects on pathogens are produced: they bind to microorganisms to facilitate phagocytosis (opsonation), attract phagocytes to the place of infection in a process called chemotaxis or even destroy invading cells. Other peptides enhance blood circulation and permeability of capillaries for molecules at the place of reaction (Roitt, Brostoff and Male 1991).

Acute phase proteins are enhanced in concentration as long as the infection persists. The C-reactive protein is, like the mannose binding lectin, dramatically increasing and binds to the membrane of microorganisms to facilitate opsonation by the complement system (Roitt, Brostoff and Male 1991).

Phagocytes possess pattern recognition receptors (PRRs) like the toll-like receptor (TLR) that make it possible for them to discriminate between self-components and infectious pathogens by recognizing conserved pathogen-associated molecular patterns (PAMPs) expressed by pathogens. Once recognised the ingestion phase is initiated and the pathogen rendered harmless by different microbicidal mechanisms. Reactive oxygen intermediates, reactive nitrogen intermediates or preformed antimicrobials finally kill the microorganisms (Delves 2006). Reactive oxygen intermediates are used in a myeloperoxidase-H_2O_2-Cl^- system to form hypochlorous acid (HOCl) that converts amino acids to amino acid chloramines (see 2.2.5III) (Harrison et al. 1975). This mechanism is dedicated to the killing of extracellular organisms after engulfment. Microbes invading the cytosol are killed by the $NO^{\cdot}$ mechanism. $NO^{\cdot}$ is formed by $NO^{\cdot}$-synthase and the antimicrobial system ends up with the production of the toxic $^{\cdot}ONOO$ radical. An oxygen-independent mechanism is based on preformed molecules with amphipathic structures (defensins) which insert into microbial membranes to form destabilizing voltage-regulated ion channels. Further destruction of the bacterial membrane is done by transfer to BPI (bactericidal permeability increasing protein), by the neutral

proteinase cathepsin G, lysozyme and lactoferrin. The last-named two enzymes are working synergistically and can function under anaerobic conditions (Delves 2006).
Natural killer cells (NK) are huge granulated lymphocytes which are able to recognise changes in the surface of cells being infected by viruses or are perniciously degenerated. In a process called cytotoxicity they bind to these cells and kill them (Roitt, Brostoff and Male 1991).

II Specific acquired immunity

The specific acquired immunity is evolutionary developed and is the supplementation to the innate immunity. The innate immunity is an effective defence system as long as phagocytes recognise invading microorganisms. Problems arise when appropriate receptors are missing or when the complement system is not activated by pathogens (Roitt, Brostoff and Male 1991). Through such mutations microbes evade the innate immunity and the specific acquired immunity is activated. Antibodies are evolutionary developed adapter molecules that have three main functions: communication with complement and the phagocytes and binding to individual microorganisms (Delves 2006).

Lymphocytes are the cells of the specific immunity, special types of leukocytes. Their stem cells differentiate either in the bone marrow to B-lymphocytes or within the milieu of the thymus gland to T-lymphocytes. B-lymphocytes are programmed to produce specific antibodies in response to contact with antigen (generates antibodies). They place it on their outer surface as receptors for antigen binding. B-cells produce only one specific antibody which then proliferates by clonal selection. The cell undergoes many divisions to form a large clone of plasma cells which either mature to effector cells (primary immune response) or to non-dividing memory cells. Memory cells enable the response to any subsequent exposure to the specific antigen (secondary response) (Delves 2006).
T-lymphocytes are responsible for the cell-mediated immunity. In contrast to B-lymphocytes they are not able to recognise free antigens but operate against microorganisms living in host cells such as viruses. T-cells have T-cell receptors (TCR) on their surface and bind to MHC (major histocompatibility complex) - cell markers on the host cell's surface. T-lymphocytes are divided into T-helper cells and cytotoxic T-cells. T-helper cells help infected macrophages by binding to a class II MHC molecules complexed with an antigenic fragment and produce different kinds of cytokines. The cytokines then activate the macrophages microbicidal

mechanisms. Cytotoxic T-cells recognise class I MHC molecules in association with antigen on the host's surface and kill the infected cells. Finally there are T-cells termed suppressor cells which are part of the control system (Delves 2006).

Antibodies of B-lymphocytes and T-cell receptors of T-lymphocytes as antigen recognising molecules belong both to the family of immunoglobulins. Whereas two types of T-cell receptors, TCR 1 and TCR 2, are known there are five different classes of antibodies: immunoglobulin G (IgG), IgA, IgM, IgD and IgE. In contrast to the T-cell receptors antibodies are soluble molecules and are able to recognise free antigens (Roitt, Brostoff and Male 1991).

2.2.2 Normal wound healing

A wound is defined as a pathological state of disrupted and/or destroyed tissue that is associated with a loss of substance and a restriction of functions. This defect is repaired mainly by reparation, the replacement of lost tissue by unspecific elements of the connective and supporting tissue, leading to the formation of a scar (Asmussen and Söllner 2005).
The first to happen after an injury is the reaction of blood vessels. Injured vessels contract to limit blood loss until blood clots are developed. In the following vasodilatation (dilatation of vessels) blood circulation in the site of injury increases and therefore the skin temperature raises. The permeability of vessels is enhanced for components of the blood in this phase. Due to decreased oxygen supply in the tissue, the CO_2-pressure is increasing and, as a consequence, the pH-value is decreasing. Collagen is depolymerising in the acid milieu and looses its ability to bind water. The liquid leads to a swelling of collagen and the process ends up in the wound oedema (Asmussen and Söllner 2005).
Acute wound healing is normally characterised by four distinct but overlapping phases: hemostasis, inflammation, proliferation and remodeling. Numerous cell-signalling events enable this efficient and highly controlled repair process to take place (Diegelmann et al. 2004).
As soon as blood components enter the site of injury platelets start sticking to the vessels and are thus activated. They change their shape from flat discs to spherical forms with pseudopodia (little feet) and clot. During this process called platelet aggregation clotting factors such as fibrinogen and prothrombin, the precursors of fibrin and thrombin, are released. Thrombin catalysis the conversion of fibrinogen and the resulting fibrin monomers polymerise to seal the wound (hemostasis) (Asmussen and Söllner 2005). Platelets also

release cytokines and growth factors (see 2.2.3) to initiate the healing process. Growth factors like the platelet-derived growth factor (PDGF) or the transforming growth factor-beta (TGF-β) initiate chemotaxis of neutrophils, attract macrophages and also stimulate the synthesis of new tissue. Neutrophils are the first to arrive at the site of injury in the following inflammatory phase. Their main function is to remove foreign material: potential pathogens, non-functional host cells and damaged matrix components. Neutrophils are also attracted by chemical signals released by bacteria and phagocytosis is initiated. Within 48 hours after injury the concentration of neutrophils decreases and blood monocytes mature to wound macrophages. They are responsible for removing foreign material and bacteria-filled neutrophils. Like neutrophils they release growth factors that further attract fibroblasts and smooth muscle cells (Diegelmann et al. 2004). The inflammation phase is triggered independently of invading microorganisms. An open wound is yet usually contaminated so that not only the innate immunity but also the acquired immunity is activated. While catabolic processes are predominant in the inflammatory phase to clean the wound the proliferation phase is more or less anabolic (Asmussen and Söllner 2005). Growth factors like TGF-β, vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF) regulate matrix synthesis and angiogenesis. TGF-β is released by platelets, lymphocytes and macrophages and mediate fibroblast functions such as the synthesis of collagen, proteoglycan and fibronectin to create the new matrix. It also reduces the secretion of proteases and stimulates their inhibitors to prevent the new tissue from degradation. Sufficient blood supply is essential for anabolic processes. Especially collagen-synthesis needs aerobic conditions and depends on vitamin C and trace elements as coenzymes and cofactors respectively for enzymes. Fibroblasts, macrophages, epidermal cells and vascular endothelial cells release VEGF, bFGF and TGF-β which stimulate the formation of new vessels (angiogenesis, vascularisation) (Diegelmann et al. 2004). Endothelial cells of intact vessels produce enzymes to degrade parts of the basal membrane. New vessels are generated by merging endothelial cells and differentiated to arteries and veins (Asmussen and Söllner 2005).

In the last phase of the wound healing process, the remodelling phase, collagen fibres are cross-linked by lysyl oxidase and also partly degraded by collagenases released by fibroblasts, neutrophils and macrophages (Diegelmann et al. 2004). Fibroblasts lose their mitotic activity and convert to fibrocytes and myofibroblasts. Like muscle cells they possess contractile elements and tighten the new tissue. In the end, ephidelial cells migrate and proliferate to close the surface of the wound (Epithelisation) (Asmussen and Söllner 2005).

2.2.3 Cytokines and growth factors

Cytokines, growth factors and chemokines are involved in the complex signaling network that is essential for wound healing. They are biologically active polypeptides and coordinate growth, differentiation and metabolism of cells. They are released by platelets, macrophages, neutrophils, fibroblasts, keratinocytes, endothelial cells and smooth muscle cells and act by binding to specific cell surface receptors or ECM proteins. The most important growth factors and cytokines for wound healing are the epidermal growth factor (EGF) family, fibroblast growth factor (FGF) family, transforming growth factor beta (TGF-β) family, platelet derived growth factor (PDGF) family, vascular endothelial growth factor (VEGF), interleukin (IL) family and tumor necrosis factor alpha (TNF-α) family (Barrientos et al. 2008).

Table 1: Important growth factors and cytokines and their function in wound healing

growth factor/cytokine	function
EGF	reepithelialisation
FGF-2	granulation tissue formation
	reepithelialization
	matrix formation and remodelling
TGF-β, PDGF	inflammation
	granulation tissue formation
	reepithelialisation
	matrix formation and remodelling
VEGF	granulation tissue formation
IL-1, IL-6, TNF-α	inflammation
	reepithelialisation

2.2.4 Delayed wound healing - chronic wounds

Wound healing is a complex cascade of events. The successful proceeding of each stage depends on accurately coordinated biochemical processes of the former stage. Disruption of these processes delays or even inhibits wound healing and can lead to the formation of chronic wounds.

Chronic wounds seem to remain in the inflammatory phase. Neutrophile granulozytes accumulate in the wound and release excessive amounts of proteases such as matrix metalloproteinases (MMPs) and neutrophile elastase. In acute wounds proteases are

responsible for degrading the extracellular matrix and foreign material and they are in balance with cytokines and growth factors. High levels of protease in chronic wounds cause the degradation of cytokines and growth factors such as PDGF, TGF-β, EGF (epidermal growth factor) and bFGF which are responsible for the mediation of the molecular processes of wound healing (Lobmann et al. 2005).

Matrix metalloproteinases are about 20 mammalian neutral pH proteinases. They are very prominent in chronic wounds, especially levels of collagenases (MMP-1, MMP-8 and MMP-13) and gelatinases (MMP-2 and especially MMP-9) are significantly increased (Yager et al. 1999). Collagenases are capable of degrading triple helical fibrillar collagen whereas gelatinases digest partial denatured and nonfibrillar collagens. Stromelysins (MMP-3, MMP-10 and MMP-11) degrade proteoglycans, non-fibrillar collagens and non-collagen components but their activity is generally enhanced later in wound repair (Lobmann et al. 2005). Pro-inflammatory cytokines like interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) stimulate the production of MMPs. They are apparently not degraded by proteases and show an extremely elevated level in non-healing wounds in contrast to acute wounds. The resulting activity of MMPs is therefore enhanced up to 30 fold (Trengove et al. 1999).

Beside the imbalance of key proteases, cytokines and growth factors there are reduced levels of tissue inhibitors for MMPs termed TIMPs. They are balanced with the proteases in good healing wounds but their reduced levels in chronic wounds and the enhanced activity of MMPs seem to contribute to a delayed wound healing. Ladewig *et al*. showed an inverse correlation of the MMP-9/TIMP-1 ratio with healing of pressure ulcers. MMP-9 as well as MMP-8 is produced by inflammatory cells and they normally decrease in acute wounds and healing proceeds to the proliferation phase. Persistent elevated levels of MMPs indicate that the healing process is stuck in the inflammatory phase (Lobmann et al. 2005).

Senescent cells also seem to be important in chronic wounds. Dermal fibroblasts loose their responsiveness to growth hormones when getting older and may further delay wound healing for this reason (Harding et al. 2002).

Apart from these imbalances of the immune response the invasion of microorganisms in the wound may influence the healing process. Every wound is contaminated with microbes. Especially devitalised exposed tissue facilitates the colonisation of microorganisms originating from the environment, the surrounding skin and endogenous sources. Their variety enhances the longer the tissue is exposed. The microbial population consists of aerobic and anaerobic microbes as well as facultative anaerobes. Due to poor blood supply and cell

metabolism the local pO_2 is decreasing and anaerobes proliferate when the residual oxygen is consumed by facultative microorganisms. Either a wound is infected or not depends on different microbial and host factors like the quantity of microbial load, the expression of virulence factors, parameters of the wound and the immune status of the host. Aerobe pathogens such as *Staphylococcus aureus* or *Pseudomonas aeruginosa* as well as anaerobes like *Prevotella* are known for the production of virulence factors but the composition of the polymicrobial wound flora seems to be more important for wound healing. Aerobic-anaerobic synergistic interactions may increase or even enable pathogenic effects (Bowler et al. 2001).

In addition, the formation of biofilms in infected wounds has been proposed to influence pathogenesis. Biofilms are bacterial communities embedded in a self- and host-produced extracellular matrix (ECM) of secreted exopolymeric substances (EPS). The matrix consists of biopolymers (polysaccharides) that also contain extracellular DNA, proteins and cellular debris. The ECM adheres to the wound surface and serves as a protection system. Bacteria within biofilms exhibit a reduced susceptibility to antibiotics compared to planktonic (free-floating) bacteria (Davis et al. 2008) as well as an extreme tolerance to the activities of the immune defense. Resistances are at least partly regulated by the cell-cell communication system termed quorum sensing (QS). It governs the regulation and expression of virulence factors in accordance with the present habitat. Bjarnsholt et al (2008) investigated *Pseudomonas aeruginosa* biofilms and found out that QS products eliminate PMNs to protect the bacteria from phagocytotic activity.

2.2.5 Bacteria and enzymes present in wound fluids

Aerobic as well as anaerobic bacteria are present in wound fluids. Literature (Brook et al. 1991; Ehrenkranz et al. 1990) and the results of the Lidwine project (see Table 19) were in agreement, the predominant bacteria in Decubitus Ulcer wounds are shown in Table 2 and Table 3. The most common aerobic gram positive bacteria are *Enterococcus species* and *Staphylococcus aureus*, the most common gram positive aerobes *Pseudomonas aeruginosa* and *Proteus species*. Predominant anaerobes are *Peptostreptococcus spp.* and *Proteus spp*.

Table 2: Aerobic bacteria present in wound fluids

aerobic bacteria

gram positive	gram negative
Corynebacterium spp	*Klebsiella pneumoniae*
Enterococcus spp	*Proteus spp*
Streptococcus group	*Pseudomonas aeruginosa*
Staphylococcus aureus	*Morganella morganii*
Staphylococcus epidermidis	*Escherichia coli*

Table 3: Anaerobic bacteria present in wound fluids

anaerobe bacteria

gram positive	gram negative
Peptostreptococcus spp.	*Bacteroides sp.*
Clostridium spp.	*Prevotella*
Eubacterium sp.	*Fusobacterium*

Enzymes present in wound fluids originate from the immune response, are secreted by invading bacteria or are of both origins. An overview is given in Figure 1. Immune response enzymes such as lysozyme, myeloperoxidase, cathepsin G and MMPs derives from the granules of phagozytes. Bacteria in infected wounds secrete enzymes such as β-lactamase or hyaluronidase as virulence factors.

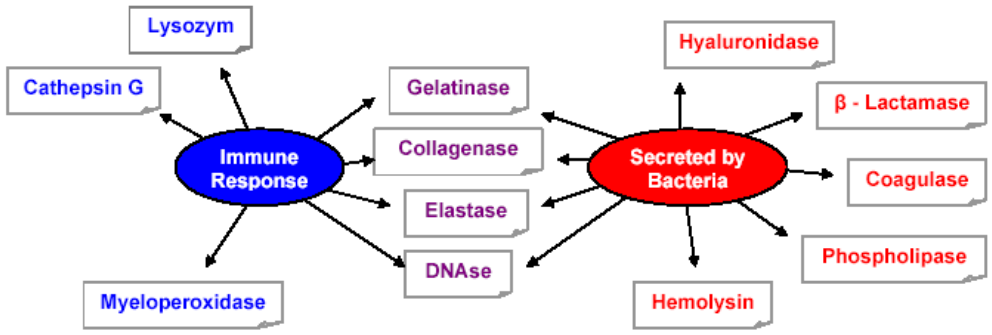


Figure 1: Enzymes present in wound fluids

Lysozyme, collagenases, myeloperoxidase and β-lactamase were used in this work as triggers for diagnostic tools. There activity in wound fluids was measured while their function is described in the following.

I Collagenases

In the inflammatory phase and during tissue remodelling the extracellular matrix (ECM) is degraded mainly by members of the matrix metalloproteinase (MMP) family. MMP-1 (interstitial collagenase, fibroblast collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase 3) possess collagenolytic activity and are therefore capable of degrading triple helical interstitial collagen being the major constituent of the connective tissue (Iyer et al. 2006; UniProtKB). The human gelatinases MMP-9 (92 kDa type IV collagenase, 92 kDa gelatinase, gelatinase B; UniProtKB Acc: P14780) and MMP-2 (72 kDa type IV collagenase, 72 kDa gelatinase, gelatinase A; UniProtKB Acc: P08253) also cleave different types of collagen (UniProtKB).

Collagenases are Zn^{2+} dependend endopeptidases and consist of the catalytic domain, a short linker region and a C-terminal hemopexin domain. Collagen is composed of three left-handed α-chains forming a right-handed superhelix. The catalytic domain alone exhibits proteolytic activity on noncollageneous proteins but needs the hemopexin domain to allow entrance of triple helical collagen to the catalytic centre. The superhelix is locally unwound and cleaved after Gly in a repeating Gly-Xxx-Yyy sequence into ¾ and ¼ fragments of the protein as shown in Figure 2 (Chung et al. 2004).

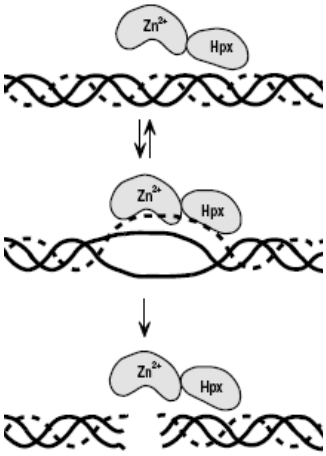


Figure 2: Locally unwinding of triple helical collagen by collagenase (Chung et al. 2004)

The catalytic domain contains two zinc ions, one catalytic and one structural as well as three or four calcium ions as cofactors. The catalytic Zn^{2+} coordinates three histidin residues and a water molecule in the active enzyme. The water molecule displaces a cystein residue of the inactive proenzyme and is again displaced by the substrate for hydrolysis of the peptide bond. The proton of the leaving water is drawn by the carboxylat group of the catalytic Glu200 in the active site and the arising hydroxyl ion breaks the peptide bond by nucleophilic attack on the carbonyl carbon (Nagase et al. 2005).

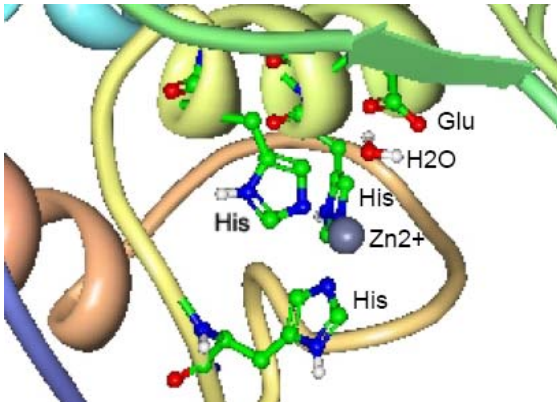


Figure 3: Catalytic center of fibroblast collagenase (PDB code 1cgl)

II Lysozyme

Lysozyme is a lysosomal enzyme of leukocytes of the innate immune system. In the inflammatory phase it is released by living cells but its main function is the intracellular digestion in leukocytes. Polymorphonuclear neutrophils and mononuclear phagocytes contain incredible amounts of lysozyme which is released due to cell death or phagocytosis of neutrophils by macrophages (Kajiki et al. 1988).

Lysozyme is a β-glycosidase (Vocadlo et al. 2001) as well as a muramidase and digests the murein layer (peptidoglycan) of gram positive bacterial cell walls. The cell wall of gram negative bacteria contains less peptidoglycan and is therefore degraded by lysozyme only in combination with complex forming agents (EDTA). The structure of peptidoglycan is shown in Figure 4. It is a heteropolymer consisting of oligosaccharides crosslinked by peptides. Alternating N-Acetylglucosamine (GlcNAc) and N-Acetylmuraminic acid (MurNAc) are 1,4-β-glycosidical linked to form the oligosaccharide chain. The glycosidic bond is cleaved by the action of lysozyme as shown in Figure 4 (Schlegel 1992).

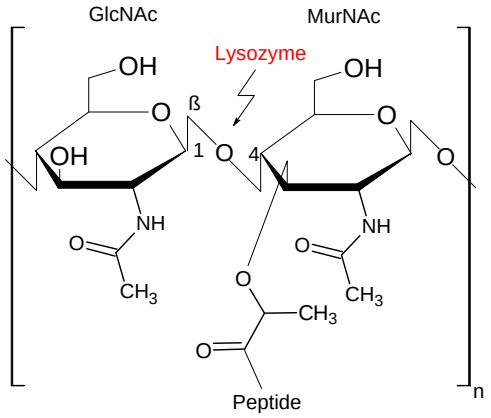


Figure 4: Structure of peptidoglycan

The catalytic mechanism is shown in Figure 5. It was first described for hen egg-white lysozyme by Phillips (Phillips mechanism). Catalysis is performed by the formation and breakdown of an oxocarbenium ion intermediate in the transition state. Catalytic Glu35 transfers a proton to the glycosidic oxygen while Asp52 stabilises the charge of the developing oxonium ion on C1 of N-Acetylmuranimic acid via electrostatic interactions. MurNAc is undergoing a conformational change to form a half chair conformation. The intermediate is hydrolysed then by the addition of water retaining the stereochemistry at C1 (Vocadlo et al. 2001).

A covalent intermediate instead of the ion pair is described by Koshland and is shown in Figure 5 (path A). Nucleophilic attack of the anomeric C by Asp52 forming a covalent acylal ester seems to be more likely. Vocadlo et al. (2001) adopted this mechanism of a covalent intermediate as well as the half chair conformation proposed by Phillips and further suggest that the anomeric C electrophilic migrates below the ring plane for hydrolysis.

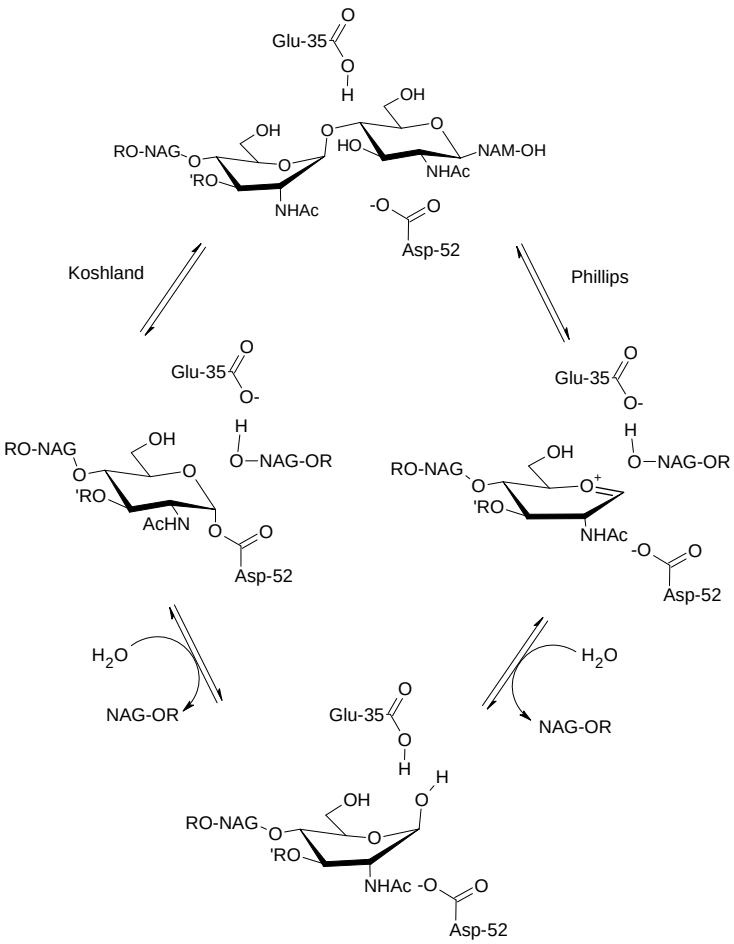


Figure 5: Lysozymes catalytic action: Koshland (path A) and Phillips mechanism (path B) (Vocadlo et al. 2001)

III Myeloperoxidase

Human myeloperoxidase (UniProtKB Acc: P05164) is a peroxidase (oxidoreductase). It is an essential part of the innate immunity of polymorphonuclear leucozytes. During phagozytosis it acts against a wide range of microorganisms. (UniProtKB)

Myeloperoxidase (MPO) consists of two heavy (α) subunits and two light (β) subunits. The heavy subunits are linked by disulfide bonds and contain covalently bound carbohydrates and, as catalytic compound, two hemes (Klebanoff 2005).

Myeloperoxidase forming the MPO-H_2O_2-halide system plays an essential role in the innate immune response described in 2.2.1I. Human neutrophils and monocytes but not mature macrophages contain high concentrations of MPO of about 1-2% of the cells dry weight in their granules. After engulfment of microbes granule compounds including MPO are released into the phagosome. The process of ingestion also activates the NADPH oxidase which is

responsible for the respiratory burst providing hydrogen peroxide for the enzymatic reaction as shown in equation 1.

$$NADPH + O_2 \rightarrow \cdot O_2^- + NADP^+ + H^+ \quad [1]$$

The superoxide anion ($\cdot O_2^-$) is further converted to H_2O_2 and O_2 either spontaneously or by superoxide dismutase (SOD) (Klebanoff 2005).
The enzymatic action of MPO generates hypochlorous acid (HOCl) as shown in equations [2], [3] and [4]. The formation of an MPO-I-Cl^- intermediate is suggested by Furtmüller et al. (2000).

$$MPO + H_2O_2 \rightleftharpoons MPO\text{-}I + H_2O \quad [2]$$

$$MPO\text{-}I + Cl^- \rightleftharpoons MPO\text{-}I\text{-}Cl^- \quad [3]$$

$$MPO\text{-}I\text{-}Cl^- \longrightarrow MPO + HOCl \quad [4]$$

Hydrogen peroxide reacts with the heme iron so that oxygen is bound by a double bond to the iron forming compound I (MPO-I). In the following two-electron reaction of MPO-I with the chloride ion hypochlorous acid is generated while the native Fe^{3+} MPO is regenerated (Klebanoff 2005).
HOCl seems to be generated in sufficient amounts to kill ingested microbes. Additionally, further products of this antimicrobial system such as chlorine, chloramines, hydroxyl radicals ($\cdot OH$), singlet oxygen ($^1O_2$) and ozone (O_3) may contribute to the killing as well as to the pathogenesis of wounds when released to the outside of the cell (Klebanoff 2005).

IV β-Lactamase

β-Lactamases are hydrolytic enzymes and their expression in bacteria is apparently the main mechanism providing resistance to β-Lactam antibiotics. According to sequence, there are four groups of β-Lactamases. Class A, C and D are known as serine β-Lactamases because they use an active site serine for hydrolysing the amide bond of the β-Lactam ring. Class B enzymes are metallo-β-Lactamases having bound one or two catalytic Zn(II) ions in their active site (Morán-Barrio et al. 2007).
Among the serine β-Lactamases, class A enzymes are the most common β-lactamases. Their genes are chromosomal as well as located on plasmids and therefore move through horizontal

gene transfer among species genera and even bacterial kingdoms. The coding gene is found in the gram-positive *Staphylococcus aureus* as well as in gram-negatives like *Pseudomonas aeruginosas* and shows resistance to the common antibiotics penicillin and cephalosporins that is further evolving (Hall et al. 2004).

Serine β-Lactamases are supposed to originate from penicillin binding protein (Suvorov et al. 2007) as they share the same conserved Ser-X-X-Lys motif in their active site and undergo acylation and deacylation on the catalytic Ser^{70} during substrate turn-over (Golemi-Kotra et al. 2004). The mechanism of a class A enzyme is shown in Figure 6. Acylation is accomplished by nucleophilic attack of the side chain oxygen of Ser^{70}. Eight key residues in the active site and two water molecules are involved in hydrogen bonding to the substrate. One water molecules, W-65, is hydrogen bonded to Ser^{70} and Glu^{166} and deacylates Ser^{70} after hydrolysis of the lactam ring (Wang et al. 2006).

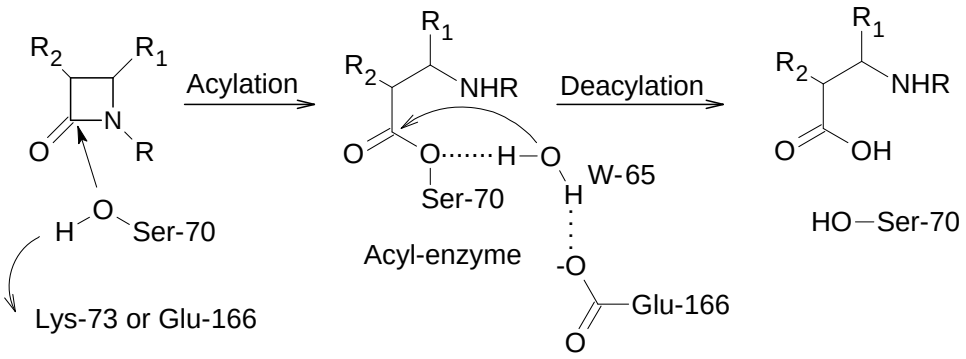


Figure 6: Mechanism of serine β-lactamase

Metallo-β-Lactamases (MβLs, class B enzymes) seem to be more severe concerning the problem of antibiotic resistance. Their β-lactame substrate specificity is unusual broad and they are not susceptible to inhibitors that work with serine β-lactamases. MβLs bind to one or two Zn(II) ions in their active site. The active site consists of the 3H metal binding site (His^{116}, His^{118} and His^{196}) and the DHC site (Asp^{120}, Cys^{221} and His^{263}) (Llarrull et al. 2007). In general, monometallic enzymes bind Zn2 in their DHC site whereas dimetallic forms bind Zn1 in the 3H site as active site and Zn2 in the DHC site that vary to the DHH site (Cys residue is substituted by His) depending on the subclass. A water molecule is bridging the two metal ions (Xu et al. 2005).

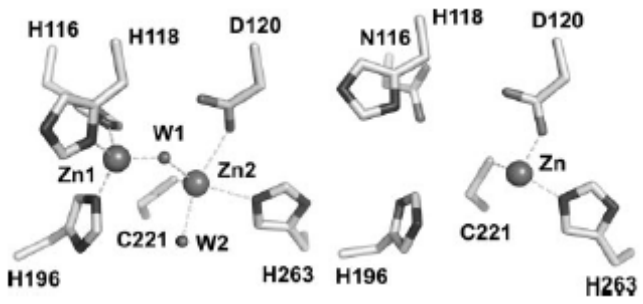


Figure 7: Metallo-β-lactamase active sites: dimetallic B. cereus BcII (PDB code 1bc2, left) and monometallic A. hydrophila CphA (PDB code 1x8g, right) (Morán-Barrio et al, 2007)

Catalytic mechanisms of MβLs are divers and not yet fully understood as well as the metal content of MβLs in vivo (Llarrull et al. 2007).

β-Lactamase producing bacteria (BLPB) in polymicrobial infections may significantly influence the wound healing process. They survive antibiotic therapy and may also protect susceptible bacteria by degrading the antibiotic (Brook 2004).

2.3 Matrix polymers

The biopolymers alginate, agarose and gelatin are used as matrix polymers. They are biocompatible as they are already used as wound dressings (alginate) and in the food industry. They form stable gels and are either enzymatically degradable itself (gelatin) or they act as structural polymers (alginate, agarose) blended with degradable substrates.

2.3.1 Alginate

Alginate is extracted as sodium alginate from seaweed and is a copolymer consisting of linear chains of (1-4) linked β-D-mannuronic acid monomer blocks (M) and blocks of α-L-guluronic acid monomers (G). The number of monomers forming the blocks depends on the type of alginate and provides different physical characteristics to the polymer. The G-blocks are bent and allow polymerisation of two chains with divalent cations such as calcium in aqueous solution. The greater the content the higher the stability and strength. The M-blocks are flat and are not reacting with calcium. They thus enhance flexibility and diffusivity with increasing content. The linear chain of alginic acid is shown in Figure 8 (Amsden et al. 1999).

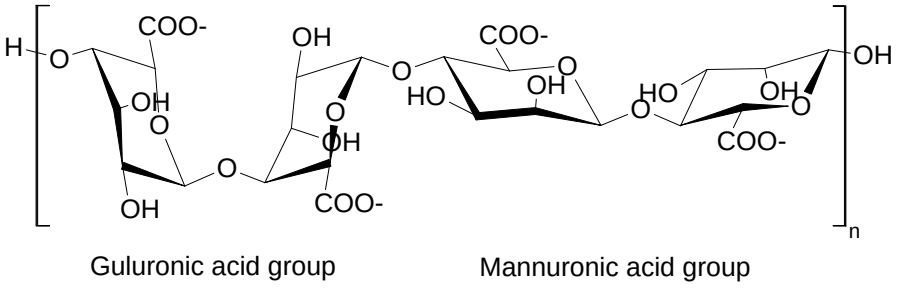


Figure 8: Linear chain of alginic acid

The conformation of polymerised G-blocks reminds of an egg box (Belitz, Grosch & Schieberle, 2001) and is shown in Figure 9.

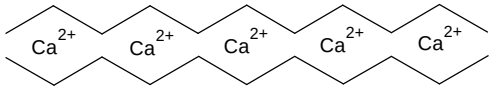


Figure 9: Polymerisation of G-bocks

Purified alginates are biocompatible as they are used in bioengineering (wound dressings, surgical sponges) (Becker et al. 2001) as well as in the food industry (stabilizer, thickening agent) (Belitz, Grosch and Schieberle 2001).

Alginolytic enzymes (alginases) digest alginate. They have been found in marine molluscs, bacteria, fungi and marine brown algae and have been characterised as EC 4.2.2.3 alginate lyases. Depending on their substrate specificity they are classified into polyG lyases and polyM lyases, either digesting the polyG or the polyM block of the polysaccharide. PolyM lyases were found in *Haliotis*, *Photobacterium* sp. and *Pseudomonas aeruginosa*, PolyG lyases in *Klebsiella aerogenes* and *Pseudomonas alginovora*. They all produce an unsaturated uronic acid at the non-reducing end after cleavage. (Sawabe, Ohtsuka and Ezura 1997)

2.3.2 Agarose

Agarose is a non-fibrillar polysaccharide and is extracted from red macroalgae, mainly from the genera *Gelidium* or *Gracilaria*. The linear chain consists of alternating 3-linked β-D-galactopyranosyl (A) and 4-linked α-L-3,6-anhydrogalactopyranosyl (B) units (Figure 10) (Rodríguez 2009).

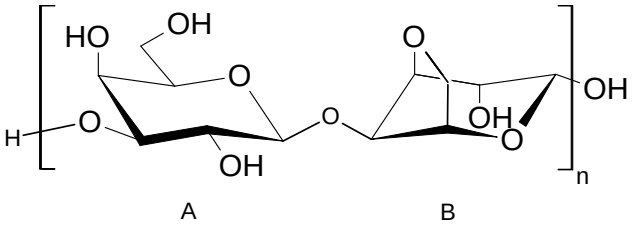


Figure 10: Molecular structure of agarose

The algae extract consists of various sulfated galactans like carragenaan (1,3-β-D-galactose and 1,4-α-D-galactose) and agar (1,3-β-D-galactose and 1,4-α-L-galactose). Agarose is the main component of agar and is the anhydrous form of the sulphated agaran (Rodríguez 2009).

Above the melting temperature (85-95°C) agarose is in the sol phase and undergoes the sol-gel transition at 30 – 40°C. During cooling random coils are forming double helices and bundles of helices leading to very hard gels at relatively low concentrations. It is therefore used for electrophoretic separations, cell immobilisation as well as in the food industry as gelling agent (Guiseley 1989).

Agarolytic enzymes (agarases) are capable of degrading agarose and are found in bacteria like *Pseudomonas*, *Pseudoalteromonas*, *Alteromonas* and *Vibrio*. They are mainly isolated from marine environment but also from soils, rivers and sewage. Agarases are divided into α-agarases (EC 3.2.1.158) and β-agarases (EC 3.2.1.81) depending on their enzyme substrate. α-agarases cleave the α-L-(1,3) linkage of agarose whereas β-agarases cleave the β-D-(1,4) linkage of the polysaccharide. Their degradation products are neoagaro-oligosaccharides which inhibit the growth of bacteria as well as stimulate macrophage activity.

2.3.3 Gelatin

Gelatin is the denatured form of collagen, mainly type I collagen, which derives from animal bones and skin. Triple helical, highly crosslinked water insoluble collagen is converted to water soluble gelatin by chemical-thermal processing. Collagen and also so gelatin consist of Gly-X-Y tripeptide units and contain the unusual aminoacids 4-Hydroxyprolin and ε-Hydroxylysin in relatively high contents as well as 0.5% (w/w) carbohydrates. Its ability to gelation is based on thermoreversible sol/gel transition below 35-40°C. Gelatin chains rewind to triple helices and intra- and intermolecular stabilization is made by hydrogen binding (Babel 1996).

Different molecular weights of gelatin result from different methods of processing of the row material and influence the gel properties. Low molecular weight gelatin forms soft gels whereas high molecular weights contain more crosslinked chains and thus form stronger gels (Elharfaoui et al. 2007).

3 Methods

The experimental part of the work is described in this chapter. In preliminary experiments wound fluids were screened for various enzyme activities (chapter 3.2). Collagenase was further detected using zymography and western blotting (chapter 3.3). The enzymes lysozyme and collagenase were investigated to be present in infected wound fluids in enhanced levels but low in wound fluids of recovering wounds and blister fluids. They were thus used as triggering enzymes for the release system described in 3.1.

3.1 Release system

The enzymatically controlled drug release system was constructed of a polymer hydrogel matrix (alginate, agarose or gelatine; see 2.3) with imbedded peptidoglycan (micrococcus cell wall) as substrate. The substrate is digested by the enzyme lysozyme and released by diffusion. Partial degradation of the polymer matrix is supposed to enhance the release rate.

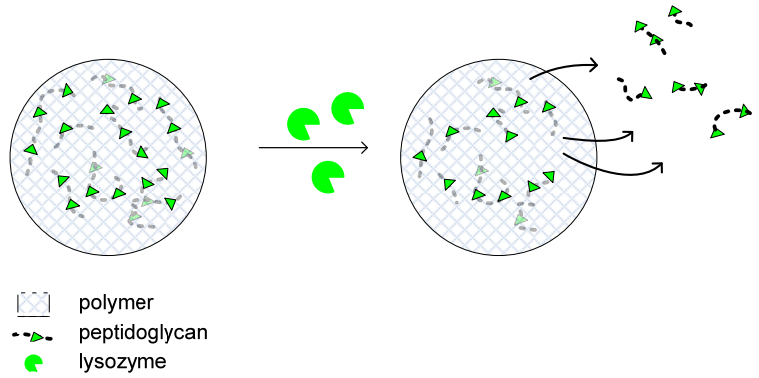


Figure 11: Enzymatically controlled drug release system

Two different kinds of release systems were performed as application for diagnostic tool. The matrix consisted of an agarose/substrate blend in the first approach and the experiment was carried out in Greiner well plates (chapter 3.1.3). The second application implied alginate blended with gelatin and/or substrate which polymerises in solutions of divalent cations forming beads and incubation was thus conducted in eppendorf tubes. Beads containing gelatin were co-incubated with microbial collagenase to enable partial degradation of the matrix (chapter 3.1.4). The enzymatic release of the substrate induced either an increase in transparency (density) of the matrix or a colouring of the incubation solution and was photometrical quantified with the TECAN platereader.

In a similar system antibiotics were embedded in alginate beads blended with gelatin and released by microbial collagenase degrading the gelatin part of the matrix. The release was photometrical quantified with the TECAN platereader.

3.1.1 Preparations

Preparations needed for the fabrication of the polymer matrix, buffers and enzyme solutions are listed in Table 4.

Table 4: Preparations needed for the release systems

component	composition
phosphate buffer 1M; pH 7.0	19.5 ml NaH_2PO_4 solution 0.2 M (27.8 g/l) + 30.5 ml $Na_2HPO_4*7H_2O$ solution 0.2 M (53.65 g/l)
HEPES buffer, 0.1M; pH 7	11.9 g HEPES dissolved in 500 ml dH_2O
collagenase buffer	EnzChek buffer diluted 1:10 (see 3.2.5)
agarose solution 1% (w/v)	1 g agarose dissolved in 100 ml of 0.1 M potassium phosphate buffer
gelatin solution 2% (w/v)	10 g gelatin dissolved in 500 ml ddH_2O
alginate solution 2% (w/v)	10 g alginate dissolved in 500 ml ddH_2O
$CaCl_2$ solution 5% (w/v)	30 g $CaCl_2*2H_2O$ dissolved in 600 ml ddH_2O
lysozyme stock solution	4.0 mg of solid lysozyme (20,000 U/mg) dissolved in 1.03 ml HEPES buffer (80,000U/ml), diluted to 500 U/ml
collagenase 1 stock solution	34.7 mg of solid collagenase 1 (288 U/mg) dissolved in 1.0 ml of collagenase buffer (10,000 U/ml)
collagenase 4 stock solution	50.3 mg of solid collagenase 4 (199 U/mg) dissolved in 1.0 ml of collagenase buffer (10,000 U/ml)

3.1.2 Staining of peptidoglycan

Staining of peptidoglycan (PG) was conducted according to Brückner (2008). It took place in 2.0 ml Eppendorf-tubes. As reactive dyestuffs, Remazol Brilliant Blue (RBB) and Alcian Blue, were used.

Table 5: Staining solutions for RBB-peptidoglycan

solution 1	solution 2
0.5% RBB-solution or	2.5% $NaSO_4$ (0.5 g per 5 ml)
0.5% Alcian Blue (0.2g in 10 ml)	1% $NaCO_3$ (0.2 g per 5 ml)

Required staining solutions were prepared using ddH_2O as described in Table 5. 50 mg of peptidoglycan were dissolved in 0.5 ml of staining solution 2, an equal volume of staining solution 1 was added and mixed in the thermal shaker for 10 minutes at 25°C. The temperature was raised then to 65°C and the reaction was continued for another 45 minutes while shaking.

The reaction solution was centrifuged for one minute (13000 rpm) and several washing steps using 1.5 ml of ddH_2O had to be carried out until the supernatant was colourless. The stained peptidoglycan was stored in 0.2 ml ddH_2O in the freezer.

Determination of the amount of bound RBB

Ten parallel staining experiments were carried out. Washing solutions of the several washing steps were photometrically measured at 600 nm. They were pooled then and the pooled solution was measured as well. Via a calibration line the mass of bound dyestuff was determined. Therefore 0.2 g Remazol Brilliant Blue were solubilised in 10 ml ddH_2O to obtain a 20 mg/ml stock solution. Via a dilution series, concentrations of 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, 0.0195 and 0.00975 mg/ml were prepared and the absorbance was measured.

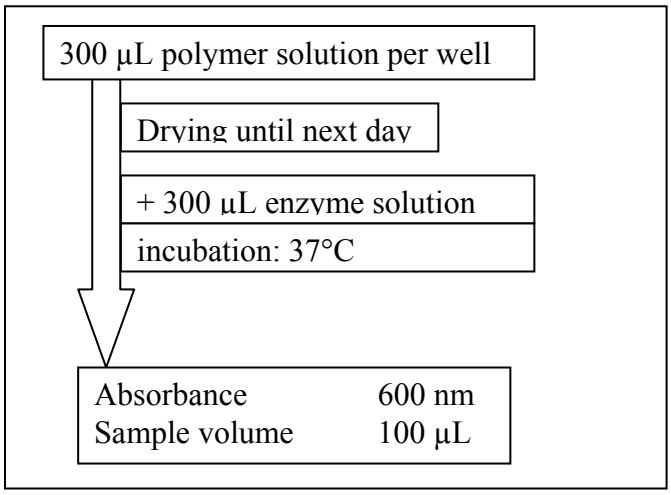
3.1.3 Diagnostic tool – Agarose release systems prepared in well-Plates

In this experiment an agarose matrix was blended with stained and unstained peptidoglycan respectively as substrate. The substrate was degraded and released during incubation with lysozyme. The release was photometrically measured. RBB-stained degraded peptidoglycan was quantified in the supernatant at 600 nm equating an increase in absorbance with an increase in release. Unstained peptidoglycan was digested due to lysozyme activity, resulting in an increase in transparency and was measured at 450 nm.

I Stained peptidoglycan as substrate

1% agarose was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) (see 3.1.1) in the microwave. 25 mg per 10 g stained peptidoglycan was added to the hot agarose-solution and mixed properly. In preliminary experiments, different concentrations of agarose had been tried, but the 1%-solution highlighted to be the best.

The experiment was carried out in 48 well plates. 300 µl of hot polymer solution (preparation see above) were pipetted per well and dried over night. The experiment was started by adding 300 µl of lysozyme solution to the polymer. A lysozyme stock solution (Table 4) was diluted with 0.1 M phosphate buffer. Enzyme activities were ranging from 10 to 10,000 U/ml. Directly after incubation start 100 µl of the supernatant were taken, the absorbance of t=0 was measured at 600 nm and the sample was pipetted back for incubation at 37°C. Further samples were measured every 60-90 minutes as well as on the next day. The procedure is shown in Flowchart 1.



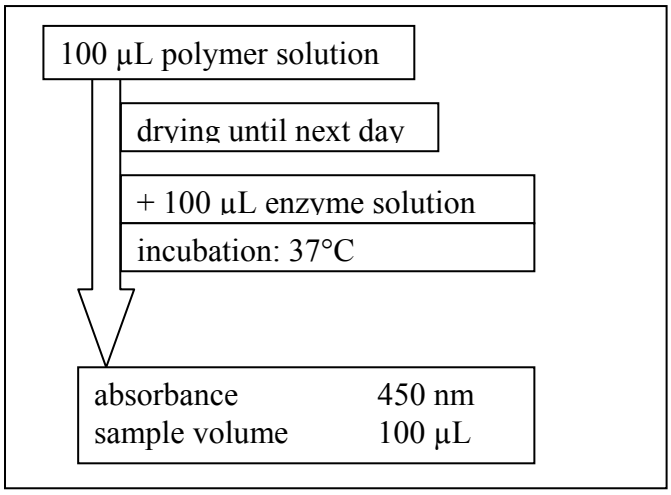
Flowchart 1: Release of RBB stained peptidoglycan from an agarose matrix

II Unstained peptidoglycan as substrate

1% agarose was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) (see 3.1.1) in the microwave. 50 mg per 10 g peptidoglycan were added to the hot polymer and mixed properly. For further experiments the concentration of peptidoglycan was varied from 15 to 60 mg per 10 g agarose solution to check out the possibility of observing the increase in transparency of the polymer blend with the naked eye.

The experiment was carried out in transparent 96 well plates. The procedure is shown in Flowchart 2. 100 µL polymer solution was pipetted per well and dried over night in the fridge.

100 µl of enzyme solution (lysozyme activities from 10 – 10,000 U/ml) were added to the polymer for incubation. The arising increase in transparency (density) due to digestion of peptidoglycan was measured at 450 nm. Measurements took place directly in the reaction plate and were done at incubation start and every following 30 to 90 minutes.



Flowchart 2: Release of unstained peptidoglycan from an agarose matrix

On the one hand the activity of lysozyme was quantified by photometric measurement of the decrease in density. On the other hand the rough amount of lysozyme in wound fluids was determined by observing the increase in transparency for fast diagnostic decision.

3.1.4 Diagnostic tool – Alginate beads as release system

Beads as release system consisted of alginate or an alginate/gelatin copolymer blended with substrate (blue stained peptidoglycan) or antibiotics (ciprofloxacin or tetracycline). Incubation with lysozyme induced the hydrolysis of stained peptidoglycan and thus the release of fragments colouring the supernatant blue. Collagenases were also supplied. They were supposed to enhance the release by digesting the gelatin part of the matrix. Beads containing antibiotics were incubated only with microbial collagenase triggering the release.
The alginate/gelatin ratio was varied as well as two different types of microbial collagenase, type I and type IV, were supplied for incubation.

I Fabrication of beads

For the production of (co-)polymer/substrate and co-polymer/antibiotics blend beads a 2% alginate and a 2% gelatin solution were prepared. The composition of the solutions is shown

in 3.1.1. Alginic acid was dissolved at 100°C while solid gelatin had to be solubilised at 50°C in ddH_2O. Alginate was blended in different ratios with gelatin to obtain final gelatin proportions of 20% (w/v) and 50% (w/v). Stained peptidoglycan and antibiotics (tetracycline or ciprofloxacin) respectively were solubilised then each in 50 g of the (co)polymer and mixed properly until a homogenous blend was obtained.
Dyeing of peptidoglycan with Alcian Blue was carried out as it is described for Remazol Brilliant Blue (see 3.1.2). The composition of the different blends is shown in Table 6.

Table 6: Composition of alginate blend beads

polymer	ratio (w/v)	substrate
alginate (2%) / gelatin (2%)	80/20	RBB – peptidoglycan 0,5% (w/w)
	50/50	RBB – peptidoglycan 0,5% (w/w)
alginate (2%) / gelatin (2%)	80/20	ciprofloxacin 0,1% (w/w)
	50/50	ciprofloxacin 0,1% (w/w)
alginate (2%) / gelatin (2%)	80/20	tetracycline 0,4% (w/w)
	50/50	tetracycline 0,4% (w/w)
alginate (2%)	100/0	RBB – peptidoglycan 0,5% (w/w)
alginate (3%)	100/0	Alcian Blue – peptidoglycan 0,5% (w/w)

Beads were formed by dropping the polymer solution into a 5% (w/v) $CaCl_2$ solution via a pump to permit cross-linking. The size of beads could be varied by regulation of the pump flow. The fabrication of beads is shown in Picture 3.

Picture 3: Fabrication of beads (picture kindly provided by Konstantin Schneider)

Beads were washed with distilled water and were either dried at room temperature or stored in buffer in the fridge. HEPES buffer was used for storing alginate/substrate beads and a 1:1 (v/v) HEPES buffer/collagenase buffer mix for alginate/gelatin/substrate beads.

II Balance of Remazol Brilliant Blue

40 g of polymer blend containing 50 mg/10 g RBB-peptidoglycan were polymerised. To determine the loss of polymer and dyestuff respectively in the equipment, dropping funnel and tube were weight before and after fabrication. The amount of RBB bound to peptidoglycan was determined in 4.1.1 and considering the loss of polymer solution the amount of RBB per bead for one charge was calculated.

III Stability test

Copolymer beads containing 20% (w/v) and 50% (w/v) gelatin and RBB-stained peptidoglycan as substrate were used for testing the stability. Five beads each were weight in 2.0 ml Eppendorf tubes. They were separately incubated in 400 µl HEPES buffer, 400 µl collagenase buffer and in 400 µl of a 1:1 HEPES/collagenase mixed buffer at 37°C in the thermomixer while shaking at 600 rpm. The experiment lasted for three days. Samples were taken at incubation start and every 30 to 90 minutes the first day, only once a day the second and the third day. 100 µl were photometrical measured in a 96 well plate at 600 nm. After measurement the sample was pipetted back into the tube not to reduce the reaction volume.

IV Release test

The release test was conducted with the enzymes lysozyme and collagenase as well as with two different samples of wound fluids. The release of degraded stained peptidoglycan depending on time was investigated. The digestion was induced by the action of lysozyme. The co-incubation of collagenase was expected to enhance the release as collagenase digests the gelatin part of the matrix. Incubation with wound fluids was done to test the feasibility as diagnostic tool.

An enzyme-overview for the different experimental approaches is given in Table 7. Lysozyme was dissolved in HEPES buffer, collagenases I and IV in collagenase buffer to prepare stock solutions (see 3.1.1). Enzymes were diluted to an activity of 250 U/ml. An incubation experiment with both enzymes was also carried out but with a supplied activity of 500 U/ml

to obtain a final activity of 250 U/ml in the incubation solution. All dilutions were prepared with a 1:1 HEPES/collagenase mixed buffer. In a parallel experiment lysozyme was diluted only with HEPES buffer to test the influence of buffer. As blank, beads were incubated with 1:1 HEPES/collagenase mixed buffer.

Incubation experiments with wound fluids included an infected wound fluid as well as a blister fluid as reference. The wound fluid was diluted 1:2 for incubation to a lysozyme activity of 1,970 U/ml. Their enzyme activities were determined in previous assays (see 3.2.2 and 3.2.5).

Table 7: Enzymes for the release test

sample		V_{inc} [µl]	activity [U/ml]	activity [U/400µl]
lysozyme		400	250	100
collagenase		400	250	100
mixed enzymes	lysozyme	200	500	100
	collagenase	200	500	100
wound fluid	lysozyme	400	1970	790
	collagenase/gelatinase		8.4	3.4
blister fluid	lysozyme	400	190	76
	collagenase/gelatinase		0.6	0.2
buffer		400	0	0

Beads not containing gelatin and beads containing 50% (w/v) gelatin were used for this experiment. Five beads per 2.0 ml Eppendorf tubes were weight and incubated with 400 µl of incubation solution. For the co-incubation experiment with both enzymes 200 µl per enzyme solution containing 500 U/ml were supplied.

Incubation was carried out at 37°C and 60°C respectively in the thermoshaker. The absorbance was measured in 100 µl of the supernatant at the according wavelengths that had been determined in wavelength scans. The scans are shown in Figure 18 and the wavelengths of maximum absorbance in Table 17 in chapter 4.1.3I.

The experiment lasted for about twelve hours, samples were taken at incubation start, after about 30 to 90 minutes of incubation and on the next day.

3.2 Activity of biomolecules in wound fluids

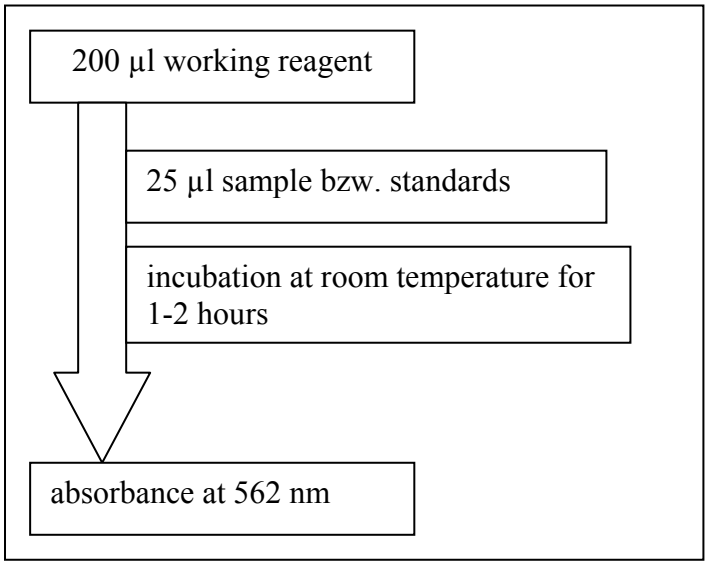
Assays for identification and quantification of enzymes present in wound fluids are described in this chapter. The total amount of protein is determined via the BCA assay. Lysozyme, myeloperoxidase, β-lactamase and collagenases/gelatinases are further quantified with the according assays.

3.2.1 BCA Assay

The Bicinchoninic Acid (BC) Protein Assay is conducted for quantifying the total amount of protein in an unknown sample and is provided as kit by Upitma. The assay is based on the formation of a Cu^{2+}-protein complex under alkaline conditions. The peptid bond of the protein reduces Cu^{2+} to Cu^{+} which forms a purple-blue complex with BC in alkaline environment. The absorbance of the Cu^{+}-BCA complex can be photometrically measured at 652 nm.

The quantification kit includes reagent A, reagent B and an Albumin standard (BSA at 2 mg/ml). The working reagent consists of reagent A and reagent B in a ratio of 50:1 (A:B). Linear concentration of the assay ranges from 20 – 2000 µg/ml whereas Bovine Serum Albumin (BSA) serves as standard.

The realisation of the assay is shown in Flowchart 3. 250 µl solution B were mixed with 12.25 ml solution A obtaining the working solution. 200 µl of the working solution were mixed with 25 µl of sample (wound fluids, standards and blank) in a Greiner 96 well plate. The colour developed during incubation at room temperature for 1-2 hours and was measured at 562 nm against the blank (working reagent + water).

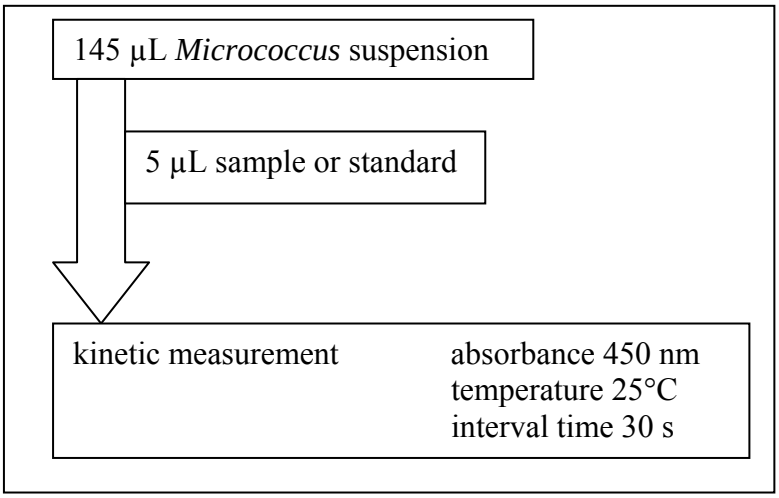


Flowchart 3: BCA Assay

The BSA stock solution was diluted with ddH_2O in a 1:2 dilution series to reach concentrations of 1, 0.5, 0.25, 0.125, 0.062, 0.031 mg/ml BSA for the standard curve. Wound fluids were diluted 1:20 and 1:40 respectively in an additional assay to reach concentrations in the linear range.

3.2.2 Lysozyme Assay

The lysozyme assay is a modified form of the assay suggested by Shugar et al. (1952). Lysozyme digests the cell wall of gram positive bacteria (see 2.2.5II) such as *Micrococcus lysodeikticus* which is the substrate in this assay. The activity of lysozyme is determined as described in Flowchart 4.



Flowchart 4: Lysozyme Assay

A cloudy suspension of 11 mg/15 ml *Micrococcus lysodeikticus* cell wall in 0.1 M potassium phosphate buffer (pH 7.0) was prepared. The assay was performed in a Greiner 96 well plate. Per well 145 µl of *Micrococcus* suspension were mixed with 5 µl sample (wound fluids, standards and blank). The enzyme activity was determined by kinetic measurement of the increase in transparency at 450 nm.

Lysozyme provided by AppliChem was used as standard. A stock solution of 100,000 U/ml was prepared in potassium phosphate buffer and diluted to activities of 800, 600, 500 400, 300, 200, 100, 50 and 0 U/ml for the standard curve. Samples were measured undiluted, 1:2, 1:5, 1:8 and 1:50.

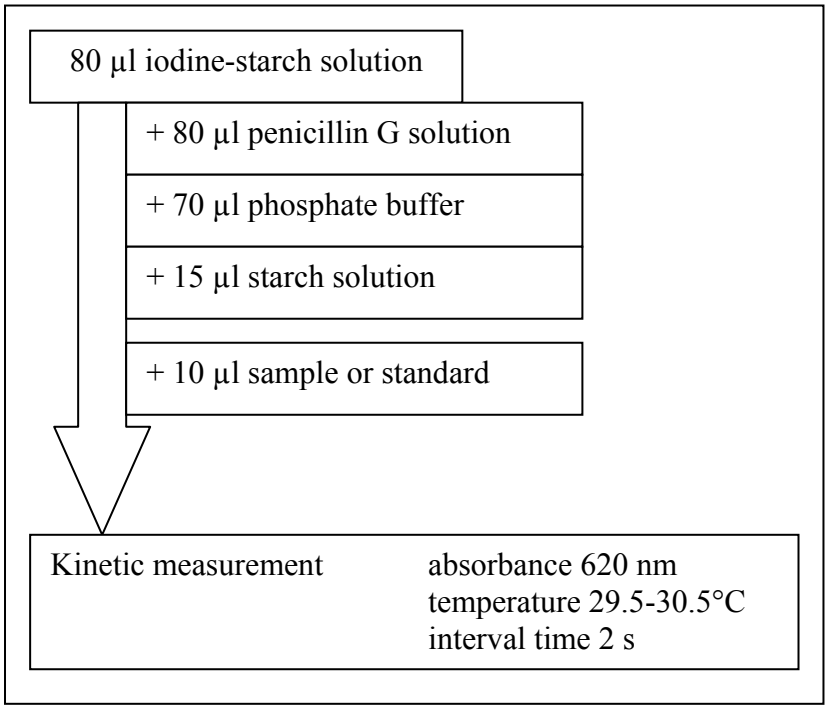
3.2.3 β-Lactamase Assay

β-Lactamase activity was determined as suggested by Novic (1962). Preparations are listed in Table 8.

Table 8: Preparations for the β-lactamase assay

component	composition
starch solution 2% (w/v)	1 g starch in 50 ml potassium phosphate buffer (pH 5.8)
iodine solution	0.08 M I_2 in 3.2 M KI
potassium phosphate buffer (pH 5.8)	6.25 g/l KH_2PO_4 and 0.696 g/l K_2HPO_4
penicillin G solution (2.5 mM)	1.86 mg in 20 ml buffer
β-lactamase stock solution	16.8 U/ml

An iodine-starch solution (120 µM) was prepared by mixing 0.3 ml of iodine solution with 180 ml phosphate buffer and 20 ml starch solution. The realisation of the assay is shown in Flowchart 5. The assay was performed in a Greiner 96-well plate. 80 µl iodine-starch solution, 80 µl penicilin G solution, 15 µl of potassium phosphate buffer and 15 µl starch solution were mixed per well and tempered at 30°C for 5 minutes in the TECAN platereader.



Flowchart 5: β-Lactamase assay

The reaction was started by adding 10 µl of standard and sample respectively and the activity of β-lactamase was determined by kinetic measurement at 620 nm.

The β-lactamase stock solution was diluted with potassium phosphate buffer in a dilution series to activities of 8.4, 4.2, 2.1, 1.0, 0.75, 0.5 U/ml for the standard curve. Samples were measured undiluted.

3.2.4 MPO Assay

Myeloperoxidase from human polymorphonuclear leukocytes was provided by Planta Natural Products and the MPO assay was conducted according to Sigma-Aldrich. The principle of the assay is shown in equation 5.

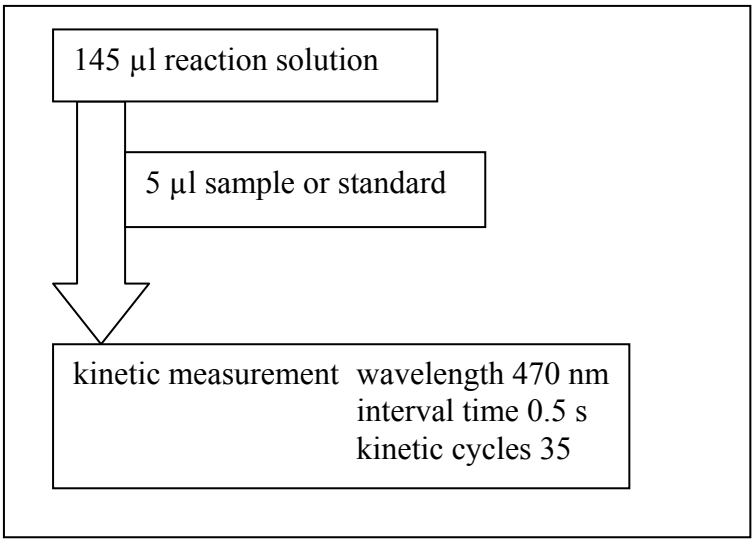
$$2 \text{ guajacol} + H_2O_2 \xrightarrow{Myeloperoxidase} 2 \text{ oxidised guajacol} + 2\ H_2O \qquad [5]$$

The assay was conducted in a Greiner 96 well plate as described in Flowchart 6. Preparations for the assay are listed in Table 9.
A 10 U/ml myeloperoxidase stock solution was prepared in ddH_2O and diluted to activities of 2, 4, 5, 6, 7, 8 and 10 for the standard curve. Samples were measured undiluted. Instead of sample or standard ddH_2O was used as blank.

Table 9: Preparations for the MPO assay

component	composition
potassium phosphate buffer, 1 M, pH 7.0	19.5 ml 1 M KH_2PO_4 and 30.5 ml 1 M K_2HPO_4
reaction solution	99 ml 50 mM potassium phosphate buffer, 0.96 ml guajacol (99% solution), 60 µl H_2O_2 (70% solution)
standard	myeloperoxidase stocksolution 1,350-1,75 U/mg dissolved in 1 ml ddH_2O

5 µl sample, standard or blank were pipetted per well. 145 µl of the reaction solution were added and mixed. In a kinetic measurement the absorbance at 470 nm is measured every 20 seconds until constant with the TECAN plate reader.



Flowchart 6: Myeloperoxidase Assay

3.2.5 EnzChek Assay

The EnzChek®Gelatinase/Collagease Assay Kit (E-12055) was provided by Molecular Probes Europe BV. Components provided by the kid are shown in Table 10.

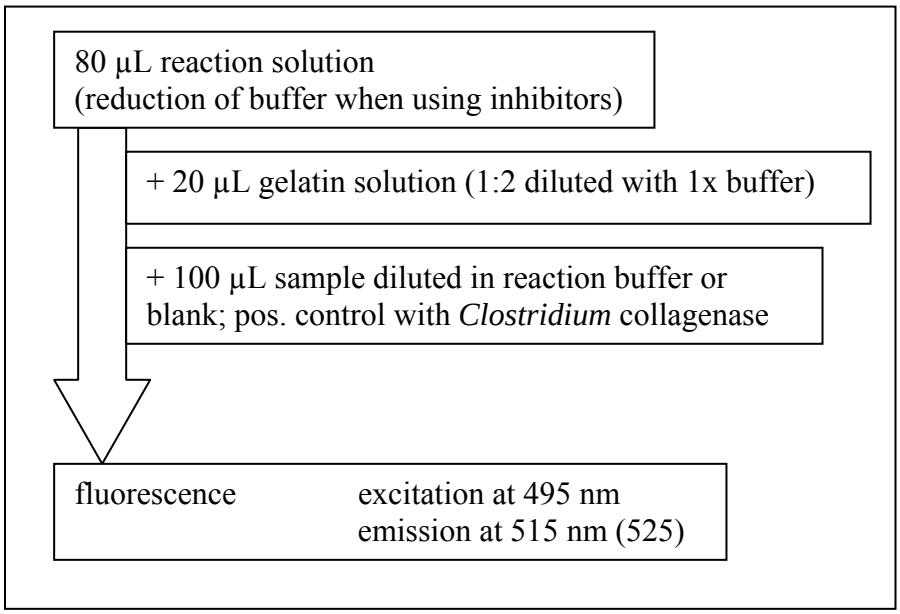
Table 10: Components provided by the EnzChek®Gelatinase/Collagenase Assay Kit

component	name	composition
A	DQ gelatin from pig skin, fluorescein conjugate	vial containing 1 mg substrate lyophilized from 1 mL of phosphate-buffered saline (PBS) pH 7.2
B	10x reaction buffer	0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM sodium azide, pH 7.6
C	1,10-phenanthroline, monohydrate	~30 mg (MW 198.2)
D	collagenase, type IV from *Clostridium histolyticum*	500 U per vial

Fluorescein-labelled but concentration-quenched gelatin (component A) is digested by gelatinases and collagenases present in wound fluids. Proteolytic activity leads to a proportional increase in fluorescence and can be photometrically measured. Component D serves as control enzyme.

Stock solutions were prepared. Gelatin was dissolved in 1 ml ddH_2O per vial to reach a concentration of 1 mg/ml and was stored at 4°C in the dark. A stock solution of *Clostridium histolyticum* of 1,000 U/ml was prepared by adding 0.5 ml of ddH_2O to a vial of component D. The reaction buffer was diluted 1:10. The *Clostridium histolyticum* stock solution was diluted with 1x reaction buffer to reach concentrations of 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.006 U/ml as dilution series. Samples were diluted 1:20 and 1:40.

The assay was carried out in a 96 well plate. The procedure is shown in Flowchart 7. 80 µl of reaction buffer were provided per well. 20 µl of 0.5 mg/ml gelatin solution were added and dispersed by sampling. The enzyme reaction was started by adding 100 µl sample and control enzyme respectively. After 2, 6 and 21 hours of incubation at 37°C the fluorescence is measured photometrical at 515 nm after excitation at 495 nm.



Flowchart 7: Enzchek Assay

3.3 MMP detection by westernblotting and zymography

3.3.1 Western blot

Western blotting is a method for detecting specific proteins and was performed for detecting the matrix metalloproteinases MMP-2 and MMP-9 in wound fluids. Via sodium dodecyl sulphate – polyacrylamid gel electrophoresis (SDS-PAGE) proteins are separated by molecular weight. They are then transferred to a membrane and detected by protein-antibody interaction. The target protein specifically binds to a primary antibody which further reacts

with a Horseradish Peroxidase (HRP) labelled secondary antibody. The subsequent chemiluminescent reaction, a HRP/H_2O_2 catalysed oxidation of luminol, can be detected by exposing the membrane to an autoradiography film.

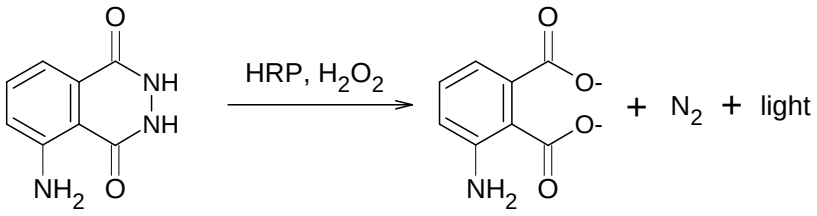
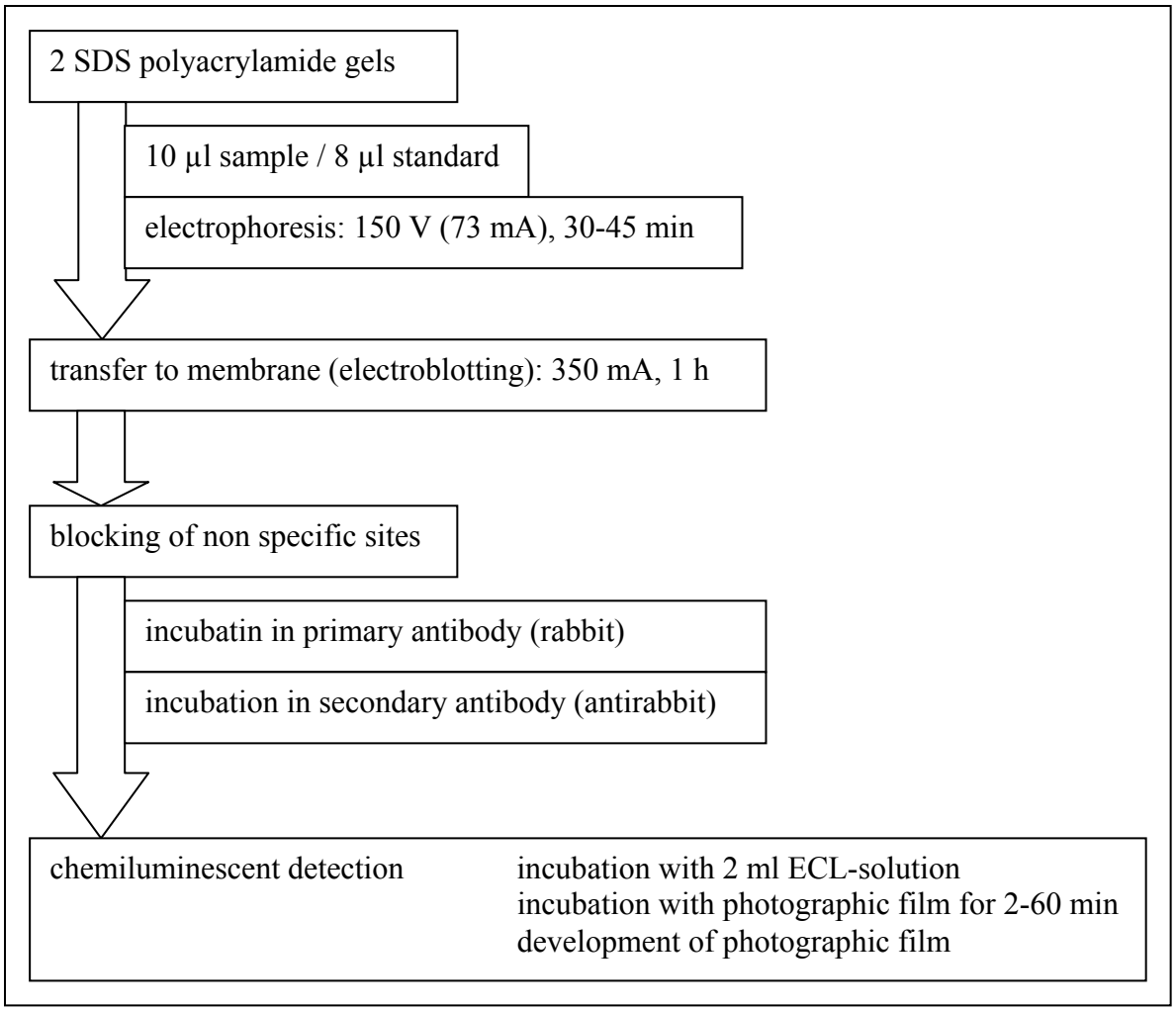


Figure 12: Chemiluminescent oxidation of luminol

The realisation of the western blot is shown in Flowchart 8.



Flowchart 8: Western blot

The composition of SDS polyacrylamide gels and the amounts for 4 gels (0.75 mm) are listed in Table 11. Two gels per target protein were prepared. 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 MMPs (*Chemicon* Gelatinase kit, see below) and standards (broad range, low range) were heated at 95°C in the eppendorf Thermomixer for 5-10 minutes. 10 µl sample and 8 µl standard respectively were loaded per slot. The protein molecular weights of the standards are listed in the Appendix (Table 24). Electrophoresis is conducted at a voltage of 150 V (73 mA) with the BIO-RAD apparatus. The composition of electrophoresis buffers is listed in Table 12.

Table 11: Composition of 7.5% SDS polyacrylamide gels (4 x 0.75mm)

	separating gel [ml]	stacking gel [ml]
ddH_2O	9.3	3.05
30% acrylamide	5	0.65
1.5 M TRIS pH 8.8	4.9	-
0.5 M TRIS pH 6.8	-	1.25
10% SDS	0.8	0.05
10% ammonium persulfate	0.1	0.025
TEMED	0.02	0.00375

Table 12: Composition of buffers for SDS-PAGE

buffer	composition
sample buffer	3.2 ml 10% SDS, 0.8 ml 2-mercaptoethanol, 2 ml 0.5 M Tris HCl (pH 6.8), 1.6 ml glycerine, 0.05% bromphenol blue in ddH_2O (4mg/8ml)
running buffer	3.0 g TRIS, 14.4 g glycine, 1.0 g SDS diluted in 1000 ml ddH_2O

The ECL™ Western blotting Analysis System was provided by Amersham. The kit includes detection reagents 1 and 2, HRP-linked whole secondary antibodies (Mouse IgG and Rabbit IgG) and the blocking reagent. Primary polyclonal antibodies to MMP-2 (gelatinase A, 72 kDa type IV collagenase) and to MMP-9 (gelatinase B, 92 kDa type IV collagenase) were provided by the *Chemicon* Gelatinase Kit as well as purified human MMP-2 and MMP-9 proenzymes as standard. The composition of further required buffers is shown in Table 13.

Table 13: Composition of buffers required for western blotting

buffer	composition
TST buffer (10x)	1.5 M NaCl, 1% tween 20 (v/v), 500 mM Tris HCl (pH 7.5)
CAPS buffer	2.21 g (10 mM) CAPS, 100 ml (10%) methanol, 900 ml H_2O (pH 11.0)
phosphate buffered saline (PBS) pH 7.4	11.50 g Na_2HPO_4 (80 mM), 2.96 g NaH_2PO_4 (20 mM), 5.84 g NaCl (100 mM) in 1000 ml dH_2O (pH 7.4)
blocking reagent	2.5 g non-fat dried milk powder in 50 ml TST buffer (0.5%)

The proteins on the gels were blotted onto the membrane in the blotting cell (BIO-RAD) with 350 mA for 1 hour while stirring using CAPS buffer as transfer buffer. Non-specific binding sites were blocked in 0.5% non-fat dried milk over night in the fridge. The membrane with the proteins on the upper side was washed using PBS buffer and incubated in 25 ml primary antibody solution (*Chemicon* Gelatinase kit, diluted 1:5000) for 90 minutes. The membrane was washed for 4 x 5 minutes with PBS to remove non-specific binding sites for the secondary antibody. The secondary antibody (antirabbit IgG) was diluted 1:15000 with TST buffer. The membrane was incubated in this solution for 90 minutes at room temperature and washed for 3 x 5 minutes.

An equal volume of detection solution 1 and 2 of the ECL^{TM} Western blotting kit was mixed for chemiluminescent detection and pipetted on to the membrane (protein side up). The detection reagent was gently drained and the membrane was placed between the two wraps of an autoclavable disposal bag (Carl ROTH GmbH). Air bubbles were smoothed out and the standard bands were marked on the plastic wrap. The blots were placed protein side up in an X-ray film cassette and covered with half a sheet of an autoradiography film. The blots were exposed to the film for 1 - 60 minutes in the closed film cassette and the film was developed afterwards.

3.3.2 Gelatin/Collagen Zymography

Zymography is a modified method of SDS gel electrophoresis. The gel contains either gelatin or collagen which serves as substrate for collagenases after separation by electrophoresis. Enzyme activity can be detected as clear band on the gel against a dark background.

The reagents required for zymography and their composition are listed in Table 14.

Table 14: Reagents required for gelatin/collagen zymography

component		composition
SDS-gel	separating gel (4 x 1.5 mm)	16.0 ml gelatin/collagen solution (2% (w/v)), 13.2 ml acrylamide, 10.0 ml TRIS 1.5 M (pH 8.8), 0.2 ml 10% APS, 0.8 ml 10% SDS, 0.028 ml TEMED
	stacking gel (4 x 1.5 mm)	1.33 ml 30% acrylamide, 0.4 ml 10% SDS, 2.5 ml 50 mM TRIS-HCl (pH6.5), 6.1 ml H_2O, 0.05 ml 10% APS, 0.01 ml TEMED
sample buffer		2.8 ml dH_2O, 1.0 ml 0.5 M TRIS-HCl pH 6.8, 3.2 ml 10% SDS solution, 7.0 ml glycerol
running buffer pH 8.3		72.05 g glycerine, 5.0 g SDS, 15.0 g TRIS in 1000 ml dH_2O
renaturating buffer		12.5 g triton X in 500 ml dH_2O (2,5%)
incubation buffer (10x conc.) pH 7.6		60.57 g TRIS-HCl (0.5 M), 87.66 g NaCl (1.5 M), 7.35 g $CaCl_2$ (50mM)
staining solution		0.5 g (0.25%) Coomassie brilliant blue R-250, 11 ml (5.5% (v/v)) acetic acid, 100 ml (50%) ethanol, filled up to 200 ml with dH_2O
destaining solution		300 ml (30%) ethanol, 100 ml (10%) acetic acid, 600 ml (60%) dH_2O

Collagenase I and collagenase IV (GIBCO^{TM}) solutions were prepared in a dilution series to obtain enzyme activities of 500, 250, 100 and 50 U/ml (stock solutions see Table 4) with collagenase buffer (incubation buffer, see Table 14). 15 µl of each solution were mixed with 15 µl of sample buffer. Samples and 900 µl of running buffer were cooled down at 4°C for 30 minutes to prevent enzyme activity during electrophoresis. 10 µl of sample were pipetted per slot and electrophoresis was conducted with 60 V (1 gel) and 100 V (2 gels) respectively. The gel was transferred into renaturating buffer for renaturation of the enzymes for 2 x 30 minutes and incubated afterwards in incubation buffer at 37°C over night. For visualisation of the bands the gel was stained with Coomassie Brilliant Blue and destained with destaining solution.

4 Results

4.1 Release system

In the first stage, an agarose system was realised in well plates. The agarose matrix was blended with peptidoglycan as substrate for lysozyme digestion. Peptidoglycan was applied either unstained or stained with Remazol Brilliant Blue (RBB) in two different approaches (chapter 3.1.3). In the second stage, an alginate bead release system was constructed with RBB-stained peptidoglycan blended to the matrix (chapter 3.1.4).

4.1.1 Determination of the amount of RBB bound to peptidoglycan

The calibration line for Remazol Brilliant Blue is shown in Figure 13. The concentrations of dyestuff in the washing solutions were calculated via the equation of the linear fit [6].

$$c[mg/ml] = \frac{abs - 0.0162}{1.8059} \qquad [6]$$

abs absorbance

c[mg/ml] concentration of Remazol Brilliant Blue

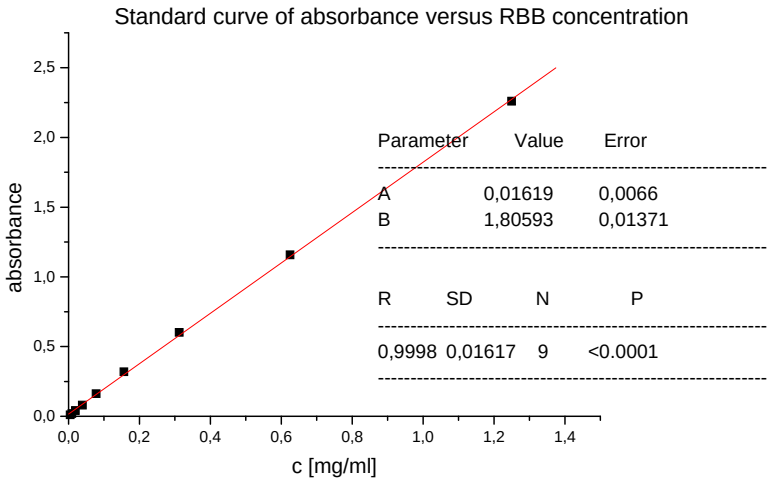


Figure 13: Calibration line for Remazol Brilliant Blue

The calculated concentrations of the separately measured washing solutions were summed and compared with the concentration of the pool. The arithmetic mean ($\bar{m}$) and the standard

deviation (SD) of the concentrations of the ten samples were determined. They are shown in Table 15.

Table 15: Amount of dyestuff washed out with washing solutions

	$\overline{m}$ [mg]	SD [mg]
sum of separately (measured washing solutions)	2.91	0.05
pool of washing solutions	2.42	0.13

Dying of 50 mg of peptidoglycan was conducted in 0.5 ml of a 20 mg/ml RBB-solution (see 3.1.2). The amount of dyestuff not being bound to peptidoglycan was determined to be 2.91 mg so it results that 7.09 mg of Remazol Brilliant Blue are bound to 50 mg of peptidoglycan. It was further calculated that every 8th monosaccharide of the polysaccharide backbone of peptidoglycan was dyed with RBB (calculations in the appendix).

4.1.2 Diagnostic tool – Agarose release system prepared in well plates

I Stained peptidoglycan as substrate

Figure 14 shows the increase in dyestuff in the supernatant containing different activities of lysozyme. Samples were taken after incubation for 0, 42, 82, 112, 192, 252, 310, 369 and 1,379 minutes. The correlation between absorbance of RBB and incubation time is shown. Blanks (absorbance of pure buffer) were subtracted.

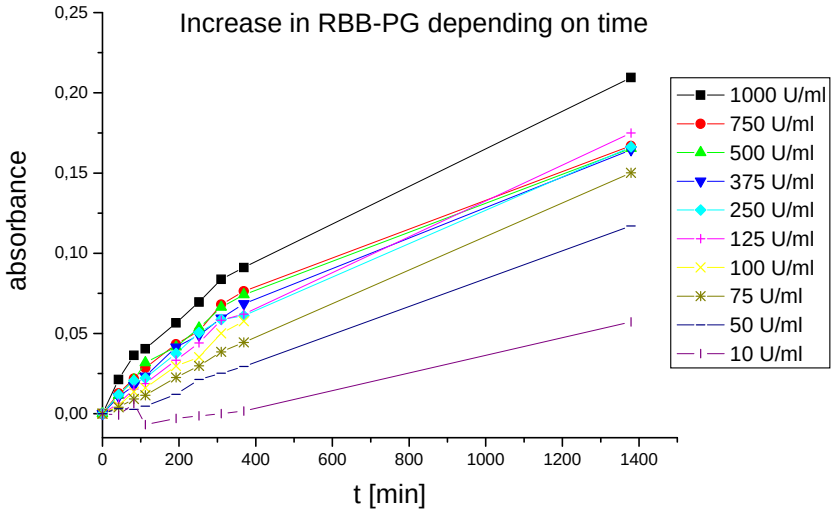


Figure 14: Agarose release system - increase in RBB-peptidoglycan depending on time

The conversion of RBB-PG by lysozyme was calculated as the absorbance per hour. It was calculated for each incubation period first as shown in [7].

$$conversion[\Delta abs/h] = \frac{\Delta abs}{\Delta t} = \frac{abs_{t2} - abs_{t1}}{t_2 - t_1} \cdot 60 \qquad [7]$$

Δabs	change (increase) in absorbance
Δt	incubation time [min]
t_2	incubation time measuring point 2 [min]
t_1	incubation time measuring point 1 [min]

For the final conversion the mean value in the linear range was determined. The increase in dyestuff in the supernatant per hour depending on the supplied lysozyme activity is shown in Figure 15. Linear correlation exists only for activities less than 125 U/ml. Non-linearity for enzyme activities higher than 125 U/ml is certainly not a problem of photometrical measurement since a linear absorbance of RBB was measured for higher concentrations. Enzyme inhibition can also be excluded. For higher as well as for lower enzyme activities non-linearity arises in the absorbance-time correlation for longer periods of incubation. It is very likely that the enzyme is deprived of substrate when incubating with higher enzyme activities or lower activities but longer incubation periods due to the construction of the blended matrix. Stained peptidoglycan may orient in the outer layers of the matrix and the surface of beads is limited so that non-linearities arise due to mechanical reasons.

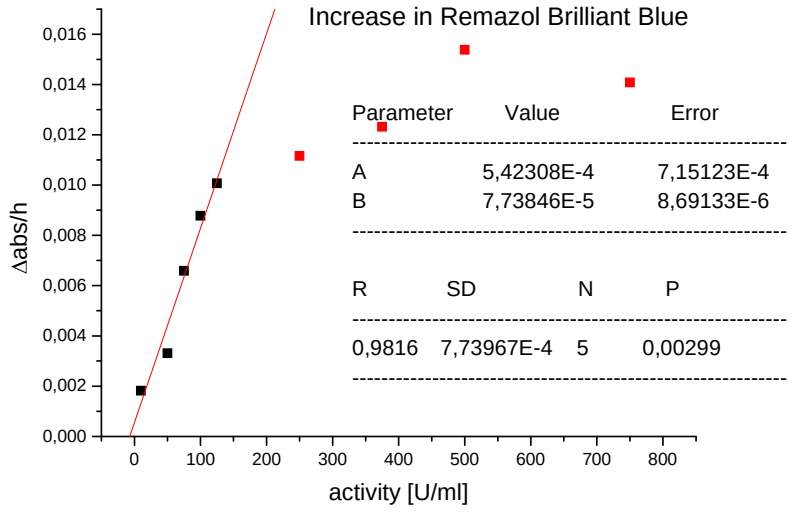


Figure 15: Agarose release system – conversion of RBB-peptidoglycan vs supplied enzyme activity

II Unstained peptidoglycan as substrate

Figure 16 and Figure 17 show the results of the agarose release system measuring the decrease in density, i.e degradation of unstained peptidoglycan during incubation with different concentrations of lysozyme. The absorbance depending on time shows a positive correlation between supplied enzyme activity and the loss in density (see Figure 16). The transparency was not changing during incubation with buffer; the increase in transparency was thus triggered only by lysozyme but not by diffusion.

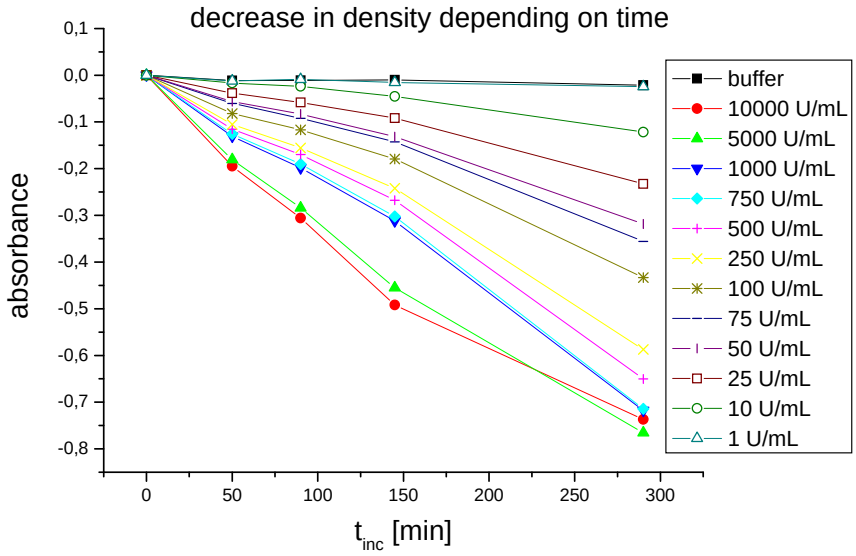


Figure 16: Agarose release system - decrease in density depending on time

The conversion of peptidoglycan by lysozyme was calculated as shown for stained peptidoglycan (see chapter I). Figure 17 shows the absorbance per hour depending on the supplied lysozyme activities. Linear correlation ranges only to 100 U/ml. For the same reasons as it was for stained peptidoglycan embedded in the matrix (see chapter I) the non-linearity results from the construction of the polymer blend. Oligosaccharides can only be released as long as there is peptidoglycan available for lysozyme. Due to the restricted surface and the distribution of substrate mainly in the outer layers of the polymer non-linearity arises for higher enzyme activities.

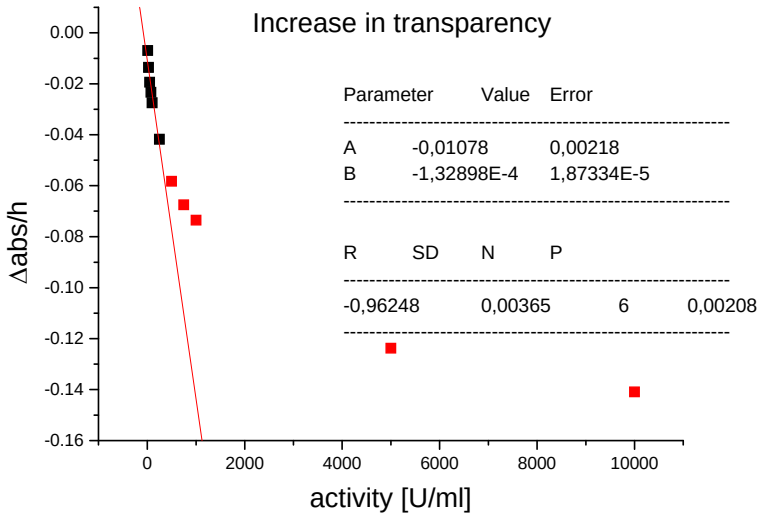
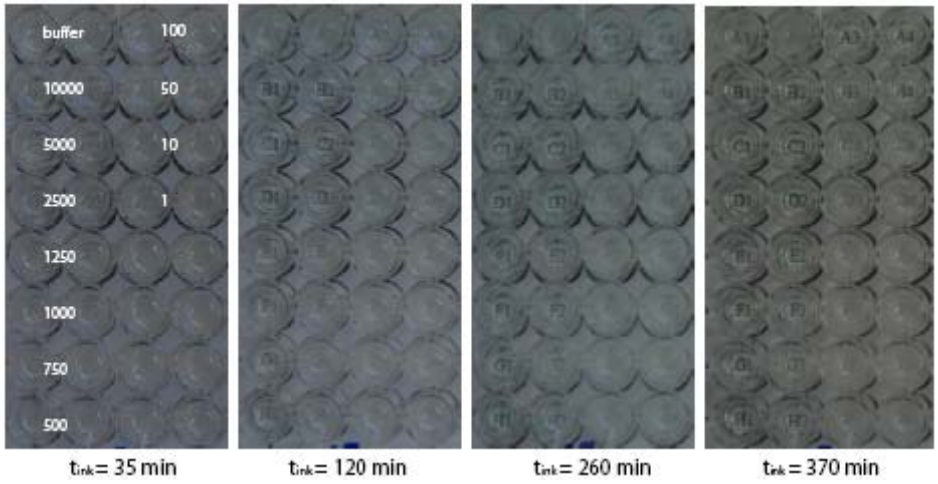


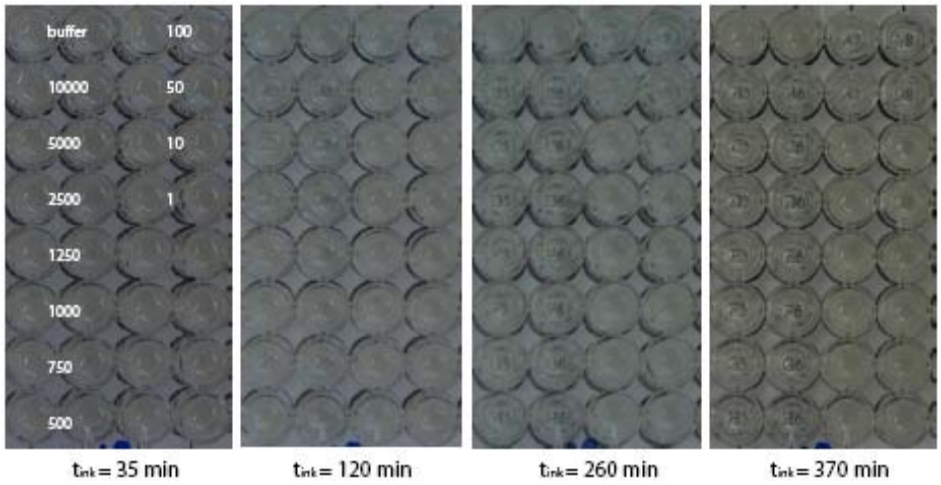
Figure 17: Agarose release system - increase in transparency vs supplied enzyme activity

The concentration of the substrate for photometric measurement (Figure 16 and Figure 17) was 50 mg/10 g (0.50%) peptidoglycan. The concentration had been optimised for observing the transparency with the naked eye. 15, 20, 25, 40, 50 and 60 mg peptidoglycan per 10 g agarose were incubated with lysozyme and 25 mg/10 g PG turned out to be the best. The increase in transparency of 15 mg (0.15%), 25 mg (0.25%) and 50 mg (0.50%) peptidoglycan per 10 g agarose after incubation with lysozyme for 35, 120, 260 and 370 minutes are shown in Picture 4, Picture 5 and Picture 6. Lysozyme activities were ranging from 10,000 – 1 U/ml and are written on the according wells (2 wells per activity).

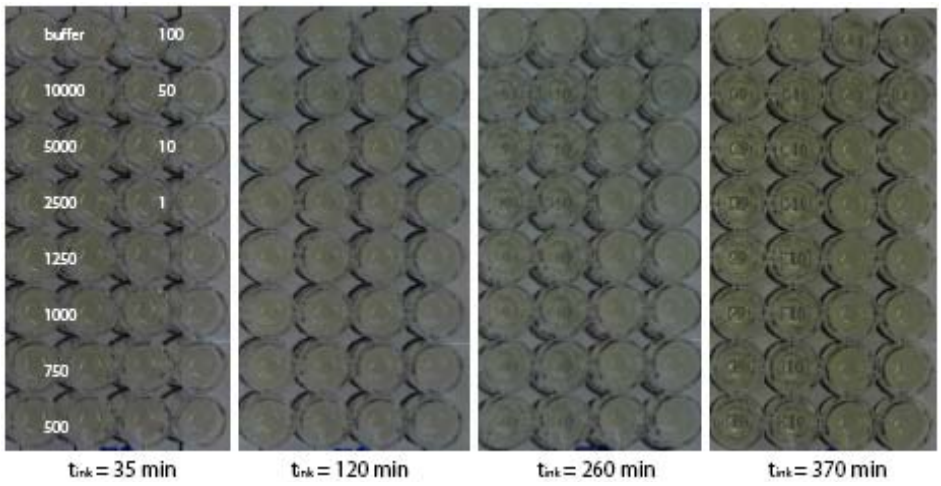
0.15% peptidoglycan in agarose (Picture 4) was not applicable for differentiation of lysozyme activities since the polymer was already slightly transparent before incubation. Polymers incubated with 10,000 – 50 U/ml became transparent at once already after 120 minutes of incubation. Polymers containing 0.5% peptidoglycan (Picture 6) are not applicable as well. There wasn't any change in transparency after 120 minutes, not even for the highest lysozyme activity. Due to this temporary delayed increase in transparency, differentiation of activities is not possible as early as it is for 0.25% peptidoglycan in agarose (Picture 5).



Picture 4: Increase in transparency of 0.15% peptidoglycan in agarose (activity of lysozyme: 1-10,000 U/ml)



Picture 5: Increase in transparency of 0.25% peptidoglycan in agarose (activity of lysozyme: 1-10,000 U/ml)



Picture 6: Increase in transparency of 0.50% peptidoglycan in agarose (activity of lysozyme: 1-10,000 U/ml)

The incubation times for a visible increase in transparency of 0.25% peptidoglycan in agarose are listed in Table 16.

Table 16: Incubation times for a visible increase in transparency of 0.25% peptidoglycan in agarose

Lysozyme activity [U/ml]	t [min]
10,000 – 2,500	120
2,500 – 500	260
500 - 50	370

After 120 minutes of incubation with lysozyme activities of 10,000 – 2,500 U/ml the polymer density apparently decreased and became transparent after 260 minutes of incubation. Differentiation to activities of 1,250 – 500 U/ml and 100 - 50 U/ml was possible after 260 and 370 minutes respectively.

4.1.3 Diagnostic tool - Alginate beads as release system

I Fabrication of beads

Substrates to be embedded into the polymermatrix were dissolved in water before fabrication of beads to determine the wavelength of their maximum absorbance. The wavelength scans are shown in Figure 18 and the wavelengths of maximum absorbance for the different substrates are listed in Table 17.

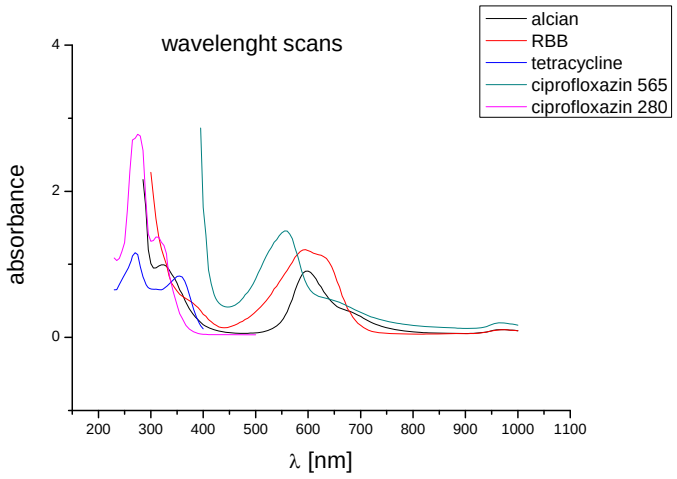


Figure 18: Wavelength scans of substrates

Table 17: Wavelength of maximum absorbance for substrates

substrate	λ [nm]
RBB – peptidoglycan	600
alcian blue - peptidoglycan	600
tetracycline	270
ciprofloxacin	280, 565

Alginate/peptidoglycan and alginate/gelatin/peptidoglycan blend beads were successfully produced. The $CaCl_2$ solution remained colourless during cross-linking of the polymer so it was concluded that all of the stained peptidoglycan was embedded in the beads. This was not the case for ciprofloxazin and tetracycline. Unlike peptidoglycan these antibiotics are small molecules so they diffused through the pores of the polymer and coloured the $CaCl_2$ solution yellow.

Due to the fact that it was not possible to keep all parameters of the bead production system constant from one charge to the next, beads of different size were fabricated. Per charge the arithmetic mean of masses ($\overline{m}$, formula [8]) as well as the standard deviation (SD, formula [9]) were calculated. A varying number of samples $n_{samples}$ consisting of either 4 or 5 beads per sample (n_{beads}) were used. The statistic characterisation of some charges is shown in Table 18,

$$\overline{m} = \frac{1}{n}\sum_{i=1}^{n} m_i = \frac{m_1 + m_2 + \ldots + m_n}{n} \qquad [8]$$

n number of beads

m_i mass of beads of sample i

$$SD = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}\left(m_i - \overline{m}\right)^2} \qquad [9]$$

Table 18: Mass of beads – mean values and standard deviation

charge	$n_{samples}$	n_{beads} per sample	$\overline{m}$ [mg]	SD [mg]	m per bead [mg]
1	36	4	50.81	1.58	12.70
2	37	5	64.58	1.48	12.92
3	18	4	50.36	1.27	12.59
4	30	5	61.10	1.12	12.22
5	36	5	59.72	1.45	11.94

II Balance of Remazol Brilliant Blue

The amount of RBB bound to peptidoglycan was determined to be 7.09 mg RBB per 50 mg peptidoglycan in 4.1.1. 40 g of polymer solution contains 0.2 g RBB-peptidoglycan and 28.36 mg RBB respectively. 5.9 g of polymer solution were lost during fabrication so 34.1 g of polymer solution containing 24.18 mg of RBB were polymerised to beads. 1266 beads were produced so that a theoretic amount of RBB per bead is calculated to be 19.1 ng.

A mean value of 11.94 mg per bead was determined in Table 18. For 1,266 beads a mass of 15.12 g of polymer is resulting. Compared to the mass of polymer solution used for bead polymerisation half of the weight got lost.

III Stability test

The stability of beads was tested. The results of beads containing 20% gelatin are shown in Figure 19. Beads were incubated in HEPES buffer (b1), in Collagenase buffer (b2) and in a 1:1 buffer mix (b1+2) at 37°C for 3 days. The three parallel experiments were done in triplicates and the results are demonstrated as mean value.

Beads were stable for the period of the experiment as they had an absorbance below 0.04 in each of the incubation buffers compared to incubation with lysozyme (l) showing an absorbance of 0.18 after 3 days (Figure 19, left). The conversion of peptidoglycan was calculated as described in equation 3 in chapter 4.1.2I and is shown in Figure 19 (right). There was some diffusion of stained peptidoglycan during incubation in buffer solutions leading to an absorbance per hour below 0.0005 compared to 0.0128 for incubation with lysozyme. Digestion of peptidoglycan by lysozyme thus induced a 26 fold higher absorbance than incubation in buffer solution.

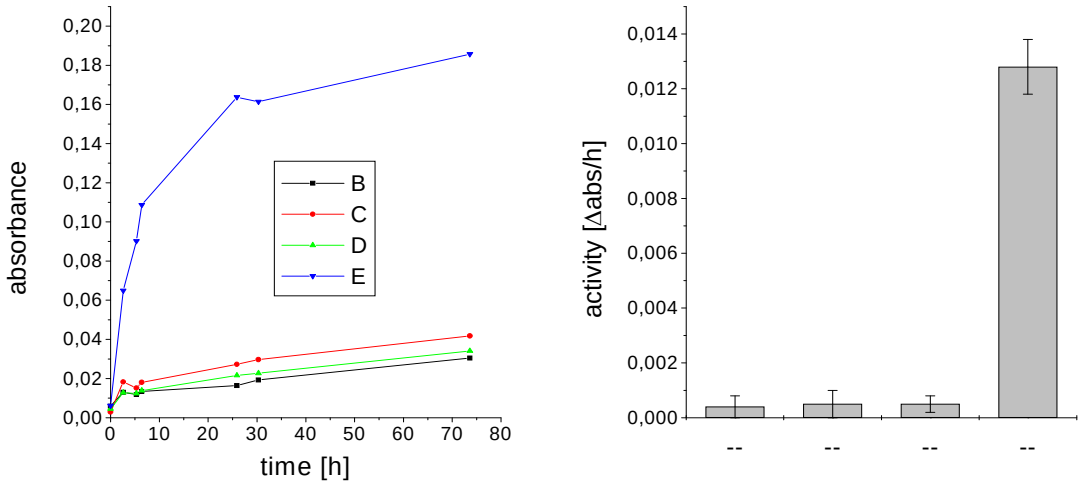


Figure 19: Stability test of beads containing 20% gelatin: absorbance vs incubation time (left) and conversion of peptidoglycan per hour (right). (HEPES buffer (b1), Collagenase buffer (b2), both buffer (b1+2), lysozyme (l))

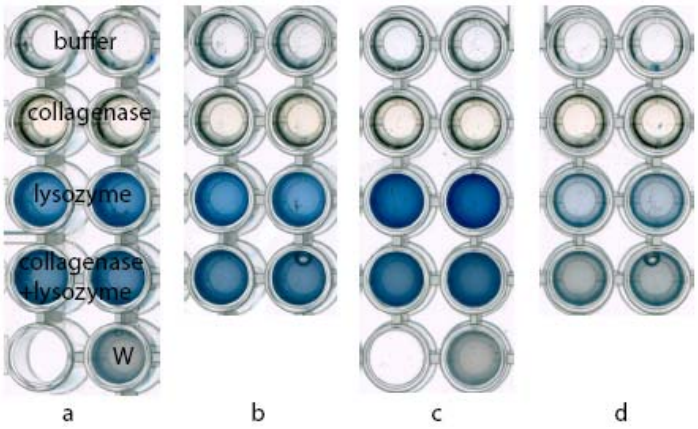
The same results were obtained for incubation of beads not containing gelatin. Beads consisting of 50/50 (w/v) alginate/gelatin showed different results when incubating for a longer period of time. After one week of incubation the absorbance was drastically increasing. The incubation solution became cloudy and the increase in density was measured as well at 600 nm. Due to non-sterile fabrication and working conditions it was assumed that the gelatin part of the matrix was contaminated since gelatin is not autoclavable for reasons of temperature.

IV Release test

The release test was carried out at 37°C and 60°C. 37°C is the normal human body temperature and thus the temperature of wound fluids whereas 60°C is the temperature optimum for lysozyme activity. For both incubation temperatures the same reaction characteristics were obtained but when incubating at 60°C the reaction rate was accelerated. Picture 7 represents the incubation solutions after incubation for 200 min at 60°C. Beads were containing peptidoglycan stained with Remazol Brilliant Blue (a-c) or Alcian Blue (d) and different amounts of gelatin. Remazol and Alcian are both feasible dyestuffs as they covalently bind to peptidoglycan and remain stable up to at least 60°C. Since Remazol Blue stained beads yield a stronger colour RBB was used for the subsequent experiments.

In preliminary zymography experiments collagenase IV was figured out to exhibit a higher gelatin conversion rate than collagenase I (see 3.3.2).

Hence, the experiment was carried out with lysozyme, collagenase IV, with both enzymes and with a sample of wound fluid. Buffer solutions as reference remained colourless (Picture 7, row 1) whereas incubation solutions containing lysozyme became blue (Picture 7, row 3 and 4). Collagenase alone (Picture 7, row 2) did not induce any release but influenced the lysozyme release characteristic in the experiment with both enzymes (Picture 7, row 4) in some extend. The wound fluid supplied here (Picture 7, row 5) was diluted to a lysozyme activity of 40 U/ml (a) and 15 U/ml (c) respectively before incubation. Compared to 250 U/ml lysozyme activity supplied in row 3 and 4, the wound fluid induced a relatively high release. Various other enzymes than lysozyme which are present in wound fluids were supposed to contribute to the digestion of the matrix.



Picture 7: Enzyme triggered release of Remazol Brilliant Blue (a-c) and Alcian Blue (d) from alginate peptidoglycan beads. Beads not containing gelatin (a,d) and beads containing 20% gelatin (b) and 50% gelatin (c) (W wound fluid)

Beads consisting of alginate/gelatin 100/0 (w/v) and 80/20 (w/v) (a and b) appeared to have the same release kinetic in contrast to beads containing 50% gelatin (c) which show higher release rates.

The conversion rates of beads not containing gelatin and beads containing 50% gelatin are compared in Figure 20. In this second preparation, the same activities of lysozyme and collagenase were supplied but the wound fluid was diluted only to an activity of 1,970 U/ml and the additional blister fluid was supplied undiluted (see Table 7). The results represent the mean values of triplicates each of the parallel experiments.

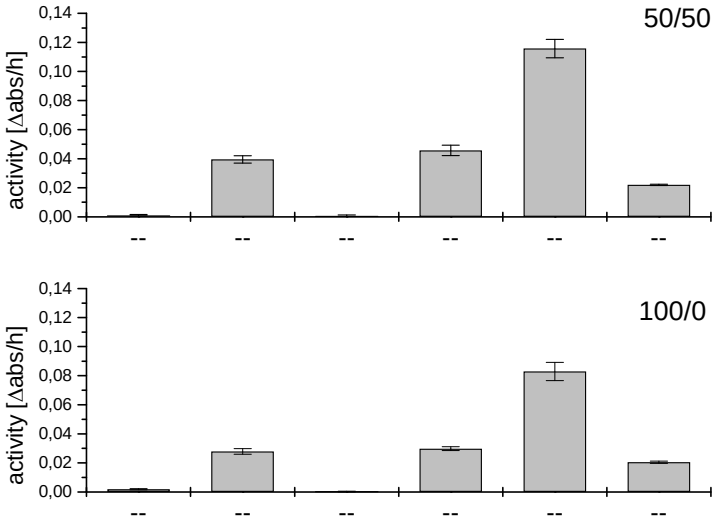


Figure 20: Release test of alginate peptidoglycan beads containing 50% (w/v) gelatin (above) and not containing gelatin (below) incubated in buffer (b), lysozyme solution (l), collagenase solution (c), lysozyme and collagenase solution (l+c), wound fluid (wf) and blister fluid (bf)

Buffer and collagenase incubation solution did not show any significant absorbance. Incubation with lysozyme (l) and both enzymes, lysozyme and collagenase (l+c), brought about a change in absorbance of 0.0395 and 0.0457 respectively per hour for beads containing 50% gelatin and 0.0279 and 0.0298 respectively per hour for beads not containing gelatin. Incubation with wound fluid leaded to a considerable high conversion rate of 0.1158 per hour (alginate/gelatin 50/50 (w/v)) and 0.0829 per hour (alginate/gelatin 100/0 (w/v)) whereas blister fluid induced less release than lysozyme.

The influence of buffer composition on the lysozyme activity was also tested. Incubation in HEPES buffer and incubation in 1:1 HEPES/collagenase mixed buffer yielded in the same enzyme conversion rate.

4.2 Detection of bacteria in wound fluids

Samples of wound fluids and blister fluids were collected in the LKH Graz. Depending on the character of the wound, samples of pure liquid wound fluid were taken as well as samples also containing pieces of dead tissue or even consisting only of dead tissue. Additionally a questionnaire (attached in the appendix) was filled for each patient concerning underlying diseases of the patient, age and stage respectively of the wound as well as physical and microbiological parameters of the wound.

Two samples each were taken. Microbiological identification was conducted by the AGES Graz. The other sample was used for enzyme identification in enzyme assays (see 3.2 and 4.3). Therefore wound fluids containing tissue were extracted with 100 µl of phosphate buffer and sterile filtered afterwards. Samples not containing tissue were sterile filtered only.
Due to the varying character of the different wound fluids and blister fluids and the fact that samples were partly containing buffer the results were statistically not significant but rough statements could be made.

Bacteria present in wound fluids were identified by the AGES Graz and are listed in Table 19.

Table 19: Bacteria present in wound fluids (iW: infected wounds, bW: blisters, rW: recovering wound fluid)

sample	inflammation	bacteria
iW 1	yes	*Proteus*
iW 2	yes	*Proteus, Streptococcus B, Pseudomonas*
iW 3	yes	Fungi
iW 4	yes	*Pseudomonas, Enterococcus*
iW 5	yes	*E. coli*
iW 6	yes	*Pseudomonas*
iW 7	yes	*Pseudomonas, Enterococcus*
iW 8	yes	*Staphylococcus epidermidis*
iW 9	yes	*Staphylococcus aureus*
iW 10	yes	*E. coli, Staphylococcus aureus, Enterococcus, Enterobacter*
iW 11	yes	*Enterococcus, Streptococcus Gr G, Proteus, Providencia*
iW 12	yes	*Enterococcus, Proteus, E. coli*
bW 1	no	
bW 2	no	
bW 3	no	
bW 4	no	
rW 1	no	*Staphylococcus epidermidis*
rW 2	no	*Staphylococcus epidermidis*, aerobe spore-forming organisms
rW 3	no	*Proteus, Enterococcus*
rW 4	no	*Proteus, Enterococcus, Staphylococcus aureus, Pseudomonas, Bacteroides*
rW 5	no	*Pseudomonas, Enterococcus*
rW 6	no	*Staphylococcus aureus*
rW 7	no	*Staphylococcus aureus*

4.3 Activity of biomolecules in wound fluids

Assays were performed to determine the protein concentration in wound fluids and the activities of the enzymes lysozyme, β-lactamase, myeloperoxidase and collagenase/gelatinase. Wound fluids of infected wounds, blister fluids and wound fluids of recovering wounds were tested.

The enzyme activities and protein concentrations respectively determined in the assays were calculated via a standard curve of the form $Y = A + B \cdot X$ whereas Y represents the measured (change in) absorbance and the emission respectively and X represents the enzyme activity or protein concentration. The results are represented as mean value of the listed wound fluids above for each assay with the according standard deviations.

4.3.1 BCA Assay

The absorbance at 562 nm was measured as repeat determination for the standard curve for protein quantification using Bovine Serum Albumin (BSA). The correlation between absorbance and protein concentration, $abs = 1.1188 \cdot c_{BSA} + 0.0028$, resulted from linear regression and is shown in Figure 21.

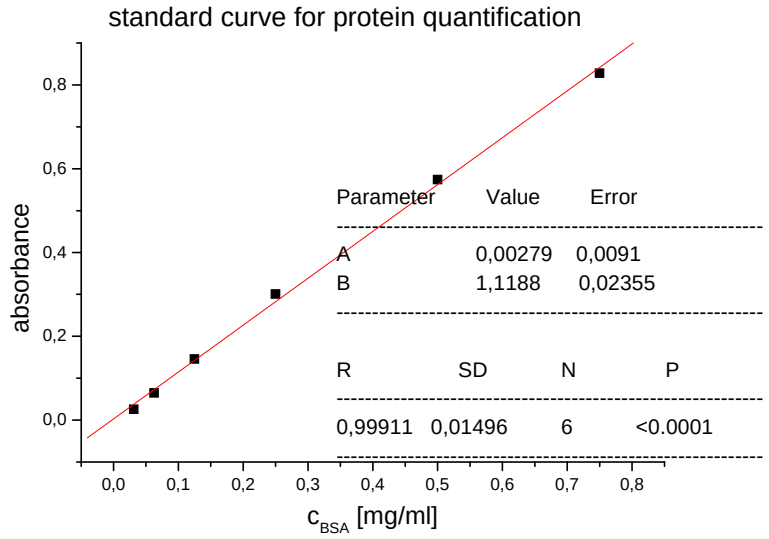


Figure 21: Standard curve for protein quantification (c_{BSA} - concentration of BSA)

The equation was used for calculating the protein concentrations in wound fluids. The results are shown in Table 20.

Table 20: Protein concentrations in wound fluids

type of wound	$\overline{m}$ [mg/ml]	SD [U/ml]
infected wounds	14.2	10.0
blister	111.5	16.3
recovering wound	16.2	4.0

In infected wounds and recovering wounds a low protein amount was found compared to blister fluids which showed a comparatively high level.

4.3.2 Lysozyme Assay

Lysozyme activity in wounds was determined by kinetically measuring the decrease in density of the *Micrococcus lysodeiktikus* dispersion at 450 nm. The slope of the absorbance-time correlation displayed the conversion of the substrate. The mean values of four parallel determinations were fitted linear (see Figure 22) resulting in the correlation $conversion[\Delta abs/\min] = -0.001 \cdot activity[U/ml] + 0.048$.

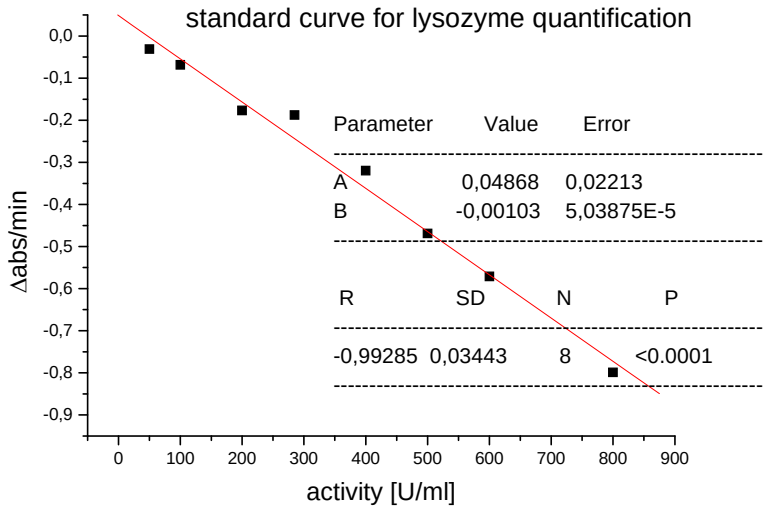


Figure 22: Standard curve for lysozyme quantification

Lysozyme activities in wound fluids determined via the standard curve are listed in Table 21.

Table 21: Lysozyme activities in wound fluids

type of wound	$\bar{m}$ [U/ml]	SD [U/ml]
infected wounds	1142	844
blister	295	175
recovering wound	310	109

Blister fluids and wound fluids of recovering wounds are low in lysozyme activity whereas a very high level of lysozyme was found in infected wounds. The statistical data of this assay are not significant as the standard deviation is very high. The samples' heterogenous character and the small sample number influenced the results.

4.3.3 β-Lactamase Assay

The β-lactamase activity was determined by kinetically measuring the decrease in absorbance of starch-iodide during penicillin hydrolysis at 620 nm. The standard curve was calculated as described for the determination of lysozyme activity (4.3.2) and resulted in the correlation of $conversion[\Delta abs/\min] = -0.0216 \cdot activity[U/ml] - 0.034$ (see Figure 23).

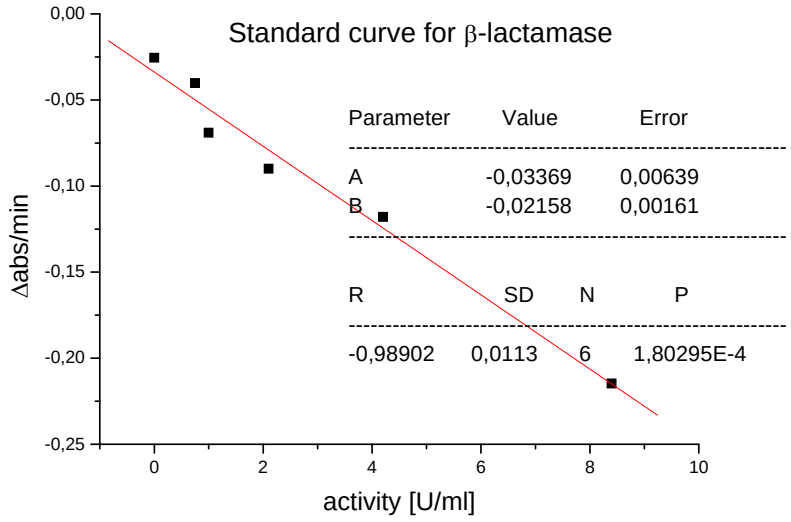


Figure 23: Standard curve for β-lactamase determination

Wound fluids of non-infected wounds were measured. Their β-lactamase activity was in the range of buffer solutions.

4.3.4 MPO Assay

Myeloperoxidase activity in wound fluids was determined by kinetic measurement of the absorbance of oxidised guajacol. The slope of the measured absorbance-time correlation reflected the oxidation rate of MPO and was used for calculating the enzyme activity. Linear regression of the standard curve (see Figure 24) resulting from repeat determination leaded to a correlation between oxidation rate and activity of $oxidation[\Delta abs/\min] = 0.034 \cdot activity[U/ml] - 0.012$.

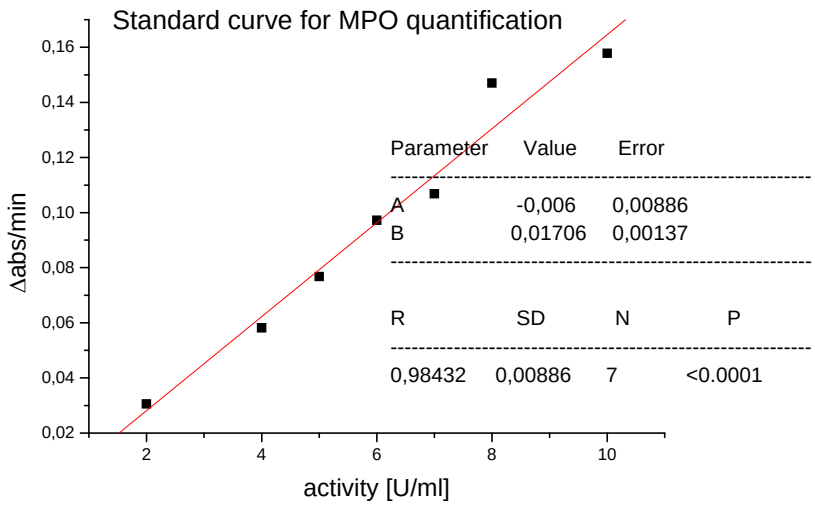


Figure 24: Standard curve for MPO quantification

Wound fluids were measured and their activity of myeloperoxidase was determined via the calibration equation. The results are shown in Table 22.

Table 22: Myeloperoxidase activity in wound fluids

type of wound	$\overline{m}$ [U/ml]	SD [U/ml]
infected wounds	171	311
blister	0	0
recovering wound	5	4

The level of myeloperoxidase found in infected wounds was high indicating the activities of the innate immunity. Blister fluids and wound fluids of recovering wounds on the contrary did not show any or very low MPO activity. As the standard deviation is very high as well for the same reasons as described in 4.3.2 the results are not statistically significant. At least an

enhanced level of myeloperoxidase in infected wounds can be stated compared to blister fluids and wound fluids of recovering wounds.

4.3.5 Enzchek Assay

The gelatinase/collagenase activity in wound fluids was determined in this assay. The fluorescence emerging from enzymatic digestion of gelatin was photometrical measured. The best results were achieved after 21 hours of incubation. Values of fluorescence of samples diluted 1:40 fitted best with the calibration area. A manual gain of actual fluorescence was setted 100.

The standard curve of the collagenase *Clostridium histolyticum* is shown in Figure 25. The resulting equation $Emission = 49275 \cdot activity[U/ml] + 717$ correlates the fluorescence emission with enzyme activity and was used for calculating gelatinase/collagenase activity in wound fluids.

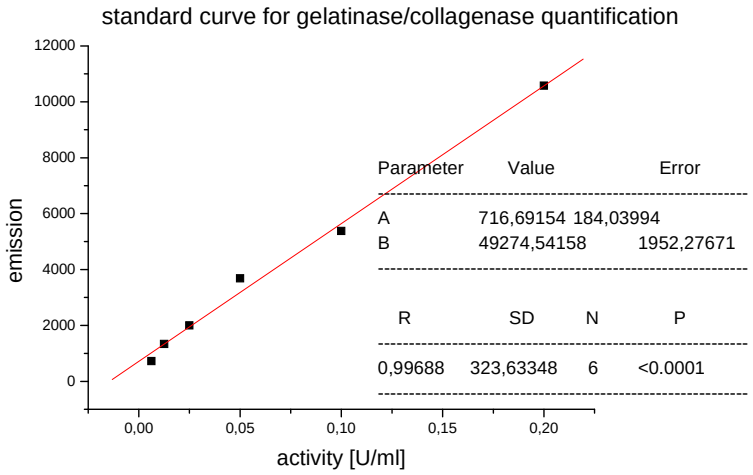


Figure 25: Standard curve for gelatinase/collagenase quantification

The determined activities of gelatinase/collagenase activity in wound fluids are listed in Table 23.

Table 23: Gelatinase/collagenase activity in wound fluids

type of wound	$\overline{m}$ [U/ml]	SD [U/ml]
infected wounds	10.5	10.8
blister	0.3	0.3
recovering wound	0.2	0.3

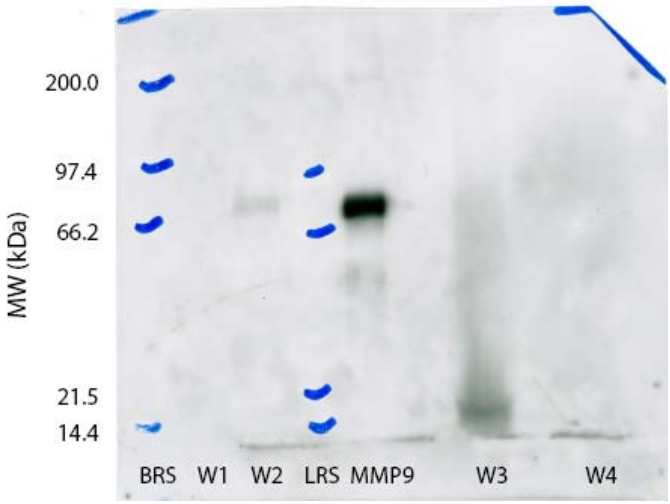
Concerning the significance of the results the same factors as described in 4.3.2 for the lysozyme assay leaded to a very high standard deviation. The gelatinase/collagenase activity in infected wounds was expected to be higher but its level is still enhanced compared to blisters and recovering wounds.

4.4 MMP detection by western blotting and zymography

Gelatinases and collagenases were further detected by western blotting and zymography. Western blotting was used for qualitative analysis of woundfluids detecting the matrix metallopeptidases MMP-9 (92 kDa type IV collagenase, gelatinase B) and MMP-2 (72 kDa type IV collagenase, gelatinase A). Gelatin- and collagen zymography was used for qualitative detection of crude microbial collagenase I and collagenase IV from *Clostridium histolyticum*.

4.4.1 Western blotting

Four different wound fluids (W1 – W4) were separated, transferred and visualised via western blotting to detect MMP-2 and MMP-9 respectively in two parallel experiments. A broad range (BRS) and a low range standard (LRS) as well as a MMP reference were blotted to enable the comparison of molecular weight. The X-ray film of the MMP-9 detection is shown in Picture 8.

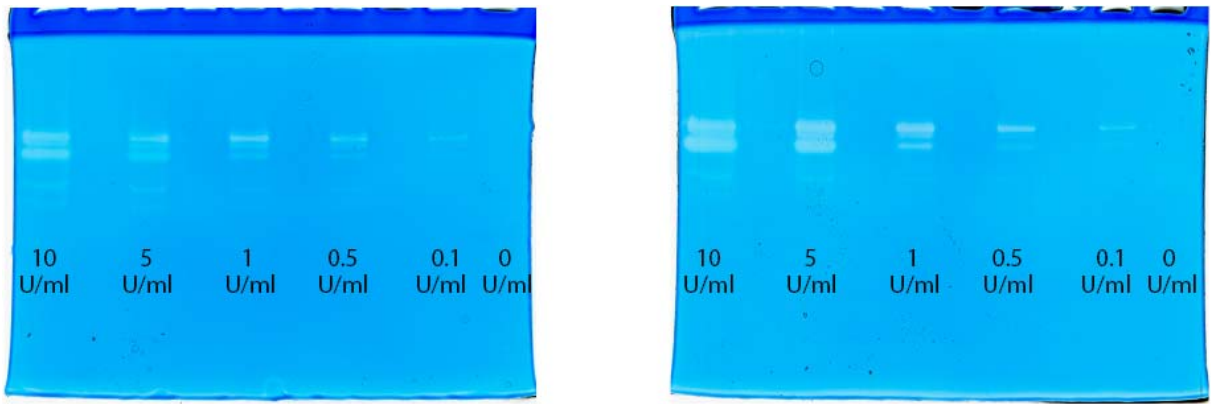


Picture 8: MMP-9 detection via western blotting (BRS: broad range standard, LRS: low range standard, MMP-9: purified MMP-9 proenzyme, W1-W4: wound fluid 1-4)

The purified human gelatinase MMP-9 was detected between 66.2 kDa and 97.4 and was thus verified as the 92 kDa MMP-9 proenzyme. A band of a similar molecular weight was identified in wound fluid 2 (W2) in lower concentration but not in wound fluids 1, 3 and 4.
The gelatinase MMP-2 (72kDa type IV collagenase) could not be detected via western blotting. Bands were not found in wound fluids and the purified human MMP-2 (standard) could not be detected neither.

4.4.2 Gelatin/Collagen zymography

Collagenase activity was visualised on gelatin- and collagen gels. Collagenases I and IV were loaded on gelatin gels with activities of 0, 50, 100, 250 and 500 U/ml. Electrophoresis did not bring about separated bands according to the enzymes' molecular weight so that collagenase activity was dispersed across the lanes. Nevertheless, the comparison showed that collagenase IV exhibits higher substrate conversion than collagenase I.
Collagenase activity was diluted to activities of 10, 5, 1, 0.5, 0.1 and 0 U/ml for collagen zymography. Picture 9 shows the different collagenase I (left) and collagenase IV (right) activities on collagen gels.



Picture 9: Collagen zymography (Collagenase I, left and collagenase IV, right) (Pictures kindly provided by Ulricke Gewessler)

Bands of different molecular weight evolved on the gels. As crude microbial collagenase was used, a number of other proteases, polysaccharidases and lipases were also included in the collagenase IV preparation (GIBCO^{TM} product information). Collagenase I was provided as mixture of collagenase, non-specific proteases and clostripain (Sigma-Aldrich product information). Crude collagenases I and IV exhibited the same band patterns. Collagenase IV

had a higher collagen conversion rate than collagenase I with the same enzyme activity supplied.

5 Discussion

The present work was part of the Lidwine project dealing with the prevention and treatment of non- and poorly healing wounds of patients suffering from Decubitus Ulcer. The objective of this work was the development of an indicator system triggered by enzymes present in infected wound fluids. The system was realised as a polymer/substrate system with stained and unstained substrates leading to a change either in colour or transparency indicating upon contact with infected wound fluids.

Various samples of wound fluids of infected and recovering wounds as well as samples of blister fluids (see Table 19) were screened for potential trigger enzymes i.e. these enzymes should enable differentiation between chronic infected and recovering non-infected wounds. Consequently, they are present in elevated levels in infected wounds but in low levels or not present in recovering wounds or blister fluids. Samples were provided by the dermatology and the plastic surgery of the LKH Graz. Due to the diversity of wounds, depending on the stage of the wounds, heterogeneous wound fluids were also used for the assays. Beside samples of pure wound exudates, samples contained or consisted of dead tissue. The tissue was extracted with a minimum volume of buffer before sterile filtration. The standard deviations of the results of the enzyme assays were comparatively high for this reason but yet clear differences were seen in infected and not infected wound samples. In accordance with the literature (Wysocki 1996; Ito, Yamada and Imoto 1992) elevated levels of lysozyme (1142 U/ml), collagenase/gelatinase (10.5 U/ml) and myeloperoxidase (171 U/ml) were found in infected wounds. Lysozyme and collagenase were thus chosen to act as trigger for the release system. Human gelatinase MMP-9 (type IV collagenase) was qualitatively detected in one of four wound fluids via western blotting. For the release system microbial collagenases I and IV from *Clostridium histoyticum* (BRENDA EC 3.4.24.3) were used instead of human collagenases MMP-2 and MMP-9 (BRENDA EC 3.4.24.24 and EC 3.4.24.35) since they all digest native collagen in the triple helix region after glycine as well as gelatin. The enzyme activity of the microbial collagenases was qualitatively compared via gelatin- and collagen zymography. Collagenase IV exhibited a higher gelatin/collagen conversion rate and was therefore used for the release experiment.

Alginate and agarose are biocompatible polymers (Becker 2001; Belitz, Grosch and Schieberle 2001; Guiseley 1989), form stable gels and are not degraded by microorganisms or enzymes found in wound fluids so that they are applicable as structural polymers. Gelatin was

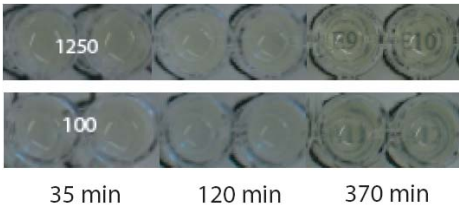
used as degradable part of the matrix as it is biocompatible as well as degradable by collagenases. Since we have chosen lysozyme as triggering enzyme, peptidoglycan (*Micrococcus lysodeikticus* cell wall) served as substrate in the release system as it is the enzymes natural substrate and was blended into the matrix.

Two different applications as release systems were performed. In the first approach the matrix consisted of agarose/peptidoglycan and was polymerised in 96-well plates whereas an alginate/peptidoglycan matrix was polymerised as beads in a $CaCl_2$ – solution in the second approach. The aim of the first application was the development of a diagnostic tool which enables fast differentiation between infected and non-infected wound fluids without photometric measurement. Stained and unstained peptidoglycan was compared as substrate. Remazol Brilliant Blue was covalently bound to peptidoglycan (see chapter 3.1.2) and incubated within the agarose matrix with different activities of lysozyme (see chapter 4.1.2I). The digested fragments of stained peptidoglycan released into the incubation solution were photometrically measured at 600 nm in addition. Supplied enzyme activity and increase in absorbance were positively correlated while incubation with buffer solution did not show a relevant absorbance. Linear correlation was restricted to enzyme activities up to 125 U/ml since the availability of substrate to lysozyme was limited due to the construction of the beads. Statistically every 8^{th} monosaccharide unit in PG carried a dye molecule. Thus, linearity is only as long possible as dyed oligomers are released.
Since a release triggered by enzymes was determined it was of exorbitant importance that the dyestuff to be measured was covalently bound to peptidoglycan and that this bond was stable under the conditions of the incubation experiment. Remazol Brilliant Blue and Alcian Blue as reactive dyestuffs were bound to the substrate according to Brückner (2008). Having passed the stability test the feasibility for the release test was compared. They were both suitable dyestuffs but the bright blue colour of Remazol Brilliant Blue appeared to facilitate differentiation compared to the somehow pale Alcian blue.
As an alternative to the release of dyed fragments, the increase of transparency was used as a parameter to monitor conversion of PG. A sheet of labelled paper was positioned underneath the transparent 96-well plate. Incubation with lysozyme, e.g. degradation of peptidoglycan induced an increase in transparency of the matrix making visible the number beneath. In addition, the density was photometrically measured at 450 nm. The measured results were the same compared to the experiment with blue-stained peptidoglycan but had inverse values as a decrease in density was measured instead of an increase. This decrease in density and increase

in transparency respectively was positively correlated with the supplied enzyme activity but, linear correlation was only up to an enzyme activity of 100 U/ml. This is most likely due to the fact that transparency correlates with polymer chain length. Thus, at a certain degree of hydrolysis the solution of resulting short chain oligomers turns completely transparent. For a fast differentiation with the naked eye the density of the matrix was optimised. 0.15% (w/w) peptidoglycan turned out to be too transparent since the numbers underneath all incubation wells with lysozyme activities ranging from 0 – 10,000 U/ml became visible at once after 120 minutes and were, in some extent, already transparent before incubation.

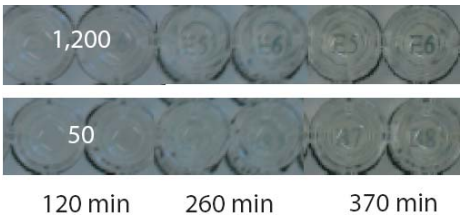
Picture 10: Agarose matrix containing 0.15 mg peptidoglycan: 120 minutes incubation with 1,200 U/ml and 100 U/ml

0.50% (w/w) peptidoglycan (see Picture 6) was too dense so that a change in transparency needed the same time for all different activities.



Picture 11: Agarose matrix containing 0.50% (w/w) peptidoglycan: 35, 120 and 370 minutes incubation with 1,250 U/ml (above) and 100 U/ml (below)

An applicable peptidoglycan concentration was found to be 0.25% (w/w) (see Picture 5). Matrices incubated with lysozyme activities of 10,000 – 2,500 U/ml became transparent after 120 minutes of incubation whereas an almost complete digestion of peptidoglycan took 260 minutes for supplied lysozyme activities ranging down to 500 U/ml. After 370 minutes lysozyme activities of 100 U/ml and 50 U/ml generated transparent matrices, activities of 10 and 1 U/ml needed even longer.



Picture 12: Agarose matrix containing 0.25% (w/w) peptidoglycan: 120, 260 and 370 minutes incubation with 1,200 U/ml (above) and 50 U/ml (below)

This transparency-time correlation is suitable for the determination of lysozyme activities in wound fluids indicating infection or not in consideration of the results of the lysozyme assay in chapter 4.3.2 (see Table 21). Activities found in blister fluids and wound fluids of recovering wounds were below 500 U/ml, in wound fluids of infected wounds on the contrary around 1,000 U/ml. Using the diagnostic tool, it is thus possible to make, in some extent, statements about the condition of a wound.

Beads were produced in a separate experiment (see chapter 3.1.4I). A semi-fluid sodium-alginate solution was blended with blue peptidoglycan as substrate for lysozyme and dropped into an ionic solution containing Ca^{2+} ions. Stable beads were formed through polymerisation. The alginate/peptidoglycan solution was further blended with different ratios of gelatin for an accelerated release. Collagenase was introduced in this experiment to degrade the gelatin part of the matrix. In a different approach peptidoglycan was implemented unstained as substrate for lysozyme and antibiotics (tetracycline or ciprofloxacin) were embedded into the alginate/peptidoglycan matrix as photometrically measurable indicator for the release. In contrast to peptidoglycan the antibiotics were not successfully enclosed into the matrix. They are small molecules and diffused into the $CaCl_2$ solution during polymerisation. Their inclusion was not optimised since it turned out that the stained peptidoglycan was as stable in the alginate matrix as it was in the agarose matrix and therefore a feasible system. Gelatin was stable as well when incubating in buffer solution for three days as proven in the stability test (see chapter 4.1.3III). The test was performed at 37°C with beads containing 20% and 50% gelatin respectively in HEPES buffer (lysozyme buffer), collagenase buffer and a 1:1 mix of both buffers. Beads were stable for three days showing only a negligible absorbance (see Figure 19). The stability test with beads containing 50% gelatin was prolonged to seven days and ended in a rapid increase in absorbance. A release of stained peptidoglycan or dyestuff by diffusion was ruled out because the incubation solution became cloudy but not blue. Fabrication of beads was not done under sterile conditions. It was thus assumed and confirmed by olfactory evaluation that gelatin is susceptible to contamination by bacteria so

that it was partly degraded after one week. Nevertheless it was used as co-polymer for the release test since the experiments were limited in time to a maximum of two days. Beads were incubated in 2.0 ml Eppendorf tubes and the absorbance of blue-stained fragments of peptidoglycan released into the supernatant was measured at 600 nm. The release test was conducted with beads not containing gelatin and with beads containing 20 and 50% gelatin. In parallel experiments they were incubated in buffer, in wound fluid, with 250 U/ml of lysozyme and with 250 U/ml of collagenase as well as co-incubated with both enzymes. Incubation was done at 37°C as it is the normal human body temperature and at 60°C as lysozyme activity rises with increasing temperature (Smolelis and Hartsell 1952) and reaches its temperature optimum at around 60°C. The same substrate conversion characteristic was observed for both temperatures but the conversion rate was accelerated when incubating at 60°C. After 6 hours of incubation at 37°C (see chapter 4.1.3IV, Picture 7) all buffer solutions were still colourless. All collagenase incubation solutions exhibited only the collagenases intrinsic orange colour. Incubation with lysozyme and co-incubation with lysozyme and collagenase brought about a release colouring the incubation solution deep blue. Beads containing 50% gelatin (Picture 7, c) showed a higher release than beads not containing gelatin and beads containing 20% gelatin (Picture 7, a and b).

Based on the results of the lysozyme assay, wound fluids were diluted with buffer to a lysozyme activity of 40 U/ml for beads not containing gelatin (a) and 15 U/ml respectively (c) for beads containing 50% gelatin. Their incubation solutions were comparable blue among themselves but lower than those of the lysozyme and lysozyme/collagenase incubation experiment. The latter ones had a supplied lysozyme activity of 250 U/ml each; the wound fluids thus triggered a comparatively high release. The same results were achieved via photometrical measurement of incubation solutions after 200 minutes of incubation at 60°C (Figure 20). A wound fluid containing approximately 2000 U/ml was introduced in this experiment as well as a blister fluid containing 187 U/ml as reference. Beads consisting of 50/50 (w/v) alginate/gelatin were compared to beads consisting only of alginate as matrix polymer. The same absorbance distribution resulted for both kinds of beads but was higher for beads containing 50% gelatin. As the release in all incubation experiments with gelatin was proportionally enhanced, not only when co-incubating with collagenase, gelatin apparently influences either the enzymatic degradation process in some way or it alters the matrix' molecular structure and thus facilitates diffusion of degraded peptidoglycan fragments. There was no significant distinction of absorbance between incubation with lysozyme and with

lysozyme/collagenase but nevertheless, a certain difference was measured in all incubation experiments. Collagenase thus exerts influence on the activity of lysozyme independently of its substrate gelatin. The absorbance of the wound fluid was significantly enhanced. Compared to 250 U/ml supplied in the lysozyme incubation solution the absorbance of the wound fluid containing nearly 2000 U/ml was still too low concerning linearity. Non-linear correlation between absorbance and higher enzyme activities was already found for the release system using an agarose matrix via a calibration line (see chapter 4.1.2I and chapter 4.1.2II). As application for diagnostic tool the detailed differentiation between lysozyme activities of enhanced levels is of no importance. Lysozyme levels of infected chronic wounds and healing wounds differ enough so that the information of either ‘enhanced’ or ‘not enhanced’ is sufficient. The blister fluid containing 187 U/ml is clearly distinguishable from the wound fluid as it is significantly lower in absorbance and even a difference to the lysozyme reference containing 250 U/ml is observable.

6 Conclusion

Two different types of triggered release system were developed to indicate wound infection. The release was triggered by the enzyme lysozyme which is present in wound fluids of chronic infected wounds in enhanced levels. Since its activity in recovering non-infected wounds was measured to be comparatively low a differentiation was possible via the release system. Positive correlation between supplied lysozyme activity and the release of degraded peptidoglycan from the agarose matrix, i.e. the increase in transparency was achieved. The alginate release system using stained peptidoglycan as substrate was realised as stable beads. It did not show any release when incubating in buffer while upon incubation with lysozyme it turned deep blue. The differentiation between a sample of wound fluid containing 2000 U/ml lysozyme and a sample of blister fluid containing 200 U/ml lysozyme was clearly possible via photometric measurement as well as with the naked eye. The addition of gelatin to the alginate matrix enhanced the release regardless of whether collagenase was present in the incubation solution or not. Gelatin was blended to the alginate matrix to be the digestable part for collagenase. The co-incubation experiment with lysozyme and collagenase did not bring about a significant enhancement of release compared to the incubation experiment using only lysozyme although degradability of gelatin and collagen by microbial collagnease I and IV was proven via zymography. Significant differences between high and low lysozyme activities were achieved after 200 minutes of incubation. Optimising the system concerning incubation time would be necessary for the application as diagnostic tool. The release test was performed with only one wound fluid and one blister fluid. Much more samples of various kinds of wound fluids have to be tested. Furthermore the standard deviation of the results of the enzyme assays has to be reduced by enhancing the number of samples. Heterogeneous wound fluids should be excluded from the assays since they are not as representative for the stage of a wound as homogeneous wound fluids are.

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8 Abbreviations

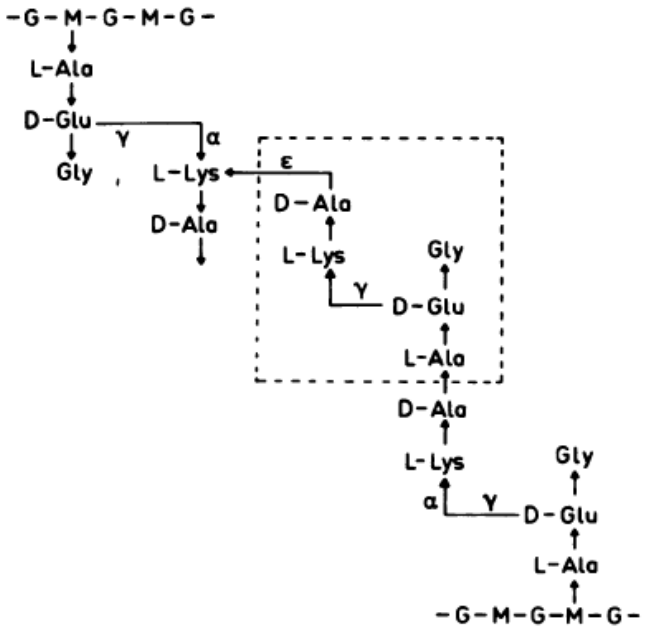
%	percent
% (w/v)	weight percent per volume
% (w/w)	weight percent
°C	degree celsius
µg	microgram
µl	microlitre
abs	absorbance
APS	ammonium persulfate
BC	bicinchoninic acid
BSA	bovine serum albumin
c	concentration
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
Da	Dalton
Δabs	change in absorbance
Δabs/h	change in absorbance per hour
ddH_2O	bidistilled water
dH_2O	deionised water
Δt	change in time
g	gram
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IgG	Immunoglobulin G
kDa	kilodalton
l	litre
M	mole per litre
m	mass
mA	miliampere
mg	miligram
mg/ml	miligram per mililiter
min	minutes
ml	mililiter

mM	millimole per liter
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MW	molecular weight
N	number
ng	nanogram
nm	nanometer
PBS	phosphate-buffered saline
PBS	phosphate buffered saline
PG	peptidoglycan
RBB	Remazol Brilliant Blue
RBB-PG	Remazol Brilliant Blue stained peptidoglycan
rpm	rounds per minute
s	seconds
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate - polyacrylamid gel electrophorese
t	time
TEMED	Tetramethylethylenediamine
t_{ink}	incubation time
TRIS	tris(hydroxymethyl)aminomethane
U	units
U/mg	units per miligran / specific activity
U/ml	units per mililiter
V	volume
V	volt
V_{inc}	incubation volume
X-ray	X-radiation, Röntgen radiation
λ	wavelength
$\overline{m}$	arithmetic mean of masses

9 Appendix

Calculation of the frequency of dyed saccharides in peptidoglycan

In the murein of Micrococcus lysodeikticus the two pentapeptides binding to the N-acetylmuraminic acid of the polysaccharide backbone are linked via an interpeptide bridge. The interpeptide bridge generally consists of one pentapeptide subunit. In case that there is more than one (up to four are possible) adjacent N-acetylmuraminic acids are not substituted (Schleifer and Kandler 1970). For the calculation of dyed saccharides it was assumed that every N-acetylmuraminic acid is substituted by a pentapeptide and that every pentapeptide is crosslinked to an opposing one by an interbridge consisting of one subunit (see Picture 13).



Picture 13: Murein structure of Micrococcus lysodeikticus (Schleifer and Kandler 1970)

The frequency of RBB-molecules bound to peptidoglycan was calculated as described in [10].

$$Disaccharids / RBB = \frac{m_{peptidoglycan} \cdot MW(RBB)}{m_{RBB} \cdot MW(Disaccharid)} = 3.94 \qquad [10]$$

$m_{peptidoglycan}$	mass of peptidoglycan [mg] (m=50mg)
m_{RBB}	mass of RBB [mg] (m=7.09mg)
MW (RBB)	molecular weight of RBB [g/mol] (MW=626.5g/mol)
MW (Dimer)	molecular weight of disaccharid [g/mol] (MW=1121.5 g/mol)

The frequency was calculated in disaccharide units. One disaccharide unit consists of the two saccharides N-acetylglucosamin (G) and N-acetylmuraninic acid (M), the pentapeptide and half of the linker unit. The proportion of masses of peptidoglycan and Remazol Brilliant Blue (RBB) in stained peptidoglycan was determined in 4.1.1.

It was determined that every 4^{th} disaccharide and every 8^{th} saccharide of the peptidoglycan backbone was stained with one molecule of Remazol Brilliant Blue.

Tables

Protein molecular weights of high range and broad range standard for Western blot:

Table 24: Protein molecular weights of standards for Western blot

Protein	Molecular Weight [Da]	Broad range	Low range
Myosin	200000	x	
β-Galactosidase	116250	x	
Phosphorylase b	97400	x	x
Serum albumin	66200	x	x
Ovalbumin	45000	x	x
Carbonic anhydrase	31000	x	x
Trypsin inhibitor	21500	x	x
Lysozyme	14400	x	x
Aprotinin	6500	x	

Questionnaire

Patient Nr.		**Keime:**
Geschlecht		
Alter		
zusätzliche Erkrankungen		
Art der Wunde		
Dauer der Wunde **erste Behandlung am** **bisherige Behandlungen** **Datum der Probennahme** **Probennahme Nr.** **Volumen der Wundflüssigkeit** **Behandlung vor Probennahme** **Wundbettbehandlung vor Probennahme** **Stadium I-IV**		
Lokalisierung der Wunde **Größe der offenen Wunde**		
Menge der Wundflüssigkeit		
nur auf Verband im Wundbett: minimal im Wundbett: mittelmäßig im Wundbett: reichlich		
Beschreibung der Wundflüssigkeit		
serumhältig blutig blutig und eitrig eitrig		
Geruch der Wunde		
kein fauler Geruch fauler Geruch		
Nekrose		
keine nur in Wundbasis am Rand der Wunde am Rand und in der Mitte		
Entzündung	**ja** **nein**	
Infektion	**ja** **nein**	
andere Auffälligkeiten		

Material

chemical	provided by
acrylamide/bis-acrylamide 30% solution	Sigma Aldrich
agarose NEEO Ultra Quality	Carl Roth GmbH
Alcian blue 8GX	Polysciences, Inc.
alginic acid sodium salt	Carl Roth GmbH
BC Assay Protein Quantification Kit UP40840A	UPTIMA
$CaCl_2$ anhydrous	Carl Roth GmbH
$CaCl_2*2H_2O$	Carl Roth GmbH
collagenase Type I	Sigma Aldrich
collagenase Type IV	GIBCO^{TM} invitrogen
coomassie Brilliant Blue R-250	BIO-RAD
ECL^{TM} Western Blotting Analysis System RPN 2108	Amersham
gelatin from bovine skin Type B	Sigma
H_2SO_4	Carl Roth GmbH
HCl 37%	Carl Roth GmbH
HEPES	Carl Roth GmbH
K_2HPO_4 anhydrous	Carl Roth GmbH
KH_2PO_4	Carl Roth GmbH
lysozyme from chicken white egg	AppliChem
peptidoglycan from *Micrococcus Lysodeikticus*	MP Biommedicals, LLC
myeloperoxidase	Planta Natural Products
Na_2HPO_4 anhydrous	Carl Roth GmbH
NaCl	Carl Roth GmbH
$NaH_2PO_4 \cdot 2H_2O$	Carl Roth GmbH
Remazol Brilliant Blue R	Sigma Aldrich
SDS pellets	Carl Roth GmbH
TEMED	Carl Roth GmbH
TRIS ultra quality	Carl Roth GmbH

Equipment

equipment	provided by
analytic balance	Sartorius 2004 MP
analytic balance	KERN PB
electrophoresis power supply	BIO-RAD, PowerPAC 1000
electrophoresis power supply	BIO-RAD, PowerPac HV
electrophoresis system	BIO-RAD, Mini-PROTEAN Tetra Electrophoresis system
magnetic stirrer	IKS RH basic 2
pH-meter	METTLER TOLEDO Seven Easy
pipette	Carl Roth GmbH
pipette	Soccorex, ACURA 825
platereader	TECAN infinite M200
scanner	Hp Scanjet 4890
shaker	Heidolph PROMAX 2020
thermomixer	Eppendorf Thermomixer comfort
vortex	JANKE & KUNKEL, IKA, VF2
well plates	Greiner bio-one
western blotting cell	BIO-RAD, Mini Trans-Blot Cell
zentrifuge	Eppendorf mini spin (F-45-12-11)