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DI Barbara THALLINGER

DISSERTATION

Functionalization of catheters with antimicrobial enzymes

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Univ. Prof. DI Dr. Georg Gübitz

Institut für Umweltbiotechnologie

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Preamble

This thesis is divided into 3 main sections. The first section (Chapter 1 and 2) provides the reader with the overall aim of this work and a general introduction to the topic, including information about pathogens colonizing biomaterials and biofilm formation, the problem with catheter associated urinary tract infection and a literature review on the current treatment methods. An extensive review about antimicrobial enzymes and their application closes the first section.

The second section (Chapter 3 to 5) lists three publications that were published during this thesis. The first one exploits the potential of Cellobiose Dehydrogenase (CDH) as antimicrobial and antibiofilm agent with a possible application in catheter lubricant formulations.

Publications two and three describe two approaches of immobilizing CDH on the polydimethylsiloxane urinary catheter surface - based on covalent binding and ultrasonic deposition of nanoparticles – and the consequent antimicrobial analysis of the created coating.

The third section (Chapter 6 and 7) closes this thesis with a general conclusion and an appendix, listing all publications, oral presentations and posters that were presented in the time course of this thesis.

Abstract

The work of this thesis was carried out within the NOVO project of the seventh framework program of the European Union, which aimed at developing novel antimicrobial coatings for urinary catheters in order to prevent the formation of pathogenic bacteria biofilms on this indwelling medical device. Biofilms forming on urinary catheters cause infections also known as catheter associated urinary tract infections (CAUTI) that account for 40% of all nosocomial infections occurring in hospitals, causing the removal of the catheter alongside intensive antibiotic treatment. Due to the difficulties faced when treating biofilm-associated infection, prevention is key in reducing the number of CAUTI. A lot of effort has been put into the development of novel antimicrobial coatings for urinary catheters in order to fight the problem. The use of enzymes with antimicrobial properties is quite a recent development and among them cellobiose dehydrogenase (CDH) which produces the strong oxidizing agent hydrogen peroxide (H_2O_2). In this study, the potential of using CDH as an antimicrobial and antibiofilm agent is investigated.

First, the conditions under which CDH exerts maximum antimicrobial activity against both gram negative and gram positive bacteria were established and then tested against clinical isolates commonly colonizing catheter surfaces. Strong effects against strains such as *A. baumanii* and the multidrug resistant *S. aureus* were demonstrated. Partially hydrolyzed exopolysaccharides, a major constituent of the biofilm matrix, were identified as potential substrates for CDH, enabling a self-sustaining antimicrobial system.

Two immobilization approaches for CDH on the polydimethylsiloxane (PDMS) urinary catheter surface were investigated. The covalent immobilization protocol developed yielded a highly stable and robust antimicrobial surface, with an enzyme remaining 20% of its original activity over 16 days. Both short term (3 hours) and long term (7 days) incubation with *S. aureus* lead to around 70% reduction of growth on the coated surface as compared to the control. The coating didn´t show any signs of toxicity against the mammalian cell lines HEK 239 and RAW 264.7 when incubated with antimicrobial effective substrate concentrations (<2 mM). With the help of high power ultrasound treatment a quick and easy deposition protocol of CDH nanoparticles on PDMS could be developed. The enzyme still produced high quantities of H_2O_2 after the treatment showing strong antimicrobial activity against *S. aureus.*

CDH is a very potent antimicrobial and antibiofilm enzyme, suitable for immobilization therefore fulfilling all requirements for a novel urinary catheter coating.

Kurzfassung

Diese Arbeit wurde im Rahmen des im 7. Rahmenprogramm der EU geförderten Projektes „NOVO“ durchgeführt. Das Ziel dieses Projekts war die Entwicklung neuer antimikrobieller Beschichtungen für Blasenkatheter um die Bildung von pathogenen Biofilmen zu verhindern. Biofilme, die sich auf Blasenkathetern bilden, führen zu Infektionen, auch Katheter assoziierte Harnwegsinfekte (CAUTI) genannt, die 40 % aller im Krankenhaus auftretenden nosokomialen Infektionen ausmachen und eine Entfernung des Katheters und eine intensive Antibiotikatherapie notwendig machen. Da es sehr schwierig ist, Biofilm-assoziierte Infektionen zu behandeln, ist Vorbeugung der einzig sinnvolle Weg um die Anzahl an CAUTI zu reduzieren. Um das Problem zu bekämpfen, wurde viel Aufwand betrieben, neue antimikrobielle Beschichtungen für Blasenkatheter zu entwickeln. Die Verwendung von Enzymen mit antimikrobiellen Eigenschaften ist eine recht neue Entwicklung auf diesem Gebiet und inkludiert Cellobiose Dehydrogenase (CDH), da es das starke Oxidationsmittel Wasserstoffperoxid (H_2O_2) produziert. In dieser Arbeit wurde das Potential der CDH für die Verwendung als antimikrobielle und antibiofilm Substanz untersucht.

Als erstes wurden die optimalen Bedingungen, die zur maximalen antimikrobiellen Aktivität gegen gram negative und gram positive Bakterien führen etabliert und folglich an Mikroorganismen getestet, die häufig Katheteroberflächen kolonisieren. Die stärksten Effekte konnten gegen den multiresistenten *S. aureus* und *A. baumanii* beobachtet werden. Partiell hydrolysierte Exopolysaccharide, ein Hauptbestandteil der Biofilmmatrix, wurden als mögliches Substrat für die CDH identifiziert wodurch ein sich selbst erhaltendes antimikrobielles System ermöglicht wird.

Es wurden zwei Immobilisierungsstrategien zur Verankerung der CDH auf Blasenkathetern aus Polydimethylsiloxan (PDMS) untersucht. Das Protokoll, das für die kovalente Immobilisierung entwickelt wurde, führte zu einer sehr stabilen und robusten antimikrobiellen Oberfläche, wobei das Enzym über einen Zeitraum von 16 Tagen noch immer 20% seiner Ursprungsaktivität behielt. Kurzzeit- (3 Stunden) und Langzeitinkubationen (7 Tage) mit *S. aureus* führten zu einer 70% Reduktion des bakteriellen Wachstums auf der Oberfläche. Die Beschichtung zeigte keine Anzeichen von Toxizität gegen die Säugetierzelllinien HEK 239 und RAW 264.7, auch nicht wenn antimikrobiell wirksame Substratkonzentrationen (<2 mM) zugegeben wurden. Mit Hilfe von Hochenergieultraschallbehandlung wurde eine einfache und schnelle Immobilisierungsmethode für CDH-Nanopartikel auf PDMS entwickelt. Das Enzym produzierte nach der Behandlung immer noch große Mengen an H_2O_2 und zeigte eine starke antimikrobielle Aktivität gegen *S. aureus*.

Die CDH ist daher ein sehr stark antimikrobiell und antibiofilm wirksames Enzym das sehr gut für Immobilisierungsprozesse geeignet ist und daher allen Anforderungen an eine neue antimikrobielle Blasenkatheterbeschichtung entspricht.

1

Aim

The work of this thesis was embedded within the “NOVO” project, funded by the seventh framework program (FP7) of the European Union. The overall aim of this project was to develop novel approaches for prevention and degeneration of pathogenic bacteria biofilms formed on medical devices e.g. catheters. In order to achieve these goals three different approaches were pursued: (i) the coating with antimicrobial active metal oxides, (ii) the coating with enzymatically modified polyphenolic compounds and (iii) the immobilization of the antimicrobial enzyme cellobiose dehydrogenase.

Due to the fact that the oxidoreductase Cellobiose Dehydrogenase (CDH), originating from the thermophilic organism *Myriococcum thermophilum*, is able to produce the strong oxidative agent hydrogen peroxide in reasonable quantities, it is a promising candidate for an antimicrobial enzyme. Therefore to achieve this aim, a number of objectives were planned including:

1. Investigating the antimicrobial and antibiofilm properties of CDH against both gram negative and gram positive bacteria colonizing catheters.

2. Developing CDH immobilization strategies on polydimethylsiloxane urinary catheters. This should ensure that the enzyme maintains its activity during the application of the enzyme functionalized catheter (Figure 1).

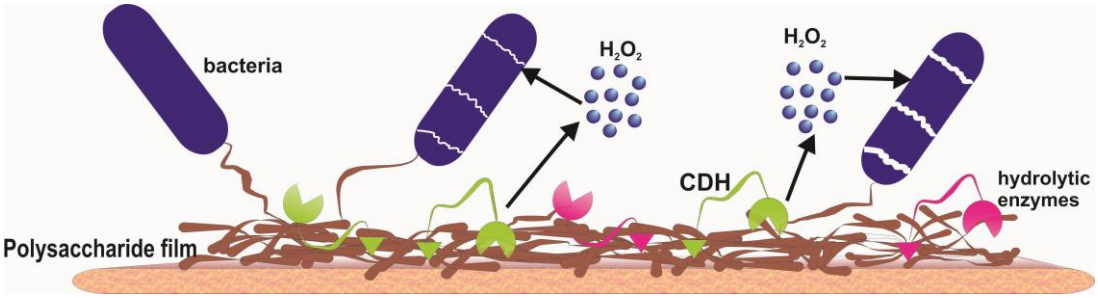

Figure 1: Mode of action urinary catheter coating containing CDH

3. Investigating the biocompatibility of CDH functionalized catheters.

4. Investigating the stability of CDH in urine.

2

Introduction

2.1 Pathogenic bacteria and their biofilms

Bacteria generally exist in two modes of growth: the planktonic mode, where they can move around freely in bulk solution and the sessile mode, where they are attached to a surface in the form of a biofilm [1]. The biofilm mode of growth offers several advantages over the planktonic state. The biofilm can act as a sorptive sponge that binds and concentrates organic molecules and ions close to the cells, which can be essential for the survival of bacterial populations [2]. It can prevent the access of antimicrobials and xenobiotics to the cells in the biofilm and offers protection against environmental stresses such as UV radiation, pH shifts and osmotic shock [3, 4]. Therefore, whenever planktonic bacteria find themselves close to a solid surface, i.e. when medical devices are implanted or inserted into the human body the consequence is the reversible attachment. This is the first and most crucial step in the formation process of biofilms, that can be seen in Figure 2 and involves a combination of electrostatic interactions, hydrogen bonds and van der Waals forces [1].

Another factor influencing adhesion is the production and secretion of extracellular polymeric substances by the bacteria, which consist of extracellular polysaccharides (EPS), proteins and nucleic acids. EPS play a crucial role in the attachment of bacterial cells to a surface as they facilitate cell adhesion to biotic (i.e. epithelial and endothelial cells) and abiotic surfaces (i.e. mineral surfaces or medical implants) as well as to each other. EPS are as structurally and functionally diverse as are the bacteria that synthetize them but can be devided into two groups: “cell-bound extracellular polymeric substances” also called capsular

polysaccharides and free EPS otherwise known as slime [5]. The biological functions of bacterial EPS include resistance to desiccation, protection against nonspecific and specific host immunity and adherence. Especially the presence of capsular polysaccharides can increase the survival of a pathogen as it acts as a permeability barrier facilitating the selective transportation of nutrients while excluding harmful substances [6-8]. EPS are composed of repeating monosaccharide units which are joined by glycosidic linkage and can take the form of either homo- or heteropolymers [9].

Within a few hours after reversible attachment of bacteria to a surface, which may be covered by a conditioning layer of proteins, irreversible binding takes place followed by multiplication of the bacteria. Once this step has occurred the bound bacteria lose their susceptibility to antibiotic treatment [10]. Microcolonies form and start to produce the highly hydrated (average water content of 95% [11]) extracellular polymeric matrix around them. The maturation of the biofilm includes the growing in thickness (up to 50 µm) and the development of mushroom- and/or tower-like structures. This is also the stage where it shows maximum resistance to antibiotics and the scientific basis is discussed in detail in below. In a last step, bacteria are released from the biofilm and spread to a different location to form a new biofilm there [12].

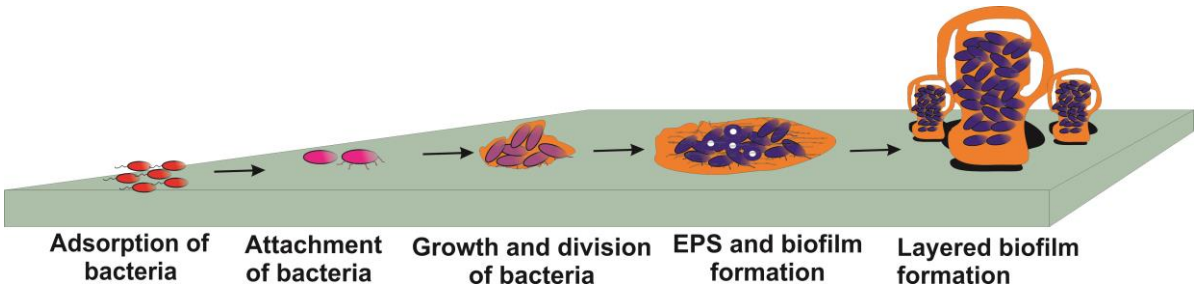

Figure 2: Scheme of biofilm formation on a solid surface

It can be stated, that microorganisms that live in matrix enclosed biofilm communities are more resistant to the host defenses as well as antibiotic therapy and are associated with the chronic nature of the subsequent infection [13, 14]. Several reasons for the resistance of bacteria living in biofilm communities are being investigated. Stewart et al. have described three possible mechanisms which are likely to be responsible for the increased antibiotic tolerance of biofilms: slow penetration of antibiotics (a), the formation of a resistant phenotype (b) and an altered microenvironment within the biofilm (c) [14].

(a) The slow or incomplete diffusion of antibiotic systems through the matrix into the biofilm has long been claimed responsible for the increased tolerance. Studies have shown that, depending on the type of antibiotic, the penetration of experimental biofilms is indeed limited. This is the case for vancomycin and ampicillin, however, other agents like tobramycin, ciproflaxin and rifampin can diffuse freely [15]. The

reason for this can be attributed to the rapid deactivation of certain antibiotics in the top layers of the biofilm, which consequently inhibits their antimicrobial effect. Results of studies don´t suggest that the exopolymeric matrix material forms a universal barrier that inhibits antimicrobial penetration [14, 15].

(b) Studies have shown that a phenotypic heterogeneity can be found within a biofilm. One subset of cells, the so-called persister cells, shows a decreased susceptibility to antimicrobials, not because they are resistant, but because they appear to be in a transient dormant state. This type of cells is responsible for the recurrence of infections related to biofilms as they can transform from the dormant to a metabolically active state causing a relapse of the infection [16].

(c) Within a biofilm, anaerobic niches, concentration gradients, local accumulation sites of acids and inhibitive waste products, zones of nutrient depletion as well as metabolically inactive zones can be found. This altered chemical microenvironment could directly antagonize the action of an antibiotic [17, 18].

As shown by the points described above, the molecular mechanisms underlying the reduced antibiotic susceptibility of biofilms are very complex and need to be studied further in order to be completely understood. Especially because the mechanisms vary depending on which types of pathogens are involved in the biofilm.

Pathogens most often associated with medical device related infections caused by biofilms are usually of commensal flora or nosocomial origin. Coagulase negative Staphylococci (CoNS) and Staphylococci spp. are the strains most frequently associated with medical device related biofilm infections [15]. Indwelling medical devices affected by bacterial adhesion include central venous catheters, urethral catheters, breast implants, cochlear implants, mechanical heart valves and many more. The principal causative pathogens are very often joined by secondary pathogens forming strong multi species biofilms. *K. pneumoniae*, *E. faecalis*, *P. aeruginosa* and Streptococcus spp. are amongst the most frequent. However, also fungal pathogens especially *Candida albicans* are known to cause biofilm associated infections especially in immune-compromised patients [15]. Other types of infections which are commonly caused by biofilms are periodontitis and chronic lung infections in cystic fibrosis patients [19, 20].

2.2 Catheters – Catheter associated urinary tract infections

Urinary catheters are usually made from latex or silicone (polydimethylsiloxane) and are inserted either through the urethra or a surgical incision made in the abdominal wall, right above the pubic bone into the bladder. Types of catheters include indwelling catheters (Foley catheters seen in Figure 3) which are secured inside the bladder by a balloon providing continuous urine drainage and suprapubic catheters (Figure 4) which are indicated if urethral catheterization is not possible. Patients are catheterized either for short-term (1-14 days) bladder drainage or for long term management of bladder dysfunction. Issues requiring short term catheterization include temporary relief of reversible bladder voiding difficulties or measurement of urine output after lower urinary tract surgery. Long-term catheterization on the other hand is indicated by chronic urinary retention or incontinence not treatable by other means [21].

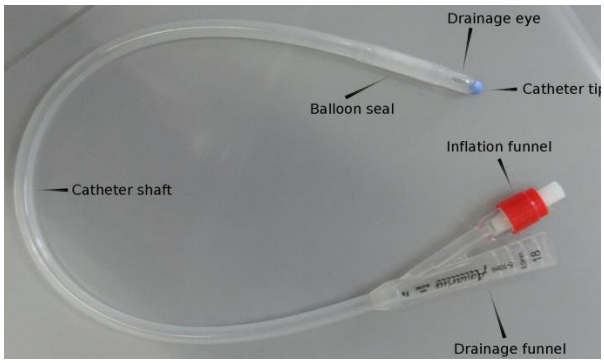

Figure 3: Set up of a non-inflated foley catheter

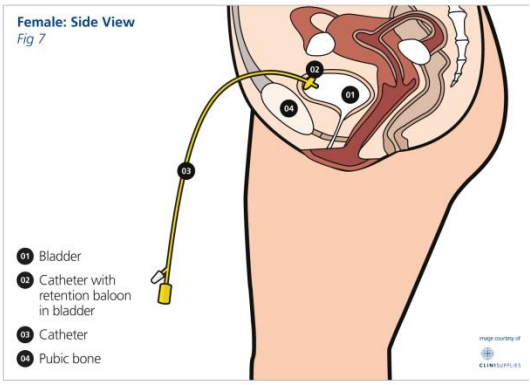

Figure 4: Set up of suprapubic catheter [22]

As urinary catheters are indwelling medical devices they offer an ideal site for bacterial attachment followed by biofilm formation consequently leading to infections in the affected area [23, 24] also because they interfere with the normal defense mechanisms of the urinary tract [25]. Urinary tract infections (UTI) account for over 40% of all nosocomial infections, which per definition are health complications that arise during therapy of a patient in a medical facility and are a consequence of microbes colonizing the surface of a polymer-based medical device [26]. These secondary infections affect an average of 8% of all hospitalized patients all over the world causing a high number of deaths and increased health care costs in the range of billion US dollars. Over 80% of UTIs are catheter associated urinary tract infections, meaning that the infection occurred as a consequence of catheterization followed by bacterial adhesion and biofilm formation [27]. The major route of infection for catheter associated urinary tract infections (CAUTIs) is ascending (i) at the time of insertion of a catheter through the urethra, (ii) via the mucosal layer between the catheter and urethra and (iii) through the catheter lumen [28, 29]. The risk of acquiring CAUTI highly depends on the method and duration of catheterization, the quality of catheter care and host

susceptibility as well as the type of catheter system used: An open system, where the urine drains into an open collection container leads to CAUTI in 100% of cases within several days. [30]. A closed system on the other hand, where the catheter empties into a securely fastened collecting bag reduces the risk as only 50% of all patients develop CAUTI when the system is in operation for more than 5 days [31, 32].

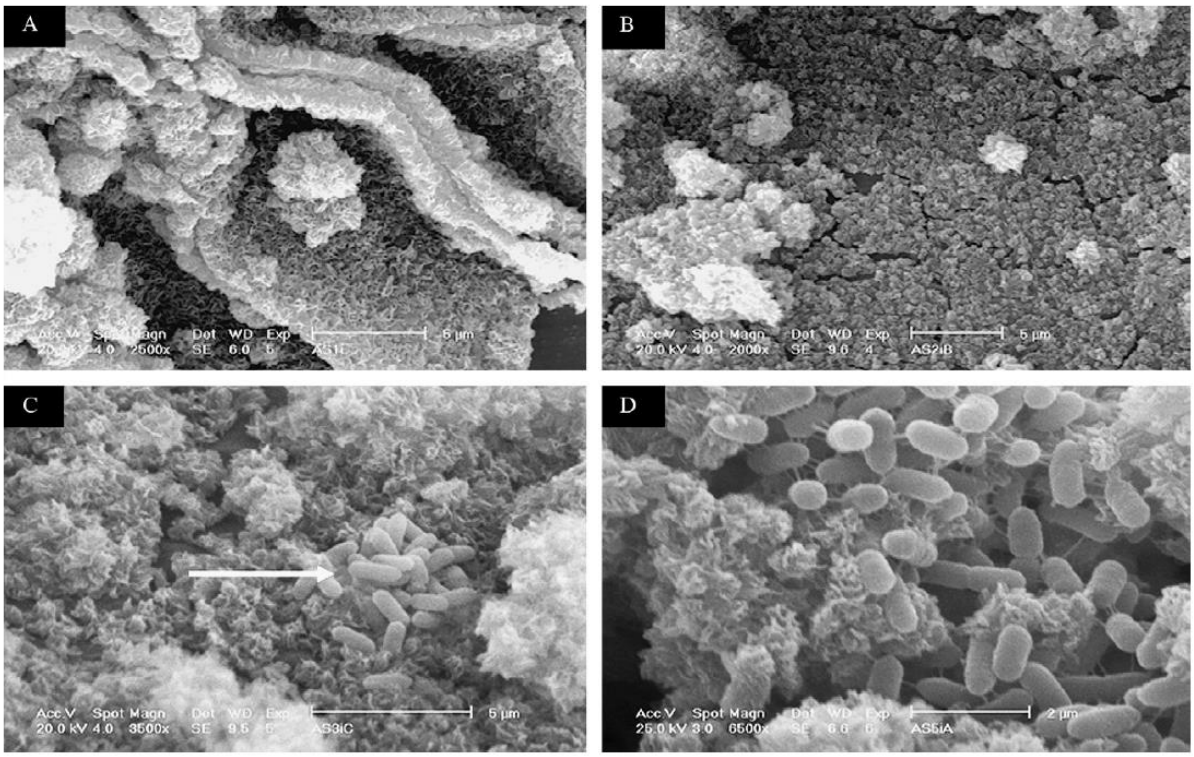

Figure 5: Examples for crystalline *P. mirabilis* biofilms formed close to the eyehole of a urinary catheter: (A) microcrystalline foundation layer after 1h, (B) foundation layer after 2h, (C) typical microcolony developing on the foundation layer after 4h, (D) mature crystalline biofilm after 18h [33]

In 90% of the cases the infections are monomicrobial with *E. coli*, *P. aeruginosa*, *Enterococci*, *Candida*, *Klebsiella* or *Enterobacter* spp. being the most frequently isolated pathogens. Certain microorganisms commonly colonizing catheters have the ability to alter the local pH through the production of urease, an enzyme that hydrolyzes the urea of the urine to form free ammonia. This in turn will increase the pH in the surroundings of the catheter and cause the precipitation of minerals such as calcium phosphate, magnesium, and ammonium phosphate [34]. The main problem about these precipitates is that they will deposit in catheter biofilms leading to encrustation and blockage of the catheter, consequently leading to the removal of the device (Figure 5). *P. mirabilis*, *M. morganii*, *P. aeruginosa*, *K. pneumoniae* and *P. vulgaris* are known to be the main urease producing organisms in urinary catheters [35].

As reports in the last few years have identified biofilm formation on urinary catheters as key in the pathogenesis of CAUTI the interest in altering the catheter surfaces to inhibit biofilm formation has arisen [29].

2.3 Treatment methods – state of the art

Once CAUTI occurs the affected catheter has to be removed and replaced while the patient has to undergo antibiotic treatment. As the treatment of biofilm associated infections is very difficult, prevention is the key to reducing the occurrence of CAUTI. The administration of systemic antibiotics is known to kill planktonic bacteria in urine and also to reduce the initial rate of catheter associated bacteriuria but fails to eradicate the sessile biofilm bacteria [36, 37]. Undertaken preventive measures in the clinical setting included the use of antiseptic lubricant gel at catheter insertion, the use of a tape seal applied to the catheter drainage tubing function, antireflux valves as well as the instillation of antiseptics in the collection bag. However, none of these measures proved to be as successful at reducing the occurrence of CAUTI as a closed drainage system [29]. Therefore a lot of intensive research has gone into altering the catheter surface with novel coatings using various antimicrobial substances, in order to prolong the time the catheter can remain in the patient without being infected. Kumon et al. examined the impact of catheter materials on biofilm development in patients with persistent urinary tract infections due to necessary long- term indwelling catheterization. They could show that silicone was the least susceptible to bacterial attachment when compared to latex and rubber [24].

Silver-coated catheters have already been studied since the late 1970 and first clinical trials gave promising results, as the incidence of bacteriuria could be decreased in patients with silver coated catheters [38, 39]. Once bigger trials were conducted and silicone was used as catheter material instead of latex, the superiority of expensive silver impregnated catheter was not supported by quality data anymore [29, 40]. Other issues with this type of coating were the fear of arising resistance to silver with widespread use and cytotoxicity problems [41].

Another approach which was investigated is the coating of catheters with hydrogels, which are macromolecular polymers that absorb large volumes of liquid [42]. The idea behind this strategy is to form a thin water film on the contacting surface of the catheter thus improving its smoothness and lubricity and therefore reducing the adhesion of both Gram-positive and Gram-negative bacteria. Again, studies didn´t give sufficient evidence that prove the effectiveness of hydrogel coated catheters [29, 43].

As results by Reid et al. showed that the pretreatment of urinary catheters with ciproflaxin significantly reduced the adhesion of *Pseudomonas aeruginosa in vitro*, various antibiotics including gentamicin, norfloxacin and nitrofurazone were investigated regarding their effectiveness in preventing CAUTI [44]. *In vitro* and *in vivo* studies of antibiotic releasing catheter surfaces gave promising results and showed that especially gentamicin and

nitrofurazone may be useful for controlling infections in patients undergoing short-term catheterization [45, 46].

A lot of effort has been put into finding the optimal coating for urinary catheters to increase their life span in patients. However, the substance/ coating strategy fulfilling all requirements has not yet been found. This is one of the reasons why the latest research in this area is focusing on creating antimicrobial surfaces using different types of enzymes, either for the immobilization of antimicrobial compounds [47, 48] or by directly immobilizing them on the silicone surface [49].

2.4 Immobilization of enzymes

In order to alter a biomedical surface and equip it with antimicrobial properties, the immobilization of enzymes is quite a novel approach. Depending on the material and the requirements for the surface coating the right immobilization strategy has to be carefully selected.

Immobilization strategies are classified in two broad categories based on the reversibility of the attachment: methods of irreversible enzyme immobilization and methods of reversible immobilization [50]. In order to provide a complete list of immobilization strategies, all available methods are listed below (Figure 6 and Figure 7), but only adsorption (Layer by Layer) and covalent binding will be described in more detail:

In the case of reversible immobilization, the protein can be detached from the surface under gentle conditions. This concept includes:

- Chelation or metal binding: transition metal salts or hydroxides are bound to organic carriers by coordination with nucleophilic groups, leaving free coordination positions for the enzyme [51].
- Disulfide bonds: thiol groups of the enzyme are used to form covalent disulfide bonds with thiol groups from the support. Although the bond is covalent, it can be broken under mild conditions.
- Adsorption: is the simplest immobilization method, where the enzymes are either attached through hydrogen bonding, van der Waals forces or hydrophobic interactions (physical adsorption) or on salt linkages (ionic binding [52]. The third class of immobilization based on adsorption is the affinity binding which is based on the complementarity of biomolecules (i.e. antibody + antigen) and therefore highly specific [53].

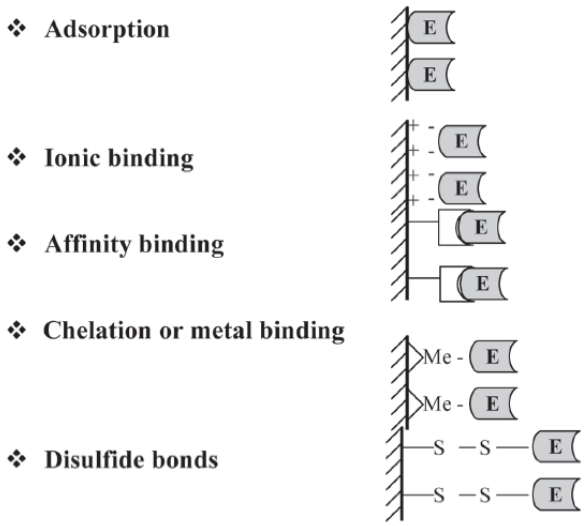

Figure 6: Overview of reversible enzyme immobilization methods [50]

Layer by Layer

One specific example for immobilization based on combination of physical adsorption and ionic binding is the layer-by-layer (Lbl) method. In this case, oppositely charged macromolecules are alternately adsorbed from their aqueous solution onto solid supports forming a multilayer construct. Depending on the isoelectric point of the enzyme of choice and the pH of the aqueous solution used, the protein can either play the part of the positively or negatively charged polyelectrolyte. Examples for macromolecules commonly used for the oppositely charged layer are listed in Table 1:

Table 1: Examples for polycations and polyanions used for Lbl technique [54]

Polycations:	Polyanions
poly(diallyldimethylammonium chloride) (PDDA)	poly(styrenesulfonate) (PSS)
poly(allylaminehydrochloride) (PAH)	poly(vinyl sulfate)
polyethyleneimine (PEI)	poly(acrylic acid)
chitosan	chitosan

Due to the wide variety of polyelectrolytes and charged supports available, more than 40 different enzymes have reportedly been immobilized using this strategy [55]. The immobilization process usually takes place under mild conditions in buffer solutions and has almost no effect on the secondary structure of the immobilized enzymes nor does the process cause denaturation of proteins [56]. One of the drawbacks of this method, and reversible immobilization in general is that there is a high chance of the leaching as the interactions between the matrix and the protein are relatively weak [50].

In the case of irreversible enzyme immobilization, the protein cannot be detached from the surface without either destroying the biological activity or the support. This concept includes:

- Cross linking: linker molecules are used to bind available functional groups of the enzyme together
- Entrapment/ Microencapsulation: the enzyme is trapped within a polymeric network that allows substrate/product exchange with the environment but retains the enzyme [57]
- Covalent binding: available functional groups of the support and the enzyme are used to form covalent bonds using a linker molecule if necessary

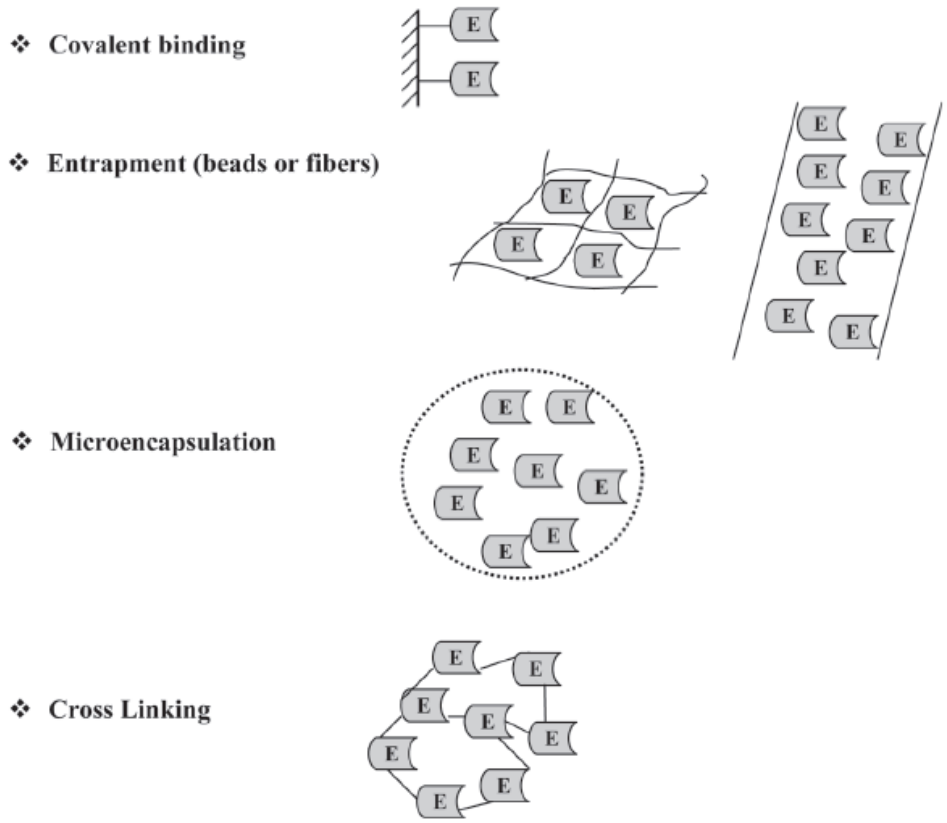

Figure 7: Overview of irreversible immobilization Methods [50]

Covalent immobilization

Covalent immobilization of proteins is among the most widely used methods to attach proteins to solid supports due to the fact that the stable nature of the bond prevents a release of the enzyme into the solution. Amino acid side chains commonly used to form the covalent bond with the support are lysine (amino group), cysteine (thiol group) and aspartic and glutamic acid (carboxylic group) yielding enzymes linked via amide, ether, thio-ether or carbamate bonds, respectively. There are many supports available that possess the right functional group for direct immobilization, but it is also possible to preactivate the matrix or to modify the polymer backbone to produce activated groups [50]. So-called linker molecules, small highly reactive chemical compounds, are often used to help with the bond formation and to act as a spacer molecule, creating more distance between the enzyme and the support, giving it more room to interact with the substrate. One point, that needs to be taken into consideration is the fact that amino acid residues important for the catalytic activity of the enzyme must not be involved in the covalent linkage as it would decrease the enzymatic activity [58].

In order to complete the introduction the types and working mechanisms of antimicrobial enzymes will be described in more detail in the following review carrying the title: “Antimicrobial enzymes: An emerging strategy to fight microbes and their biofilms”.

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Review

Antimicrobial enzymes: An emerging strategy to fight microbes and microbial biofilms

Barbara Thallinger[1], Endry N. Prasetyo[1], Gibson S. Nyanhongo[1] and Georg M. Guebitz[1,2]

[1] Graz University of Technology, Institute of Environmental Biotechnology, Graz, Austria
[2] Institute of Environmental Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

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Abbreviations: MRSA- Multidrug Resistant *Staphyloccocus aureus*; DNA- Deoxyribonucleic acid, DNAse- Deoxyribonuclease, Minimal inhibitory concentration (MIC), dsDNA- double-stranded DNA, PVOH- polyvinyl alcohol, TiO2- titanium (IV) oxide, DspB- Dispersin B**,** NADPH -nicotine adenine disphosphonucleotide, H_2O_2 -hydrogen peroxide, LPO – lactoperoxidases, GOD- Glucose oxidase, U- Units, I_2 –iodine , Cl_2 - chlorine Br_2 – bromine, SCN^--thiocyanate anion, $OSCN^-$- hypothiocyanite, MPO- Myeloperoxidase, HRP- Horseradish peroxidase, VCPO- Vanadium chloroperoxidase, AHL- acyl homoserine lactones, BLAST- Basic Local Alignment Search Tool, EU- European Union

Abstract

Several antimicrobial enzymes targeting different cellular components and biofilms are intensively being investigated with some products already commercialized in the health, food and biomedical industry. Although generally enzymes are effective as antimicrobial agents, successful removal of complex biofilms requires the use of a complex enzyme formulations containing DNases to degrade extracellular DNA, carbohydrases to hydrolyze extracellular polysaccharides, proteases to hydrolyze proteins as well as anti-quorum sensing enzymes to prevent biofilm formation. The development of such an effective complex enzyme formulation is urgently needed to deal with the problems associated with biofilm formation in industry, environment and health industries. Nevertheless, advances in synthetic biology, enzyme engineering and whole DNA sequencing technologies are showing a great potential to allow the development of more effective antimicrobial and antibiofilm enzymes. For example, applying synthetic biology approaches, an engineered bacteriophage expressing a biofilm degrading enzyme eliminated > 99.9% of bacterial cells in the biofilm matrix. These technological approaches give hope for successful development of enzyme based antibiofilm and antimicrobial systems.

Introduction

Many bacteria and their biofilms are a major cause of concern to human health, environment and industry. The biofilm is a complex extracellular polymeric material secreted by the respective microorganisms comprising of polysaccharides, proteins, glycopeptides, nucleic acids, lipids, etc. [1]. In industry, microbes colonize production lines, working surfaces and waste treatment plants[2] leading to corrosion of machinery, contamination of raw materials and products, fouling of cooling water pipeline problems in heat transfer and breakdown of machinery [3]. In the health care industry, microbial biofilms are a perpetual source of nosocomial infections accounting for > 60% of hospital acquired infections. They colonize medical devices (catheters, contact lenses, mechanical cardiac valves, endotracheal tubes, dental plaques etc. [4]. In addition to problems associated with colonization of materials, bacteria are the best known living organisms to rapidly overcome human strategies to eliminate them. As early as 1946, Alexander Fleming summed it all when he noted that, “There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring ‘fastness’ (resistance)” [5]. Over the years it has become clear that successful application of many therapeutic agents is compromised by the rapid development of resistance [6, 7] as summarized in Table 2.

Despite the increasing drug resistance, no new drug discoveries have been reported since 1987 [8], an indication we are losing the battle. Further, although many chemical disinfectants are used in health facilities and industries, many have adverse effect on equipment and also leave toxic residues [9, 10].

To overcome problems related to microbial biofilms, multidrug resistance and current disinfectants, many efforts are directed at developing bio based antimicrobial agents. Thanks to advances in biotechnology over the past three decades, the void left by traditional antibiotic producing companies is beginning to be filled. For example, the successful sequencing of whole genetic material of microorganisms has paved way for new era and hope for the discovery of new antimicrobial compounds. Equipped with these new techniques many companies have started employing genomic driven drug screening strategies targeting both microorganisms and microbial biofilms [11]. Among the emerging broad based bio solution technologies, antimicrobial enzymes are emerging as an attractive option [12].

This review therefore presents an overview of enzymes which are increasingly being exploited to fight microorganisms and their biofilms in industry, environment and in the health industry. However, for detailed enzymology and biochemistry of these enzymes, the reader is referred to existing literature.

2.5 Microbes in their cities “biofilms” and associated problems for their control

It is estimated that 99 % of the micro-organisms on earth live in biofilms [13]. Microbial biofilm formation is a complex process which starts with the adsorption of bacteria onto the surface of solid materials followed by attachment, growth and secretion of extracellular polysaccharides as shown schematically in Figure 8. This process has been subject of many comprehensive excellent reviews [14, 15] to which the reader is kindly referred.

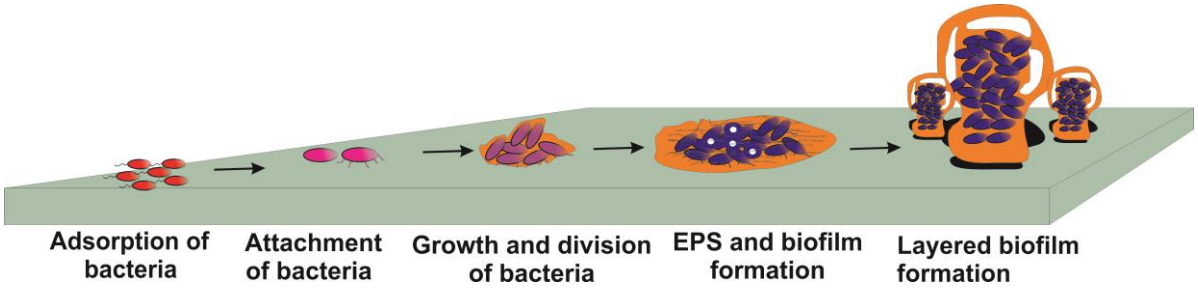

Figure 8: Shows stages of biofilm formation. Bacteria attaches onto a surface, multiply while secreting exopolymer that traps other microorganisms resulting in a complex biofilm matrix containing a diverse microbial community

Important during the formation of the extracellular polysaccharide is the release of chemical signaling molecules, the so called “quorum sensing” molecules by the bacteria [15]. Quorum sensing molecules enable bacteria to detect the presence of other bacteria and communicate with them. As the bacteria multiply, the concentration of the released quorum sensing molecules increases reaching a certain threshold at which these molecules start to affect the expression of many genes [15] including genes encoding for adhesins, exopolysaccharides (major component of the biofilm), virulent factors [14,15] etc. Other microorganisms including algae, fungi and protozoa become also trapped in the matrix resulting in the formation of a complex microbial community. The biofilm confers significant survival advantages to the trapped microorganisms including resisting dislodging, ideal ecological niche for reproduction, protection from host immune and antimicrobial agents, protection from by phages and protozoas and allows them to also exchange genetic material. Acquisition and exchange of genetic material (e.g. plasmids, transposons and integrons) lead to horizontal transfer of genes among them genes which confer the host with new phenotypic traits such as drug resistance. Thus, over the years drug resistance to the main classes of antimicrobial drugs has increased. This includes β-lactam based antibiotics and glycopeptides targeting cell wall biosynthesis, daptomycin and colistin targeting cell membranes, fluoroquinolones targeting type II topoisomerases, macrolide based antimicrobials, tetracyclines, and streptogramins targeting ribosomes, rifampicin targeting transcription as well as, sulfonamides and trimethoprim targeting folate biosynthesis. Accumulating mutations and acquisition of genetic elements in *S. aureus, P. aeruginosa, Acinetobacter baumanii, M. tuberculosis* etc. have led to multidrug resistant strains to nearly

all antibiotics [16, 17]. This an additional proof why biofilm dwelling microorganisms are more resistant to antibiotics as compared to the same free living strain [5]. Examples of the most common drug resistance mechanisms acquired through mutations and the exchange/acquisition of genetic materials include;

- Enzyme inactivating drugs (e.g. aminoglycoside-modifying enzymes, β-lactamase inactivating β-Lactams)[18].
- Acquiring genes encoding for new none susceptible proteins e.g. penicillin resistance among *Streptococcus pneumonia* and *Neisseria meningitides* were attributed to the synthesis of hybrid proteins using genes acquired from several methicillin-vancomycin-resistant Multidrug Resistant *Staphyloccocus aureus* (MRSA) strains carrying vanA gene, originating from enterococci [19].
- Modification of the target protein (e.g. resistance to vancomycin was achieved by altering the vancomycin binding site thereby reducing affinity [9, 20].
- Use of active efflux pumps to keep the drug from entering the cell.
- Preventing drugs from accessing target proteins e.g. some plasmids produce special proteins which protect DNA from drugs such as (fluoro)quinolones [9, 20].

Thus the survival strategies employed by microorganisms when challenged are many and diverse. This has severe implications in preventing microbial infectious and eradicating their negative effects in industry.

2.6 Antimicrobial enzymes and microbial antibiofilm enzymes

Antimicrobial enzymes are wide spread in nature where they play a critical role in defending living organisms from bacterial attack. These enzymes are now increasingly being exploited as antimicrobial systems based on their ability to directly attack the microorganism, interfere with the biofilm formation, destroy the biofilm and/or ability to catalyze reactions which result in the production antimicrobial compounds. As shown in Figure 9 many enzymes from different classes with different catalytic properties are used/are being investigated as antimicrobial agents. The use of these enzymes in some cases is now an established technology. More and more enzyme liquid formulations are being exploited as antimicrobial and antibiofilm enzymes for cleaning surfaces and some are incorporated or grafted into/onto polymer materials to prevent microbial colonization. The formulations may contain one or more enzymes or enzymes with other antimicrobial or antibiofilm agents [21].

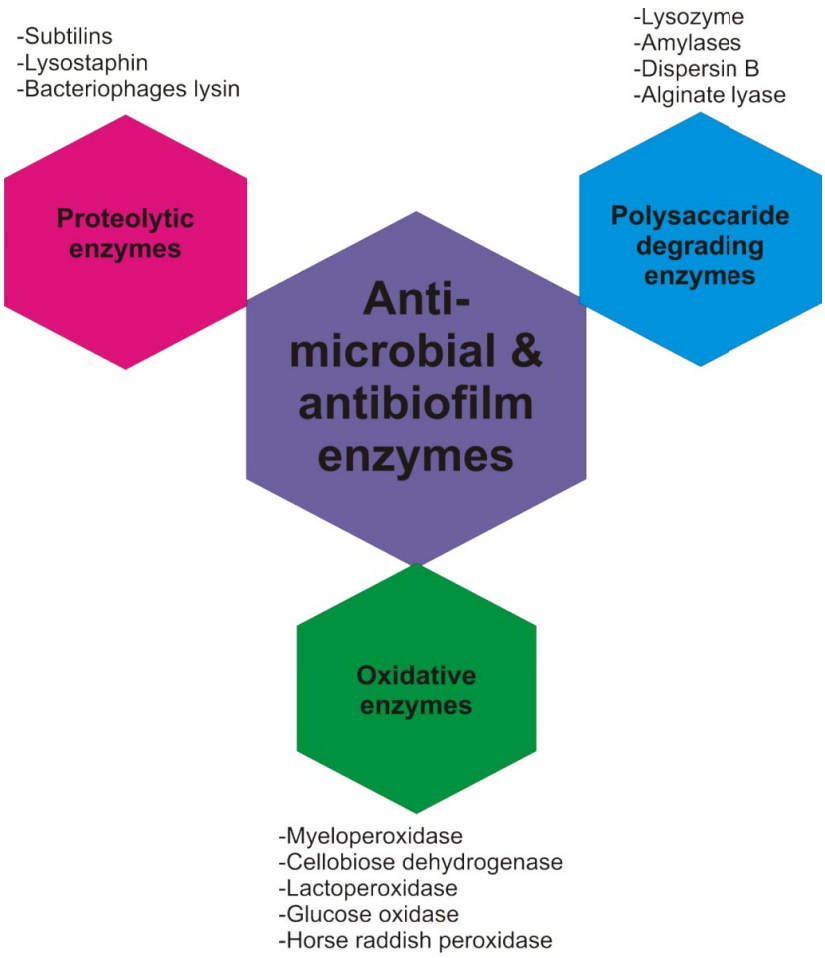

Figure 9: Some of the enzymes from different classes increasing being exploited as antimicrobial and antibiofilm systems.

2.6.1 Proteolytic enzymes

Proteases are enzymes hydrolyzing proteins. This is diverse group differing in structure, their substrates, reaction mechanism and many physicochemical properties [22]. Proteases are classified into two major groups exopeptidases and endopeptidases according to their catalytic properties and according to their pH optima into acidic, neutral or alkaline[23].

2.6.1.1 Subtilisins

Proteases especially subtilisins are the most widely used enzymes for the control of biofilm in industry [23]. Subtilisins are produced by *Bacillus* sp. And are serine proteases that cleave proteins in which serine serves as the nucleophilic amino acid [24]. Subtilisins hydrolyze adhesins (bacterial proteins essential for attachment onto solid supports) as well as prevent co-aggregation of microorganisms a condition which allows bacteria to communicate with other and participate in multispecies biofilm formation [25]. They successfully removed biofilms harboring health and industrially microbes like *Pseudomonas* spp, *Pseudoalteromonas,* and *Bacillus* [26-28] and *Listeria monocytogenes* [25, 28] and

prevented co-aggregation of *Actinomyces naeslundii*, *Streptococcus oralis*, *Porphyromonas gingivalis* [26]. A screening study of several commercial hydrolases preparations against *P. aeruginosa*, *Lactobacillus bulgaricus, Lactobacillus lactis* and *Streptococcus thermophilus* biofilms showed that Pandion, Resinase A2X, Spezyme GA300 and Paradigm were effective [23]. Recently, a new group of subtilisin-like proteases produced by hyperthermophiles (e.g. pernisine, from *Aeropyrum pernix*) with optimum operational temperature at 90 ºC [29] is being investigated for targeting biofilms in heat exchangers.

2.6.1.2 Lysostaphin

Lysostaphin, a *Staphylococcus simulans* is a metalloendopeptidase, first isolated by Schindler and Schuhardt in 1964. It cleaves the pentaglycine bridges of *Staphylococci* cell wall on the third and fourth glycine residues of the pentaglycine cross-bridge of the cell wall material of several *Staphylococcus sp*. (Figure 10) [30, 31]. This enzyme is attracting significant medical interest for the control of multidrug resistant MRSA.

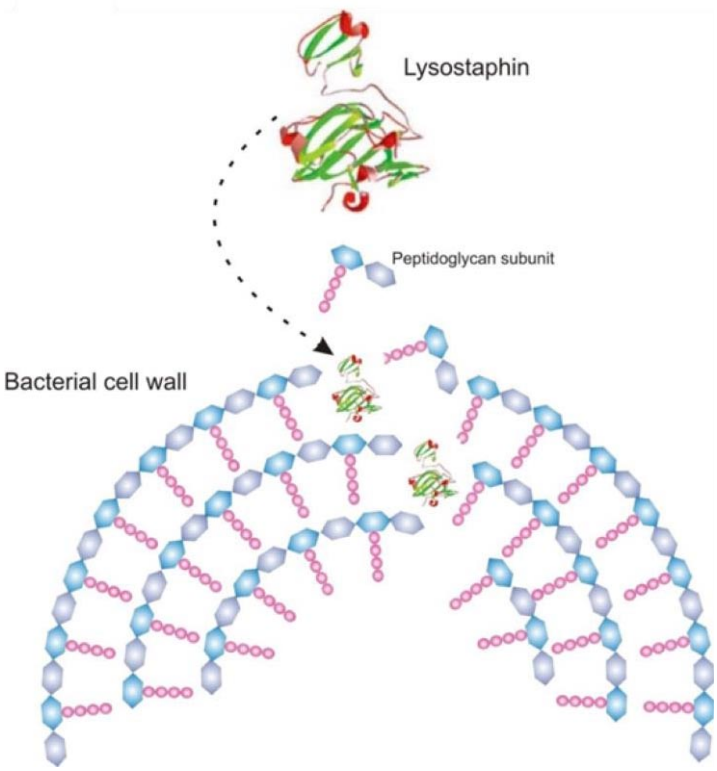

Figure 10: Simplified scheme of the hydrolysis of bacterial cell walls by lysostaphin.

Unfortunately, lysostaphin is not able to hydrolyze cell wall containing glycylserine and serylglycine peptide bonds [31]. Lysostaphin exhibits three different catalytic activities including glycylglycine endopeptidase, endo-beta-N-acetyl glucosamidase and N-acteyl muramyl-L-alanine amidase activity [32]. This makes it effective hydrolyze of *S. aureus* cell walls which are made up of β-1,4 linked *N*-acetylglucosamine, *N*-acetylmuramic acid,

muramic acid , D-alanine, D-isoglutamine and L-lysine. Lysostaphin also kills *Staphylococcus carnosus* (fermenters of meat), *Staphylococcus epidermidis* and *Staphylococcus haemolyticus (*nosocomial pathogens infecting immune-compromised patients especially those with medical implants such as prosthetic devices, surgical patients, individuals undergoing dialysis, diabetic patients), *S. saprophyticus* (implicated in 10 - 20% of urinary tract infections*), Staphylococcus xylosus* (opportunistic pathogen) [33]. Heterologously produced lysostaphins are increasing being used to treat MRSA [34]. The minimal inhibitory concentration (MIC) values of lysostaphin for penicillinase-positive strains were half that of vancomycin or methicillin. This effectiveness has witnessed lysostaphin application extending to controlling the formation of biofilms on surfaces of biomaterials including cellulose bandages [35,], porcine biomesh [36], Hernia repair meshes [37], catheters [38] and carbon nanotubes [39]. In another approach lysostaphin impregnated creams were more effective than mupirocin ointment in killing *S. aureus* and preventing the colonization of cotton rat nasal cavity [30,40] and prevent nasal colonization by MRSA [41]. This is important since multidrug resistant MRSA is a major pathogen causing sepsis in low birth weight babys and accounting for 20 % deaths [41]. Administration of lysostaphin alone or its co-administration with oxacillin or vancomycin helped enhance the clearance of MRSA [42].

Lysostaphin based formulations for direct intraperitoneal or subcutaneous administrations have also been developed. Intraperitoneal or subcutaneous administration of lysostaphin cured *S. aureus* infected mice while *S. epidermidis* isolates resistant to methicillin were effectively controlled by combining nafcillin and lysostaphin [43].

In the dairy industry, lysostaphin prevents mastitis, a disease causing inflammation of the mammalian glands, costly disease in dairy cattle [44]. Transgenic cows expressing bacteria derived r-lysostaphinsecreted in the milk inhibited several *S. chromogenes*, *S. hyicus, S. epidermidis, S. simulans*, and *S. xylosus* even when diluted 16-fold [44]. This is an interesting development since mastitis is a major cause of losses in the world estimated to be over $4 billion dollars a year in the US and European.

2.6.1.3 Bacteriophages lysins

Bacteriophages occupy every niche in the biosphere where their hosts bacteria are present [45]. Bacteriophages were first discovered by Harkin in 1896 and were used first to treat microbial infections before antibiotics were discovered. D'Hérelle treated dysentery in 1919 using bacteriophages and several companies commercialized phages during 1930s [46].

Following replication inside their bacterial host, double-stranded DNA (dsDNA) phages express two types of essential proteins, endolysins and holins, critical for host cell lysis [47]. Holins are small proteins that permeabilize the bacterial membrane allowing endolysin access the peptidoglycan layer of the host bacteria. Endolysins is a collective term used for describing bacteriophage muralytic enzymes which differ from each other on their specificities. These include enzymes such as *N*-acetylmuramidases, endo-β-*N*-acetylglucosaminidases, lytic transglycosylases, endopeptidases,and *N*-acetylmuramoyl-l-alanine amidases [47, 48]. Although it has traditionally been known that endolysins only perform one type of reaction (muramic activity, transglycosylase activity, glucosaminidase activity, etc., it is also becoming clear that some of the endolysins display two or more activities. For example, endolysins encoded by *Streptococcus agalactiae* bacteriophage B30 have both muramidase and endopeptidase activities and *S. aureus* phage φ11 exhibit both endopeptidase and amidase activities [49].

Bacteriophage endolysins are re-emerging as a new class of antibacterial agents for application in agriculture, health, food and environment. Group C streptococci C1 phage endolysin was the first to be applied topically for treating bacterial infections and is re-emerging as alternative drug to deal with increasing resistance to erythromycin and clindamycin administered to penicillin-allergic women [50]. Oral administration of one dose of bacteriophage formulation to mice infected with 10^7 Group A streptococci effectively prevented colonization of its upper respiratory system [50]. Similarly, *S. agalactiae* phage NCTC 11261 PlyGBS used to prevent infections kill *S. agalactiae* in newborns babies exposed to penicillin resistant strains [51]. *S. pneumonia*, a major cause of pneumonia, meningitis, otitis media [46] is also controlled using bacteriophages [50]. Bacteriophage endolysins Cpl-1 and Pal administered intraperitoneally conferred 100 % protection to mice infected with multidrug resistant *S. pneumoniae* [52]. In a screening study, 14 isolates/strains of *Bacillus anthracis* gathered worldwide and a *Bacillus cereus* were lysed by *B. anthracis* phage c PlyG amidase [53]. Yoong *et al.*, [54] recently reported a unique endolysin, PlyV12 amidase of the *Enterococcus faecalis* phage ϕ1 that exhibits lytic activity against several pathogenic bacterial species including vancomycin-resistant *E. faecalis*, *Streptococcus pyogenes*, and *Streptococcus*. Bacteriophages and their endolysins have also been used to prevent formation of biofilm by *L. monocytogenes* [55] and *E.coli* [56]. Applying synthetic biology approaches, a recombinant bacteriophage expressing biofilm hydrolases was developed which killed > 99.9 % of bacterial cells in biofilm [20]. Gaeng *et al.*, [57] produced a recombinant *L. lactis* cell coding for listerial endolysin gene which offered protection of dairy products against *L. monocytogenes*. Evolving new powerful lower cost and high throughput DNA sequencing and genetic material manipulation techniques will quicken the production of engineered phages for wider applications. It is agreed that the dairy industry is

leading in trying to harness phages as antimicrobial agents. Transgenic plants expressing endolysin genes have also been produced [58].

Many other proteolytic enzymes from different sources are also increasingly being investigated including proteinase K, trypsin, protease A, papain, etc. A toothpaste containing Citroxain, a mixture of papain, sodium citrate and alumina marketed as whitening toothpaste based on its ability to remove stains forming on teeth surfaces [59]. Proteases hydrolyze adhesins and other related proteins which enable bacteria to attach on to a surface. Proteolytic enzymes e.g. Travase are used to treat wound surface in order to disrupt the coagulum and expose the bacteria to antibiotics [60].

2.6.2 **Polysaccharide degrading enzymes**

Among the polysaccharide hydrolyzing enzymes; lysozymes, alginate lysases, Dispersin B and amylases are by far the most commonly exploited.

Lysozymes from chicken egg white (major industrial source of lysozyme) were the first to be described in 1909 by Laschtschenko [61] and also became the first protein to have its structure elucidated by x-ray crystallography during 1960s. In 1921 Alexander Fleming discovered that *Micrococcus lysodeikticus* was susceptible to lysozyme [62]. Lyzozyme mostly attacks gram positive bacteria although it does also hydrolyze cell walls of some gram negative bacteria and some viruses by hydrolyzing 1,4-beta-linkages in the bacterial cell walls [63].

Lysozyme is used in health and food industry to kill bacteria and it can be purchased "over-the-counter". Its pharmaceutical applications range from treatment of sore throats, decontamination and preventing microbial colonization of biomaterials. Lysozyme is used to increase the shelf life of many foods e.g. fruits, vegetables, meat, cheese etc. [64,65]. It is incorporated into infant formula, chewing gum to treat periodontitis and is co-administered with antibiotic to cancer patients [64,65]. The application of lysozyme as adjuvant in drugs and its dimeric forms are extending its application for the treatment many bacterial and viral infections.

There has been a growing interest in developing bioactive packaging materials for application in the food and health industry. Lysozyme immobilized in PVOH films [66], cellulose [67] and chitosan [68] were effective in inhibiting food spoiling microorganisms. Coating surgical steel blades with a mixture of lysozyme and silver nanoparticles reduced the viability of *Klebsiella pneumoniae*, *B. anthracis, Bacillus subtilis, S. aureus* and *Acinetobacter baylyi* [69]. Lysozyme immobilized on titanium (IV) oxide (TiO2) [70] and on wool fabrics inhibited microorganisms even after washing several times [71]. Gelatin gels made from fish skin impregnated with lysozyme increased shelf life of food products [72] and similar

lysozyme–chitosan composite films inhibited both *E. coli* and *Streptococcus faecalis* [73]. Thus a number of bioactive products are being developed for application in the food and health industry.

2.6.2.1 Amylases

Alpha-amylases inhibit the formation of biofilm and hydrolyze biofilms of *S. aureus* biofilms [74]. Nevertheless, amylases, when used in conjunction with proteases enhance removal of biofilms and prevent bacteria from adhering onto surfaces. A combination of proteases and amylases was effective in removing *Pseudomonas fluorescens* biofilm [25].

Dispersin B (DspB) is another important antimicrobial and antibiofilm enzyme. It is produced by *Actinobacillus actinomycetemcomitans.* DspB hydrolyzes β-1,6-*N*-acetyl-d-glucosamine, an important polymer needed for biofilm attachment onto surfaces [75]. Its combination with proteases, DNAses and glycolytic enzymes enhances the removal of biofilms [76, 77] although DspB alone is also able to attack biofilms of several different species of bacteria.[77]. An engineered DspB from T7 phage hydrolyzed *Staphylococcus* and *E. coli* exopolysaccharides and reduced bacterial cell counts 100 000 fold times [77]. Catheters coated with triclosan1 DspB and chlorhexidine silver sulfadiazine was effective in combating *S. aureus* although the former catheters showed broader antimicrobial activities against *S. aureus*, *S. epidermidis* and *E. coli* [77].

Other polysaccharide degrading enzymes of interest includes pectin methylesterase, an enzyme incorporated into enzymes used as disinfectants. Pectin methylesterase has been increase biofilm removal in the pulp and paper experimental setup [78].

2.6.2.2 Alginate lyase

Alginate lyase is another antimicrobial enzyme cleaving β–glycosidic bonds of bacteria alginate polymer. Alginate is a copolymer made up of alpha-L-guluronate and Beta-D-mannuronate [79]. Alginate lyases are widely distributed and have been found in algae, invertebrates and microorganisms [79, 80]. Ganeca [81] proposed the mechanism of the alginate lyase which consists of three step actions namely:

(i) removal of the negative charge on the carboxylate anion;

(ii) abstraction of the proton on C_5

(iii) ß-elimination of the 4-O-glycosidic bond (lyase) (Figure 11).

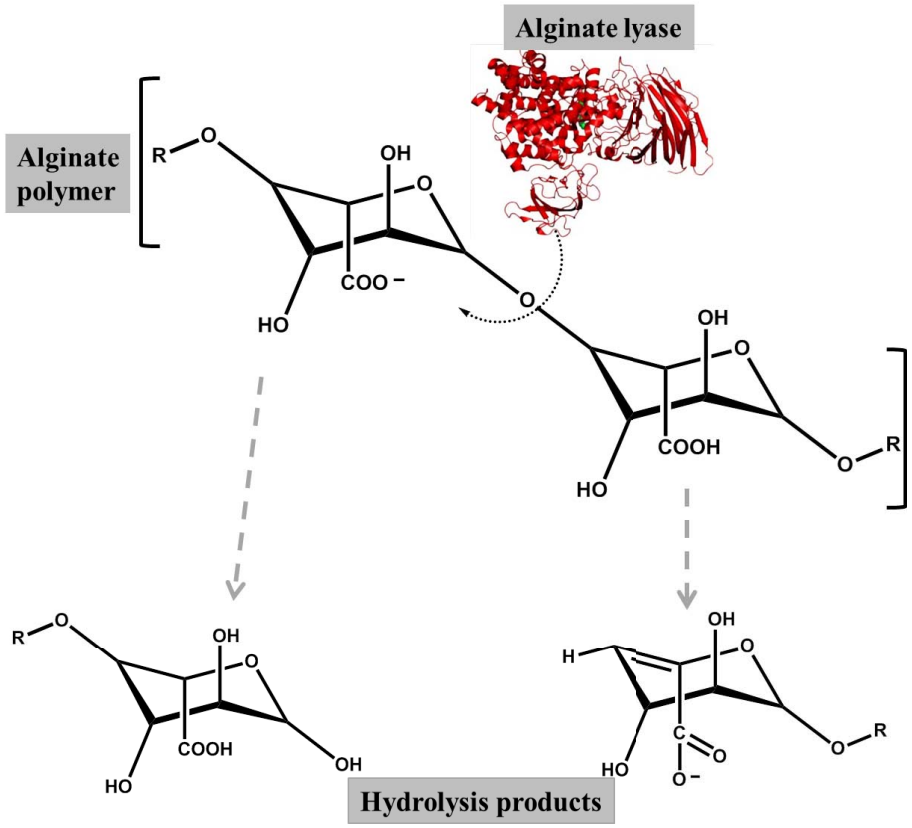

Figure 11: Alginate lyase catalyze the hydrolysis of alginate, a copolymer of _α-L-guluronate (G) and C5 epimer -D-mannuronate (M), through β-elimination.

The enzymes have been used to control *P. aeruginosa* colonizing the respiratory tracts of patients with cystic fibrosis [80]. Treating patients with a combination of alginate lyase and gentamicin is effective for treatment of cystic fibrosis [81]. A genetically engineered A1-III alginate lyase immobilized on PEG removed >90% of bacteria adherent polymers of *P. aeruginosa* [82], a notorious wound pathogen.

DNA from dead microorganisms in biofilms is an effective cross-linker of biofilms owing to its electronegativity which increases the viscosity of the mucus of patients with cystic fibrosis. Deoxyribonuclease is used to hydrolyze this DNA in cystic fibrosis mucus [83] and reports by Nijland et al [84] shows it is an effective anti-adhesins. Combining DNAses with alginate lyase leads to better results [80]. Chitinases and β-glucanases are gaining interest as antifungal agents. Chitin is the main component of the cell walls of fungi consisting of long-chain polymer of a N-acetylglucosamine. Several chitinases from both plant and microbial sources are able to hydrolyze glycosidic bonds in chitin [85, 86]. *Streptomyces* chitinases are used to hydrolyze cell walls of many plant pathogenic fungi [87].

Generally the use of a combination of protein degrading enzymes and polysaccharide degrading enzymes achieves better results when targeting biofilms. Enzyme mixtures containing alpha-amylase, β-glucuronidase, glucose oxidase, dextranase, protease and pectinase were effective in the removal of biofilms on stainless steel coupons and dispense

lines [88]. A combination of serine proteases and α-amylase was effective in removing biofilms in the food industry [77]. The use of a mixture of formulations of proteases, carbohydrases and lipases reduced biofilm formation on ships [89]. The use of whole microorganisms producing these antimicrobial and antibiofilm enzymes has been proposed as a better option and this is supported by the observation that *Aspergillus niger*, *Trichoderma viride* and *Penicillium* spp. are effective in removing biofilm [90].

2.6.3 **Oxidative enzymes**

Perhaps the most fascinating antimicrobial defense system of the human body includes the production of large amounts of superoxide by the membrane-associated NADPH oxidase. The superoxide anion is also produced by xanthine oxidase, lipoxygenases and cylooxgenases [91]. Superoxide anions are considered the ‘‘primary’’ reactive oxygen species (ROS) from which all the other *in vivo* ROS are derived from [92]. The superoxide dismutates into hydrogen peroxide (H_2O_2) which is used by peroxidases (lactoperoxidases, myeloperoxidases etc.) to destroy invading pathogens. The peroxidases (lactoperoxidases, myeloperoxidases etc.) use the H_2O_2 to oxidize halides (bromide, chlorine, iodine) and isocyanate to more potent antimicrobial compounds active against invading pathogens (bacteria, fungi and viruses). The H_2O_2 also directly reacts with metals such as iron producing hydroxyl free radicals which are stronger antimicrobial compounds [92]. The production of superoxide anion radical is tremendously produced during “respiratory burst” that reaction of the human body to invasion by pathogenic microorganisms [93]. Growing interest is aimed at mimicking this human in-built defense system to develop antimicrobial systems for application in food, environment, health industries as well as personal health care products.

2.6.3.1 Hydrogen peroxide producing enzymes as antimicrobial systems

Glucose oxidase activity was first reported in 1928 by Müller from extracts of *A. niger* [94] and has since then gained importance various technological applications. Glucose oxidase (GOD) catalyzes the oxidation of β-D–glucose to glucono-δ-lactone while simultaneously producing hydrogen peroxide [95].

The antimicrobial properties of glucose oxidase/glucose system are therefore based on the produced H_2O_2 and the accumulation of D-gluconic acid. Hydrogen peroxide kills bacterial cells through peroxidation and disruption of cell membranes, oxidation of oxygen scavengers and thiol groups and disruption of protein synthesis [96]. The D-gluconic acid lowers the pH in the environment which negatively affects bacterial growth. Low concentrations H_2O_2 (around 3%) are effective as antimicrobial agents against *E. coli* and *S.*

epidermis [97]. Tiina and Sandholm [98] demonstrated the ability of glucose oxidase to inhibit food spoling microorganisms like *S. aureus, Salmonella infantis, Clostridium perfringens, B. cereus, Campylobacter jejuni, L. monocytogenes* and *Yersinia enterocolitica* [98]. Glucose oxidase inhibits the growth of *P. fluorescens*, *Acinetobacter calcoaceticum*, *Hansenula polymorpha* responsible for the microbial spoilage of shrimps and the produced gluconic acid reduced growth of *Corynebacterium aquaticum*, another food spoilage microorganism [99]. The addition of 500 U of glucose oxidase and 0.5 g of glucose to 100 ml of liquid whole egg, killed *Salmonella enteritidis*, *Micrococcus luteus* and *B. cereus* inoculated at 10^3 cells/ml after 5 days of storage at 7°C and the system had bacteriostatic effect on *P. fluorescens* attributed to the strong catalase activity of the strain [100]. Several studies have investigated the incorporation of glucose oxidase in packaging materials such as polypropylene films [101], poly(vinylalcohol/ chitosan/ tea extract electrospun nanofibrous membranes produced a novel deoxidizer food packaging material able to remove oxygen and prolong the shelf life of food [102]. Glucose oxidase is increasingly investigated for application in the food industry to kill food spoiling microorganisms and food borne pathogens.

Another enzyme with increasing biotechnological importance is cellobiose dehydrogenase, the only known extracellular flavocytochrome produced by wood degrading fungi [103]. The versatile reactions catalyzed by cellobiose dehydrogenase which including its ability to use a variety of cellooligosacharides as electron donors to reduce quinones or generate H_2O_2 is being exploited for the development of biosensors [104], bleaching systems [105], antimicrobial systems [106] and continuous antioxidant regenerating systems [107]. Recent studies have shown its ability to completely inhibit the growth of *Stapylococcus aeureus*, *Bacillus subtilis*, *Pseudomonas putida*, *Escherichia coli* and *Cellulomonasmicrobium cellulans (*106). The authors suggested its application in bioresponsivepolymers for the management of chronic wounds [106,107].

2.6.3.2 Hydrogen peroxide using enzymes

Haloperoxidases are found in many plants, animals and humans and include enzymes like myeloperoxidase and lactoperoxidase. These enzymes are classified based on the nature of their cofactor (heme, vanadium etc.) [108] and use H_2O_2 as a substrate to oxidize halide/pseudohalide to more potent antimicrobials [109]. Apart from the halides I_2, Cl_2 and Br_2, they oxidize pseudohalide thiocyanate yielding hypothiocyanite, a potent antimicrobial agent [110].

Lactoperoxidase is the second most abundant natural enzyme in milk [110] and plays an important role in preserving milk and is found in tears and thyroid glands [111]. Its antimicrobial activity is based on the oxidation of the thiocyanate anion SCN^-. Depending on the pH two antimicrobial agents namely; hypothiocyanite $OSCN^-$ and hypothiocyanous acid

that are capable of oxidizing the sulfhydryl groups of enzymes and proteins thereby inhibiting microbial growth and is effective against many gram negative and gram positive bacteria [112]. The lactoperoxidase system is widely used for preserving food, boosting the airway host defense system of humans, plant and crop protection [112-114]. Recently the LPO system has been shown to play an important role in maintaining oral hygiene. The implementation of the system is facilitated through the natural occurrence of SCN^- ions in the saliva and the similarity of lactoperoxidase to salivary peroxidase [115]. For example, *Streptococcus mutans* and *Streptococcus sanguinis* exposed to the LPO/SCN^-/H_2O_2 system were completely killed after 15 minutes only [116-118]. The hypothiocyanite also proved effective against *C. albicans* biofilms on oral biomaterials [116 -118] in both solution and when coated on polymer surfaces [117, 118]. Ongoing studies are combining lactoperoxidase system with the glucose oxidase system (Figure 12). In this system, glucose oxidase uses glucose to produce H_2O_2 needed by lactoperoxidase to oxidize halides.

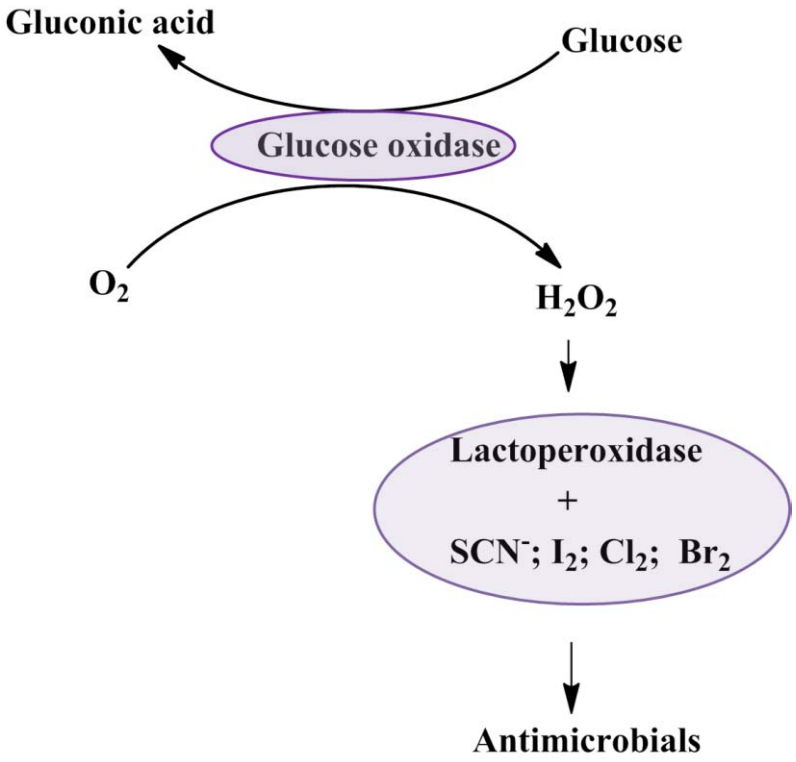

Figure 12: The glucose oxidase –lactoperoxidase antimicrobial system. Glucose oxidase uses glucose as electron donor substrate and reduces oxygen resulting in the production of H_2O_2. The H_2O_2 is in turn used by lactoreperoxidase to oxidize halides to more potent antimicrobial compounds.

This system has been found attractive in developing enzyme based mouth rinse solutions and toothpastes [119, 120]. The glucose oxidase- lactoperoxidase system has also been investigated as an antifungal agent [119] and for the removal of biofilms from polypropylene surfaces resulting in reduction of *Staphylococcus* and *Pseudomonas* biofilms and death of planktonic cells [20]. The only challenge of these antimicrobial systems is the lowering of the

pH due to the accumulation of D-gluconic acid which is potentially corrosive.

Myeloperoxidase

Myeloperoxidase (MPO) is the most common neutrophilic enzyme first isolated by Agner in 1941 [121]. It plays an important role in the human defense system by oxidizing chloride anion using H_2O_2 yielding very potent antimicrobial agents namely; hypochlorite anion or undissociated hypochloric acid depending on the pH [122]. The produced reactive species oxidize microbial biomolecules of the pathogens [122]. The naturally occurring SCN- ions in human saliva are the preferred substrate of MPO. The generated radicals by MPO inhibit the growth of bacterial but are non-toxic to the surround cells [123]. Borelli et al. [124] reported a consistent killing activity of MPO against a clinical isolate of *M. tuberculosis* in the presence of H_2O_2 and chloride ions in the assay medium [124]. MPO has virucidal effect even against human immunodeficiency Virus Type 1 [125]. Similarly, horseradish peroxidase (HRP), perhaps one of the most widely used enzyme in diagnostics combined with glucose oxidase incorporated in polyurethane electrospun fibers also proved to be a good antmicrobial enzyme [126]. The produced fibers inhibited both *E.coli* and *S.aureus* [126]. Vanadium chloroperoxidase (VCPO) from *Curvularia inaequalis* oxidizes effectively inhibited formation of *E. faecalis* biofilms [127]. *S. mutans*, another significant contributor to tooth decay was also susceptible to treatment with the oxidation products of VCPO [128]. *Curvularia* haloperoxidase produced by *Curvularia verruculosa* oxidized chloride, bromide and iodide resulting in inhibition of *Pseudomonas* spp., *Serratia marcescens*, *E. coli*, *Shewanella putrefaciens*, *Aeromonas salmonicida*, *Staphylococcus epidermidis*, yeasts like *Candida* spp. *Aspergillus* sp. and *Penicillium* spp. [129,130].

2.6.4 Anti-quorum sensing enzymes

Other emerging groups of antimicrobial enzymes include the so called anti-quorum sensing enzymes. As discussed elsewhere in this review, bacteria use quorum sensing to regulate various physiological activities, including virulence, competence, conjugation, antibiotic and bacteriocin production, motility, and spore and biofilm formation [14,131]. Among the targeted quorum sensing molecules are the acyl homoserine lactones (AHLs) which are implicated in the regulation of bacterial virulence in >50 bacterial species [131] and are the best characterized quorum sensing molecules. These quorum sensing molecules move in and out of the cell through passive diffusion or active transport [132]. However, scientists have also discovered quorum-quenching enzymes such as the lactonases which hydrolyze the ester bond of the homoserine lactone ring of acylated homoserine lactones thereby preventing AHLs from binding to their target transcriptional regulators. AHL acylase

hydrolyses the amide linkage between the acyl chain and the homoserine moiety of AHL molecules. The first quorum quenching enzyme encoded by the *aiiA* gene was identified from a soil bacterial isolate belonging to a Gram-positive *Bacillus* sp. [133,134]. Genes encoding for enzymes responsible for the degradation AHL have since been cloned even in plants [134]. The anti-quorum sensing enzymes have also been reported in porcine kidney and BLAST searches shows that porcine kidney acylase I is conserved in mice, rats and zebra fish while the paraoxonase enzyme found in human have been shown to hydrolyze the lactone ring of AHL signal molecules [135,136]. The expression of a quorum quenching enzyme, in *Erwinia carotovora* and *P. aeruginosa*, reduced the reduced potato soft [135-137]. By suppressing the virulence of pathogenic microorganisms these enzymes are likely to play major biotechnological role in agriculture and health industries.

2.7 Challenges in application of enzymes as antimicrobial and antibiofilm agents

Successful removal of biofilm requires a combination of enzymes including proteases, polysaccharide degrading enzymes, DNases and anti-quorum sensing enzymes. This makes this process relatively expensive given the current cost of enzymes especially for biomedical application where pure enzymes are required. Further, successful application of enzymes for the control of biofilms depends on the environment e.g. application of enzymes in preventing microbial colonization or destruction of biofilms on ships poses a huge challenge because of hostile environment to enzymes. Synthetic biology approaches integrating the genes coding for different antimicrobial enzymes in a single host may provide the solution as demonstrated by Lu and Collins [138].

Although resistance to antimicrobial enzymes has rarely been reported, a few studies have demonstrated the emergency of resistance to lysostaphin both *in vitro* and *in vivo* [139,140]. This has been attributed to mutations that affect the *femA gene* encoding the factor responsible for addition of the second and third glycines residues to the pentaglycine bridge of the cell wall [139,140]. This addition results in the replacement of glycine residues with serine residues which affects the binding of lysostaphin onto the pentaglycine cross-bridge and subsequent loss of lysostaphin to hydrolyze the pepetodoglycans [140]. Interestingly resistant to lysostaphin makes *S. aureus* lose their methicillin resistance phenotype [141] and therefore this drug can be used again. The presence of microorganisms like *Deinococcus spp* and *Helicobacter pylori* with inbuilt antioxidant defence mechanisms to overcome the host inflammatory response in biofilms may through “horizontal gene transfer” make other microorganisms resistant to H_2O_2. Catalase-deficient isolates of *Helicobacter pylori* were reported to be hypersensitive to H_2O_2 as compared to wild type [142]. Lactoperoxidase tolerant *Escherichia coli* transposon mutants have also been isolated and characterized [143].

Bacteriophage endolysins are usually not able to attack gram –negative bacteria since they are surrounded by an outer membrane which makes it difficult to access the peptidoglycan layer [144]. Nevertheless, some endolysins can still effectively kill gram-negative bacteria despite the presence of the outer membrane using their C-terminal membrane-active peptide sequences [144]. Decrease in bacterial susceptibility to endolysin is attributed to modifications of synthesized biomecules including increasing density of cross-linked peptidoglycan, deacetylation of amino sugars as well as increase in proteins and polysaccharides on the cell wall [144]. Despite these observed challenges generally it is generally agreed that bacteriophages have evolved remarkable strategies that are difficult for the host to evade. This makes resistance to lysins very rare. For example, the receptor for pneumococcal phage lysins is choline which is important for its surviva and polyrhamnose in Group A Streptococci is important for its growth yet it is the binding site of bacteriophage endolysin attacking it [144]. The failure of bacteria to quickly evade bacteriophages was also demonstrated by failure to produce resistant mutants after multiple exposure of *S. pneumoniae* or *B. cereus* to low doses of lysin on agar plates and in liquid cultures failed [145]. Exposure of *B. cereus* to methanesulfonic acid ethyl ester mutagens resulted in1 000-fold and 10 000-fold increase in novobiocin and streptomycin resistance, respectively but remained sensitive to endolysin [145]. This again demonstrates that the development of resistance against these enzymes is unlikely.

Table 2: Increasing multidrug resistance with time

	Drug resistance	**Ref**
1930s	pneumococci resistance to sulfonamides	[6]
1940	Streptococci were noted and resistance to penicillin	[7]
1950s	beta-lactamase-producing staphylococci	[6]
1960s	highly resistant gram-negative enteric bacteria	[6]
1970s	beta-lactamase-producing *Haemophilus influenzae* and *Moraxella catarrhalis* in the 1970s	[6]
1980s	multidrug-resistant pneumococci, *Staphylococcus aureus, Mycobacterium tuberculosis, Pseudomonas aeruginosa*	[6]

2.8 Concluding remarks

Several antimicrobial enzymes targeting different cellular components and biofilms are increasing being investigated with some products already commercialized. The success stories highlighted in this review, point to an increasing role of antimicrobial enzymes in industry, health, environment and food industry. While some enzymes are effective as standalone antimicrobial agents, it is also clear that a combination of enzymes (proteases, polysaccharide degrading enzymes, DNases and anti-quorum sensing enzymes) are needed to prevent biofilm formation or destroying microbial biofilms. This is largely attributed to the complexity of the biofilm. The challenge for the future research activities in this area is to harness all the advances in biotechnology (protein engineering, synthetic biology, domain swapping and gene shuffling, bioinformatics, metagenomics, large-scale DNA sequencing technologies) for the development of novel and more potent antimicrobial and antibiofilm enzymes.

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3

Preventing microbial colonization of catheters: antimicrobial and antibiofilm activities of cellobiose dehydrogenase

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Abstract

The ability of cellobiose dehydrogenase (CDH) to produce hydrogen peroxide (H_2O_2) for antimicrobial and antibiofilm functionalization of urinary catheters was investigated. A recombinantly produced CDH from *Myriococcum thermophilum* was shown to completely inhibit growth of *Escherichia coli* and *Staphylococcus aureus* in both liquid and solid media when supplemented with either 0.8 mM or 2 mM cellobiose as substrate. Biofilm formation on silicone films was prevented by CDH when supplemented with 1 mM cellobiose. The CDH/cellobiose system also successfully inhibited many common urinary catheter colonizing microorganisms including the multidrug resistant *Staphylococcus aureus, Staphylococcus epiderimidis, Proteus mirabilis, Stenotrophomonas maltophilia, Acinetobacter baumanii* and *Pseudomonas aeruginosa.* Interestingly, CDH was also able to produce H_2O_2 during oxidation of extracellular polysaccharides (exPS) formed by microorganisms in the absence of cellobiose. The H_2O_2 production and consequently antimicrobial and antibiofilm activities on these exPS were enhanced by incorporation of glycoside hydrolases like amylases. Hydrolysis of polysaccharides by these enzymes increases the number of terminal reducing sugars as substrates for CDH as well as destabilizes the biofilm. Further, CDH suspended in catheter lubricants killed bacteria in biofilms colonizing catheters. The incorporation of the CDH/cellobiose system in the lubricant therefore makes it an easy strategy for preventing microbial colonization of catheters.

3.1 Introduction

Indwelling urinary catheters are standard medical devices used the world over. Severe health problems can arise ranging from urinary tract infections to life-threatening complications when patients are catheterized. Catheter associated urinary tract infections (CAUTI) account for 40% of all nosocomial infections occurring in hospitals and for 80% of all nosocomial urinary tract infections [1, 2]. In order to prevent infections, catheters are replaced at regular intervals causing inconvenience for the patient and increased cost for the health care system [3]. CAUTIs are predominantly caused by *Escherichia, Klebsiella, Proteus, Pseudomonas, Acinetobacter* and *Enterobacter* species [4, 5], adhering to the catheter surface in a complex biofilm [6]. Biofilms contain extracellular polymeric substances (EPS) produced by the microorganisms, that generally comprise DNA, proteins and extracellular polysaccharides (exPS). The composition of the biofilm is highly dependent on the type of bacteria and the growth conditions and its main function is to protect the microorganisms from negative environmental influences [7]. Several reasons including acquisition of antibiotic resistant genetic elements from other bacteria and the formation of a diffusion barrier makes microorganisms in biofilms more resistant to antibiotic treatment and difficult to remove once they are formed [8]. Therefore prevention of biofilm formation and microbial colonization is a key to a successful strategy against medical device related infections. Over the past decades the development of antimicrobial coatings containing substances like antibiotics, silver, gendine and nitric oxide [9, 10] and, more recently antimicrobial peptides are intensively being explored [11]. However, most of these coatings have raised issues concerning cytotoxicity and an increase of antibiotic resistance [8].

Enzymes are gaining special interests as the new generation of antimicrobials as summarized in Thallinger et al. [12]. Among them glucose oxidase which uses glucose to produce hydrogen peroxide (H_2O_2), a well-known antimicrobial agent extensively used in the food industry [12]. H_2O_2 is a strong oxidizing agent that has been widely used as disinfectant and an antiseptic in low concentrations (0.25-3%). The action of H_2O_2 on microbes is due to the formation of radicals in solution which attack membrane lipids, DNA and other cell constituents important for the viability of the microorganism [13]. Although similar studies using glucose oxidase showed promising results, the glucose needed by the enzyme as substrate is also a very good growth substrate for most microorganisms of concern. Recently our group has demonstrated the ability of cellobiose dehydrogenase (CDH) from *M. thermophilum*, to inhibit bacterial growth [14]. CDH is an oxidoreductase, which unlike glucose oxidase can oxidize cellooligosaccharides (including cellobiose) and other oligosaccharides to produce H_2O_2 which are no growth substrates for many microorganisms causing infections [15, 16]. In addition, CDH oxidation of cellooligosaccharides produces cellobionic acid which lowers the pH thereby creating a hostile environment for bacteria [15,

17]. This study therefore investigates the possibility of using CDH as an antimicrobial and antibiofilm agent to prevent urinary catheter based infections. The CDH is incorporated into the lubricant traditionally used to minimize discomfort during catheterization [18]. Further, the potential of CDH to produce H_2O_2 for antibiofilm and antimicrobial activities directly from exPS without addition of cellobiose was assessed.

3.2 Materials and Methods

3.2.1 **Materials and microorganisms**

All chemicals used were of analytical grade. Media components, cellobiose, and dichloroindophenol (DCIP) were purchased from Carl ROTH (Karlsruhe, Germany). All other chemicals and chemicals for buffer solutions were purchased from Sigma-Aldrich (Steinheim, Germany). Commercial enzymes (alpha amylase, mannanase and pulpzyme) were purchased from Novozymes (Bagsvaerd, Denmark) and endoglucanase from Fluka (Steinheim, Germany). The recombinant *M. thermophilum* cellobiose dehydrogenase (rMtCDH) was produced as previously described [15]. Catheters and silicone sheets were provided by Degania Silicone, Israel.

Staphylococcus aureus strain ATCC 25923 and *Escherichia Coli* strain XL1 used throughout the experiments were acquired from the culture collection of the Institute of Environmental Biotechnology from Graz University of Technology. The other clinical bacterial isolates (*MRSA* U-1768/1*, Staphylococcus epiderimidis* U-333/3*, Enterococcus faecalis* X - 3196*, Escherichia coli* ESBL B-2718*, Klebsiella pneumoniae* ESBL+ Y-995/22*, Proteus mirabilis* U-1958/1*, Stenotrophomonas maltophilia* U-57/28*, Acinetobacter baumanii* U-1724/1 and *Pseudomonas aeruginosa* U-1662/63*)* were obtained from different departments (Burn, Surgical, Internal medicine and Urology) at Pirogov hospital, Sofia, Bulgaria.

3.2.2 **CDH activity assay**

The activity of cellobiose dehydrogenase was assayed according to Baminger et al. [19], and modified by Flitsch et al. [15]. Briefly, rMtCDH activity was measured by monitoring the decrease in absorbance of 2, 6-dichlorophenolindophenol (DCIP), at 520 nm (ε_{520}=6.8×10^3/M*cm), pH 4.5 and 30 °C using a Hitachi U-2900 spectrophotometer. The reaction mixture contained 3 mM DCIP and 300 mM lactose in 100 mM sodium acetate buffer, pH 4.5. The reaction was started by addition of CDH and the decrease in absorbance monitored for 2 minutes. The activity was calculated from the slope and defined in Units as the amount of enzyme reducing 1 µmol of DCIP per minute under the above reaction conditions.

3.2.3 **Antibiofilm activities of CDH**

The antibiofilm activities of CDH were investigated by incubation with *S. aureus* in the presence of cellobiose in a 96-well plate (Sarstedt, Germany). Briefly, the media containing tryptic soy broth supplemented with 0.5 % (w/w) glucose (TSB+) and TSB+ supplemented with 0.6 % (w/w) yeast extract and 0.2 % (w/w) sodium citrate was inoculated with actively growing bacteria (final OD_{600} 0.01), cellobiose (0.5 to 2.0 mM) and 0.33 U/ml CDH. An autoclaved silicone piece (diameter 7 mm) was also added to each well. The plate was

incubated for 18 hours at 37 °C without shaking. In a parallel experiment media, bacteria and silicone films were supplemented with varying amounts of pure H_2O_2 (125 to 2000 µM) and incubation carried out under the above conditions with appropriate controls. After incubation, non-attached cells were removed by washing with distilled water while the biofilms attached to the silicone sheets were fixed by drying at 60 °C for one hour followed by a staining step with 100 µl 0.1 % (w/w) crystal violet for ten minutes. The silicone sheets were then immersed in 100 µl of 30 % acetic acid for 10 minutes to solubilize the crystal violet. The supernatant (50 µl) was transferred in a new 96-well plate to measure the absorbance at 595 nm in a plate reader (Tecan Infinite M200 Pro Platereader, Switzerland). Percent biofilm formation was calculated by dividing the absorbance values of the cellobiose containing wells by the absorbance of the negative control.

3.2.4 Determination of apparent inhibitory concentration 50 (IC_{50}) in liquid medium

The apparent inhibitory concentration 50 (IC_{50}) of cellobiose (leading to equimolar H_2O_2 production upon complete oxidation by CDH) were determined by incubating different cellobiose concentrations with *E. coli* and *S. aureus* in 96 well plates. Mueller-Hinton (MH) broth medium (100 µl) was pipetted into each well followed by 100 µl of 50 mM cellobiose. Serial dilutions of cellobiose ranging from 0.1 to 25.0 mM were achieved by transferring 100 µl in each consecutive lane. A bacterial suspension containing 5 µl of 10^5 colony forming unites per milliliter (CFU/ml) bacteria was added followed by 10 µl of 1.64 U/ml CDH (final activity in each well 0.14). The respective controls e.g. CDH only, negative and sterile control were also prepared in the other lanes. Plates were incubated at 37 °C for 16 hours and optical density (OD) measured at 600 nm every 30 minutes in a platereader. The apparent IC_{50} value was defined as the concentration of cellobiose inhibiting growth by 50 % compared to the negative control. All determinations were performed in triplicates.

3.2.5 Determination of apparent IC_{50} on agar plates

The apparent IC_{50} on agar plates was assessed against *S. aureus* and *E. coli*. A 1 ml solution of CDH was added to every 100 ml of liquid MH agar (~40 °C) in order to yield an enzyme activity of 0.33 U/plate. One ml of 10^8 CFU/ml bacteria suspension was added together with different cellobiose concentrations ranging from 0.1 to 2.0 mM. Controls included plates with cellobiose only, CDH only and the bacteria only. Plates were left to stand for 30 minutes in order for the liquid to soak before turning them upside down and incubating at 37 °C for 24 hours in an incubation chamber. The CFU/ml were calculated and lowest cellobiose concentration determined which led to 50 % inhibition of growth compared to the negative control. All measurements were performed in triplicates.

3.2.6 **Antimicrobial activity of CDH against clinical isolates commonly colonizing catheters**

The antimicrobial effect of the CDH/cellobiose system on several clinical isolates (gram positive and gram negative listed in Table 3 frequently found in urinary catheter associated biofilms was tested. The isolates were obtained from different departments at Pirogov hospital as denoted by the suffix: burn unit (U), surgical unit (X), internal medicine unit (B) and urology (Y).

Table 3: Clinical isolates tested concerning the antimicrobial effect of cellobiose dehydrogenase (ESBL=Extended Spectrum beta lactamase); letters before numbers refer to isolation location at Pirogov hospital: U-burn, X-surgery, B-internal, Y-urology

Gram positive	Gram negative
MRSA U-1768/1	*Escherichia coli* ESBL B-2718
Staphylococcus epiderimidis U-333/3	*Klebsiella pneumoniae* ESBL+ Y-995/22
Enterococcus faecalis X -3196	*Proteus mirabilis* U-1958/1
	Stenotrophomonas maltophilia U-57/28 (non fermenter)
	*Acinetobacter baumanii*U-1724/1 (non fermenter)
	*Pseudomonas aeruginosa*U-1662/63 (non fermenter)

A 1 ml suspension of 10^9 CFU/ml of each clinical strain was applied onto MH agar plates containing a total of 0.33 U of CDH together with either 2 or 10 mM of cellobiose. Appropriate controls (MH agar plates without cellobiose or CDH, CDH alone, cellobiose alone) were also prepared and incubated under the same conditions. Plates were incubated for 20 hours at 37 °C followed by the quantification of the colonies. All experiments were performed in triplicates.

3.2.7 **exPS production, hydrolysis and incubation with CDH**

In order to investigate the ability of CDH to use exPS as substrate, overnight cultures of *E. coli* and *S. aureus* grown in TSB+ at 37 °C and 125 rpm were equally distributed on TSB+ agar plates supplemented with 3 % (w/w) sodium chloride. Plates were incubated at 37 °C for 24 hours to allow biofilm formation. The biofilms were harvested by carefully scratching them off with a spatula. exPS extraction was carried out as described by Pan et al. [20]. Briefly, an equal volume of 2 % (w/v) ethylenediaminetetraacetic acid (EDTA) was added to the harvested samples, followed by 3 hours of incubation on ice. Thereafter the samples were centrifuged for 20 minutes at 5600 rpm. The extraction was repeated twice to maximize yields of exPS. The supernatant was filtrated with Acrodisc® syringe filters (0.2 µm) and dialysed (cut off 3500 Da) against water for 24 hours, to remove EDTA and glucose.

The extracted *E. coli* and *S. aureus* exPS were incubated with CDH and H_2O_2 production monitored. Further, the extracted exPS were hydrolyzed separately using 3 U/ml

of either alpha amylase, endoglucanase, mannanase or pulpzyme supplemented with 500 µl of exPS. Samples were incubated at 50 °C while shaking at 400 rpm for 24 hours. The exPS hydrolysates were then incubated with CDH while monitoring H_2O_2 production using a modified Leuco Crystal Violet Assay described by Pricelius et al.[17]. Briefly, 100 µl of CDH (0.5 U/ml) was incubated with 555 µl of sodium phosphate buffer (55 mM, pH 6.5) and 100µl of hydrolysed exPS for 30 minutes at 40 °C and 600 rpm in a Thermomixer (Eppendorf, Hamburg, Germany). The mixture was then incubated at 99°C for 10 minutes to inactivate both the CDH and the hydrolytic enzymes. The H_2O_2 produced was measured as described by Pricelius et al.[17].

3.2.8 Antimicrobial and antibiofilm activities of CDH/amylase in catheter lubricant

The lubricant was made by dissolving 0.14 g of sodium alginate in 6.46 ml of H_2O_{dest} followed by the addition of 3.4 g of glycerol. This lubricant containing CDH (final activity 0.66 U/ml lubricant) was supplemented with 100 µl of 300 mM lactose and the activity monitored as described in section 3.2.2. Further, the ability of CDH and amylase (final activity 3 U/ml lubricant) to kill *S. aureus* in biofilms already formed on catheter surfaces was investigated. Catheters (1 cm x 1 cm) already colonized by *S. aureus* biofilms (biofilms were grown according to section 3.2.3) were suspended in catheter lubricant containing CDH and amylase followed by a 2 hour incubation at 37 °C as illustrated in Figure 13.

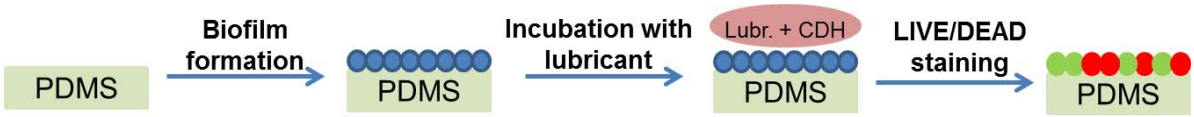

Figure 13: Layout of antimicrobial and antibiofilm studies using CDH and/or Amylase incorporated in catheter lubricant using LIVE/DEAD staining and CLSM analysis

The positive control biofilm was incubated at 65 °C for 2 hours in order to kill the bacteria whilst the negative control was incubated for 2 hours at 37°C in lubricant without enzyme. Plates were washed and then stained with the LIVE/DEAD cell viability assay (Life technologies, Carlsbad, USA). The stained biofilms were then visualized using the FluoView 1200 Confocal Laser Scanning microscope (CLSM) from Olympus (Pennsylvania, USA).

3.3 Results

3.3.1 **Antimicrobial and antibiofilm activities of CDH**

Preliminary experiments showed that oxidation of 1 mol of cellobiose by CDH leads stochiometrically to the production of 1 mol of H_2O_2 [21]. Based on these results the biofilm inhibitory activities of CDH were compared to those of added H_2O_2 against actively growing *S. aureus*. As shown in Figure 14, a similar trend on the inhibitory effect of CDH produced H_2O_2 was observed when compared to added H_2O_2. Quite expectedly the inhibition increased with increasing concentration of cellobiose or increasing concentration of added H_2O_2, respectively. Total biofilm inhibition of *S. aureus* was achieved at concentrations above 1 mM H_2O_2 or 1 mM cellobiose (Figure 14).

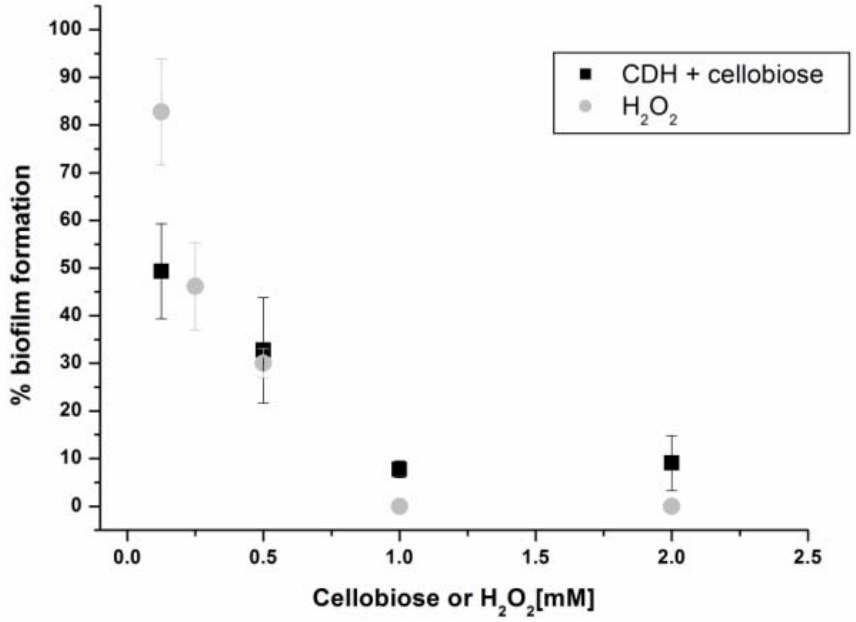

Figure 14: The biofilm inhibiting effect of added H_2O_2 (grey circles) compared to in-situ produced H_2O_2 by CDH in combination with various cellobiose concentrations (black squares).

Further studies to determine the apparent IC_{50} using different concentrations of cellobiose in liquid cultures revealed that 0.19 and 0.25 mM of cellobiose are necessary to inhibit the growth of *S. aureus* and *E. coli* by 50% in liquid cultures, respectively, in the presence of 0.33 U CDH. In both cases a complete inhibition of growth was achieved with 0.8 mM of cellobiose (Figure 15).

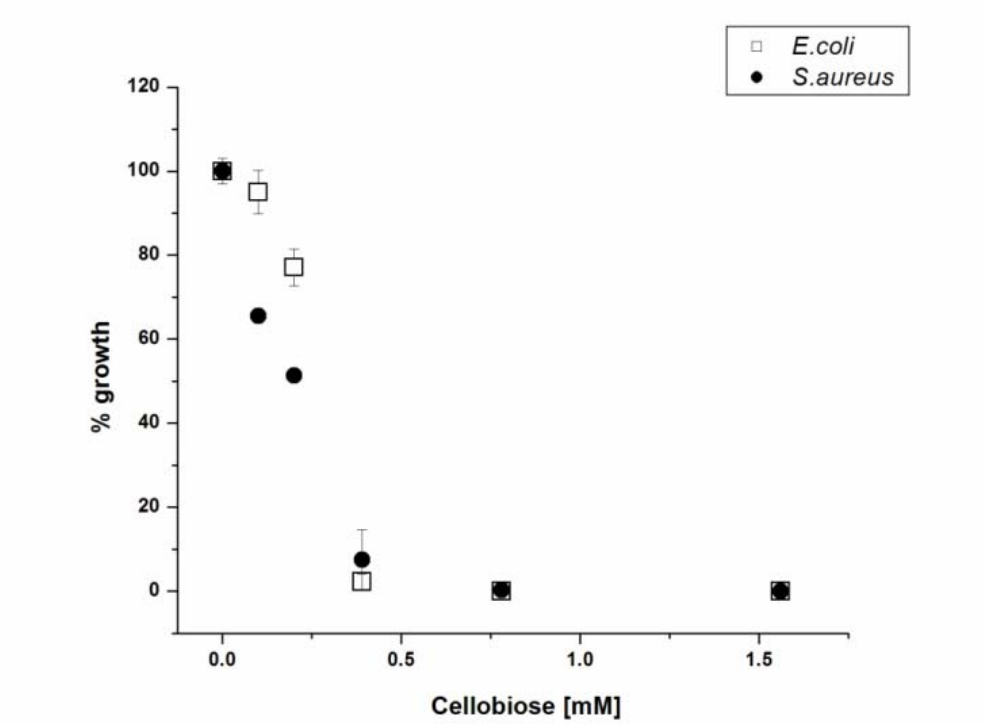

Figure 15: Growth inhibition of *E. coli* (squares) and *S. aureus* (circles) in liquid cultures as a function of cellobiose concentration in the presence of CDH.

Subsequent studies investigating the ability of CDH immobilized in agar plates to inhibit bacteria was conducted on agar plates. The MH – agar impregnated with 0.33 U of CDH supplemented with 80 µM cellobiose was able to inhibit CFU formation of *S. aureus* by 50 %. Complete inhibition of growth for both microorganisms was achieved on plates containing 2 mM cellobiose (Figure 16). There was also some growth inhibition by CDH in the absence of cellobiose which is most likely due to the presence of other carbohydrates in the cultivation medium.

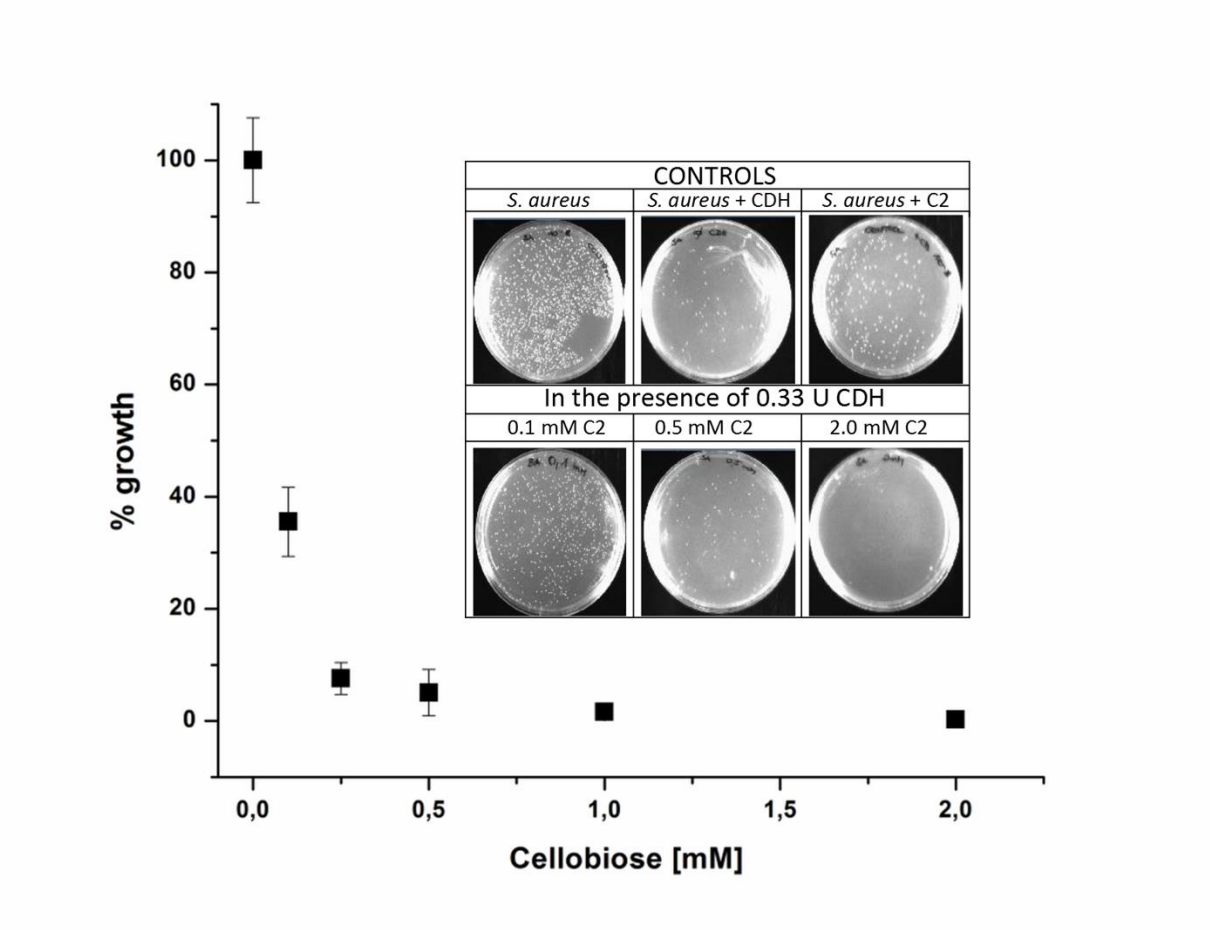

Figure 16: Growth on agar plates of S. aureus as a function of cellobiose (C2) concentration in the presence of CDH.

3.3.2 Antimicrobial activities of CDH against clinical isolates commonly colonizing catheters

Complete inhibition of the *E. coli* and *S. aureus* in liquid was achieved with 0.8 mM cellobiose and 2 mM cellobiose on solid media, the latter concentration was selected as the base for further studies with different clinical isolates. Table 4 lists the growth reduction on plates containing CDH and 2 mM or 10 mM of cellobiose. The strongest antimicrobial effects (100% inhibition) were obtained with *A. baumanii* and the multidrug resistant *S. aureus* (MRSA) in the presence of 10 mM cellobiose (Table 4). *S. epiderimidis*, *P. mirabilis* and *S. maltophilia* were moderately inhibited in the presence of 2 mM cellobiose and also significantly in the presence of 10 mM cellobiose. Surprisingly, the inhibition of *P. aeruginosa* was only noticed in the presence of 10 mM cellobiose and no significant inhibition was observed with *E. faecalis* and *K. pneumoniae*.

Table 4: Antimicrobial effect of CDH/cellobiose against clinical isolates; (0) no reduction of growth compared to control, (+) slight reduction of growth, (++) moderate reduction of growth, (+++) strong reduction of growth, (++++) no growth.

	Reduction of growth (inhibition)	
Amount of cellobiose supplemented / **Bacterial clinical isolate**	**2 mM**	**10 mM**
MRSA U-1768/1	**++**	**++++**
Staphylococcus epiderimidis U-333/3	**++**	**+++**
Enterococcus faecalis X -3196	***0***	***0***
Escherichia coli ESBL B-2718	**+**	**++**
Klebsiella pneumoniae ESBL+ Y-995/22	***0***	***0***
Proteus mirabilis U-1958/1	**++**	**++**
Stenotrophomonas maltophilia U-57/28	**++**	**+++**
Acinetobacter baumanii U-1724/1	**+++**	**++++**
Pseudomonas aeruginosa U-1662/63	***0***	**++**

3.3.3 Investigating the ability of CDH to use exPS and/or enzymatically hydrolyzed exPS to produce H2O2

Incubation of CDH with exPS from *E. coli* and *S. aureus* alone resulted in the production of low amounts (0.1 µM) H_2O_2. In order to investigate the possibility of boosting the antibiofilm properties of CDH, it was decided to evaluate the effect of glycoside which would hydrolyze the exPS generating better substrates for CDH. Different amounts of H_2O_2 were produced when CDH was incubated with exPS in the presence of different glycoside hydrolases (Figure 17). For example, exPS extracted from both *E. coli* and *S. aureus* then treated with alpha amylase lead to the production of the highest concentration of H_2O_2 of 5.8 and 4.8 µM, respectively. However, very low H_2O_2 concentrations were measured when exPS was treated with mannanase. Therefore, enzymatically hydrolyzed exPS from *E. coli* yielded the highest amounts of H_2O_2 (Figure 17).

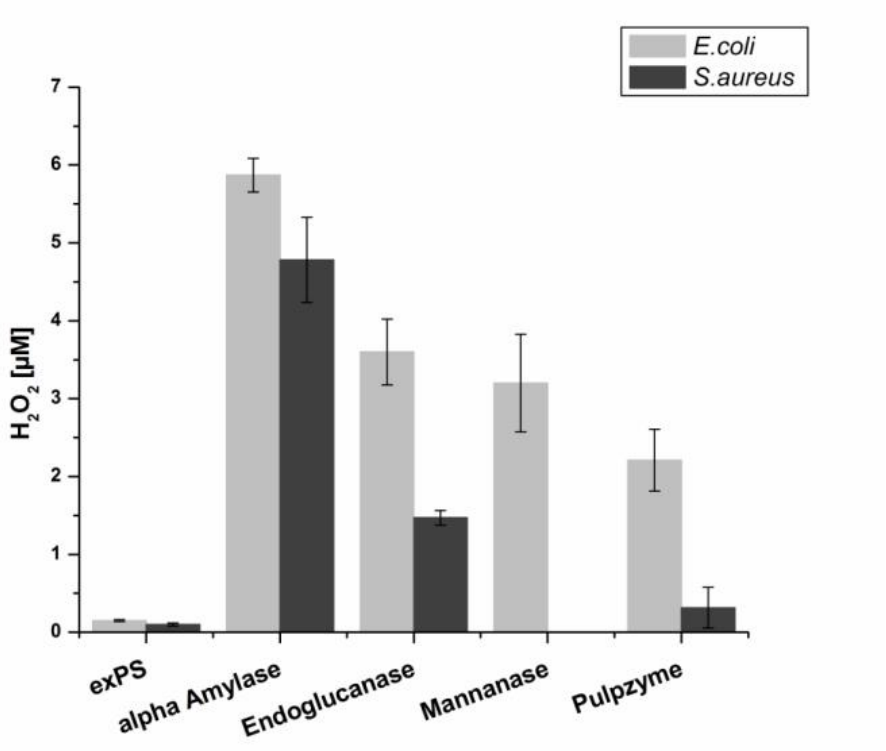

Figure 17: H_2O_2 production by CDH on exPS produced by *E.coli* (light grey) or *S. aureus* (dark grey) and hydrolyzed by four different glycoside hydrolase enzyme preparations.

3.3.4 Antimicrobial and antibiofilm activities of CDH suspended in catheter lubricant

CDH suspended in catheter lubricant showed the same activity as CDH diluted in the same amount of buffer that is 1.4 U/ml. Based on these observations, *S. aureus* colonized catheters were suspended in catheter lubricant containing CDH supplemented with cellobiose in order to investigate the ability of CDH to kill bacteria in biofilms. As shown in Figure 18, incubation of CDH in lubricants with already formed biofilms on catheters led to the extensive death of the bacteria as evidenced by the dead red cells compared to negative control which shows an abundance of live cells (green). The incorporation of amylase into the CDH/cellobiose lubricant did not enhance the antimicrobial effect under the investigated conditions.

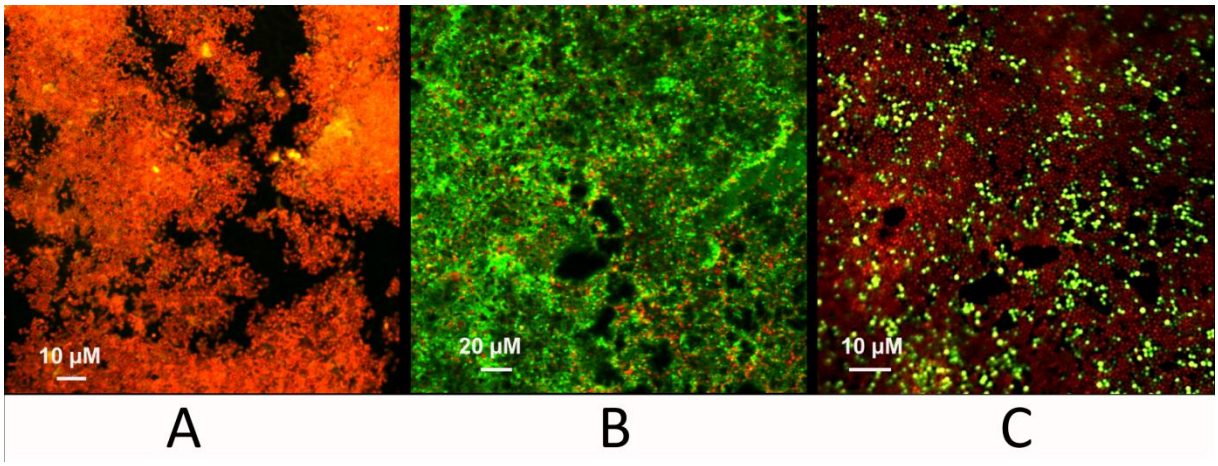

Figure 18: CLSM pictures of LIVE/DEAD stained *S.aureus* biofilms formed on silicone sheets incubated at 65°C for 2h (A), treated with lubricant at 37°C for 2h (B) and treated with lubricant containing CDH and cellobiose at 37°C for 2h (C).

3.4 Discussion

A novel *in situ* antibiofilm and antimicrobial system based on the ability of CDH to produce H_2O_2 in the presence of cellobiose was successfully developed. CDH in the presence of 1 mM cellobiose was able to completely inhibit biofilm formation by *S. aureus* while only 0.8 mM cellobiose completely inhibited bacterial growth of *E. coli* and *S. aureus* in liquid media. These results are consistent with the findings from Miyasaki et al. [22] and Watts et al. [23], who demonstrated that exposure of bacteria to 0.7 µM H_2O_2 had bactericidal effects. Interestingly, this study further shows that these H_2O_2 concentrations produced by CDH incorporated into agar were able to inhibit bacterial growth of both *E. coli* and *S. aureus.* These results suggest that when CDH is immobilized in hydrogels it can still produce H_2O_2 and have great implications in applying CDH/cellobiose system in preventing microbial colonization and killing bacteria in both the production industry and health.

Indeed application of the CDH/cellobiose system supplemented with cellobiose resulted in the inhibition of common urinary catheter colonizing microorganisms like multidrug resistant *S. aureus, A. baumanii, S. epidermidis*, *P. mirabilis*, *S. maltophilia* and *E. coli* to varying extents. However, it is important to note the ability of the CDH system to inhibit *E. faecalis, E. coli, P. mirabilis* and *P. aeruginosa* is of utmost importance in urinary catheters since these are the most commonly reported catheter colonizing microorganisms. Generally it can be stated that, out of the different classes of bacteria tested (gram positive and gram negative), the CDH/cellobiose system is most effective against gram positive bacteria considering the strong inhibition of MRSA and *S. epidermidis*. Similar findings were also observed by McDonall and Russel [13]. Despite most of the used strains being catalase positive except *E. faecalis*, they were still inhibited. However, pre-exposure of *E. faecalis* to sublethal stress levels is known to enable it to withstand harsh environmental conditions including normally lethal levels of H_2O_2, sodium dodecyl sulfate, bile salts, heat and ethanol [24]. Similarly, *K. pneumonia´s* resistance to inhibition by the H_2O_2 produced by the CDH/cellobiose system supplemented with even 10 mM cellobiose may be attributed to its well-known oxyR transcription factor responsible for the upregulation of defense mechanisms against oxidative stress [25]. This is expected since normally *K. pneumoniae* strains are found in the hostile environment of the gastrointestinal tract of patients. These observations therefore demonstrate that the inhibition of bacteria by H_2O_2 is strain dependent. Similarly, previous studies also showed that 1 mM of H_2O_2 inhibited *H. influenza [26]* while 2.1 mM were effective in inhibiting *S. aureus* and *P. aeruginosa* [27].

The CDH/cellobiose system showed another interesting feature as it was able to oxidize enzymatically hydrolysed *E. coli* and *S aureus* exPS leading to production of H_2O_2. Since CDH is able to oxidize a wide variety of oligosaccharides [28], it is believed that the enzymatically generated fragments were oxidized by CDH resulting in concomitant H_2O_2

production as evidenced by increased H_2O_2 concentration especially when amylase treated exPS were incubated with CDH. These enzymes are known to exhibit antimicrobial activities by degrading the biofilm matrix through the cleavage of alpha linked glycosides, which are also present in exPS [16]. The antimicrobial and antibiofilm activities are therefore strongly enhanced when used together with glycoside hydrolases. Further, the hydrolysis of the biofilm leads to its destabilization and failure to protect the microorganisms from antimicrobial agents. The CDH was able to produce higher H_2O_2 in the presence of exPS from *E. coli* than from *S. aureus*. This again shows that the production of H_2O_2 depends on the source of exPS which is basically determined by the chemical composition of the exPS, type and growth conditions of the bacteria etc..

Incorporation of CDH into the catheter lubricant formulation seems an easy and very effective strategy to kill bacteria colonizing catheters. Integration of antimicrobial CDH in the lubricant would be a cost effective strategy when compared to immobilization of the enzyme on catheters. Enzymes produced by microorganisms during degradation of cellulose are currently used in the Biomass-to-Ethanol process in multi-ton scale. Consequently, it is not expected that the cost of CDH will represent a limiting factor. Moreover, when a combination with glycoside hydrolases is used, H_2O_2 is produced “on-demand”, - i.e. only when biofilms/exPS are formed the enzymes become active which is more beneficial for the patients than continuous uncontrolled release of possibly irritant molecules.

3.5 Conclusion

A novel in situ antibiofilm and antimicrobial technique based on the ability of CDH to produce H_2O_2 in the presence of cellobiose or exPS suitable for preventing microbial colonization of urinary catheters was investigated. Incubation of CDH with 0.8 mM and 2 mM cellobiose completely inhibits bacterial growth of E. coli and S. aureus in both liquid media and on agar plates, respectively. The CDH/cellobiose system was effective in inhibiting many common urinary catheter colonizing microorganisms like the multidrug resistant S. aureus, A. baumanii, S. epiderimidis, P. mirabilis, S. maltophilia and E. coli. The studies also show that CDH in combination with glycoside hydrolases can exhibit antimicrobial activity (i.e. produce H2O2) on demand from exPS once produced by bacteria during biofilm generation. The ability of CDH suspended in catheter lubricant to kill bacteria in biofilms formed on catheters demonstrates the potential of this system. Based on these findings, application tests including biocompatibility have been initiated and in-vivo tests planned.

ACKNOWLEDGEMENTS

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4

Cellobiose dehydrogenase functionalized urinary catheter as novel antibiofilm system

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Abstract

Urinary catheters expose patients to a high risk of acquiring nosocomial infections. To prevent this risk of infection, cellobiose dehydrogenase (CDH), an antimicrobial enzyme able to use various oligosaccharides as electron donors to produce hydrogen peroxide using oxygen as an electron acceptor, was covalently grafted onto plasma-activated urinary polydimethylsiloxane (PDMS) catheter surfaces. Successful immobilization of CDH on PDMS was confirmed by Fourier transformed infrared spectrometry and production of H_2O_2. The CDH functionalized PDMS surfaces reduced the amount of viable *Staphylococcus aureus* by 60 %, total biomass deposited on the surface by 30 % and 70 % of biofilm formation. The immobilized CDH was relatively stable in artificial urine over 16 days, retaining 20 % of its initial activity. The CDH coated PDMS surface did not affect the growth and physiology of HEK 239 and RAW 264,7 mammalian cells. Therefore this new CDH functionalized catheter system shows great potential for solving the current problems associated with urinary catheters.

4.1 Introduction

Urinary catheters are among the most frequently used prosthetic devices in modern medicine [1]. Due to their widespread use, catheter-associated urinary tract infections (CAUTI) caused by biofilms account for 40% of all nosocomial infections [2, 3]. Indwelling catheters provide ideal conditions for microbial colonization and biofilm formation. Biofilms comprising of extracellular polymeric substances (EPS) including extracellular polysaccharides (exPS) as the main structural component offer protection to microbes from the human defense system and antibacterial agents (4,5). Since bacteria embedded in biofilms are difficult to eradicate by conventional antibiotics, this situation raises the urgent need to design catheter surfaces that can prevent pathogen colonization and biofilm formation. In recent years, a wide variety of antimicrobial coating technologies have been developed with variable success. For example, silver coated catheters have been extensively evaluated, although their efficiency has not been consistently supported by clinical data and the results have raised issues concerning cytotoxicity and bacterial resistance development. Other strategies investigated with limited success include hydrogel coating, antibiotic coatings and nitric oxide [4, 5]. Recently, enzymes have been attracting interest as new generation of antimicrobials [6, 7] targeting different aspects of microbial growth and biofilm formation. Our group developed a new antimicrobial and antibiofilm system based on cellobiose dehydrogenase (CDH) [6, 8]. This enzyme uses many structurally different cellooligosaccharides (including cellobiose) as substrates to produce hydrogen peroxide (H_2O_2), a well-known antimicrobial agent [7]. H_2O_2 is a strong oxidizing agent widely used as an antiseptic in low concentrations (0.25 – 3%) [9]. In previous studies we have demonstrated the ability of CDH to inhibit the growth of many clinically relevant microorganisms colonizing the catheters [6, 8]. Another interesting aspect of this study is the fact that CDH was able to use the bacterial exPS as substrate to produce H_2O_2 [6].The current study was aimed at covalently immobilizing CDH on polydimethylsiloxane (PDMS) urinary catheter surfaces, and evaluating the ability of the immobilized enzyme to inhibit the growth of *S. aureus* biofilm on the catheters incubated in artificial urine under static and dynamic conditions.

4.2 Materials and Methods

4.2.1 **Materials**

Media components and cellobiose were purchased from Carl ROTH (Karlsruhe, Germany). All other chemicals used were of analytical grade purchased from Sigma-Aldrich (Steinheim, Germany). The recombinant *Myriococcum thermophilum* cellobiose dehydrogenase (rMtCDH) was optimized for increased oxygen reactivity by a single mutation (N748G) and was produced in *Pichia pastoris* as previously described [10]. Catheters and films were provided by Degania Silicone Ltd (Israel). *Staphylococcus aureus* ATCC 25923 was obtained from the culture collection of the Institute of Environmental Biotechnology (Graz, Austria).

4.2.2 **Determination of H_2O_2 production by free and immobilized CDH**

H_2O_2 production by the immobilized CDH was assessed by the Amplex Red Assay [11] with some modifications. The CDH-PDMS films were incubated in a solution containing 425 µL 0.25 M Na_2HPO_4/NaH_2PO_4 buffer pH 7.4 and 75 µL 200 mM cellobiose at 37 °C for 3 min at 600 rpm to allow H_2O_2 production. After incubation, 197.5 µL of the sample were transferred into a black 96-well plate (Sarstedt, Germany) and the reaction started as described. Fluorescence was monitored at 37°C (Ex/Em 550/587 nm) on a microplate reader (Tecan Infinite M200 Pro Platereader, Switzerland) and data converted to µM H_2O_2 by using a calibration curve built with various dilutions of a 30% H_2O_2 yielding 0 – 15 µM H_2O_2 in the assay. Pristine PDMS films and films treated only with the immobilization reagents served as references. One unit of enzyme activity was defined as 1 µmol of H_2O_2 produced per min by CDH in solution or immobilized on the PDMS surface.

4.2.3 **Immobilization of CDH on PDMS films**

CDH immobilization involved 4 consecutive steps (Figure 19). PDMS urinary catheters and films were washed with 96 % EtOH and distilled water (dH_2O), and then dried at room temperature for 2 hours before functionalization. Hydroxyl groups were generated on the silicone surface using low pressure oxygen plasma in a Plasma system FEMTO timer (Diener Electronic**,** Royal Oak, USA**)** for 5 min with the generator frequency performance of 13.56 MHz set at 100 % and an oxygen flow of 20 sccm per min. The formation of new hydrophilic functional groups was monitored by measuring the water contact angle of the PDMS films (Drop shape analyzer Krüss DSA 100, Germany).

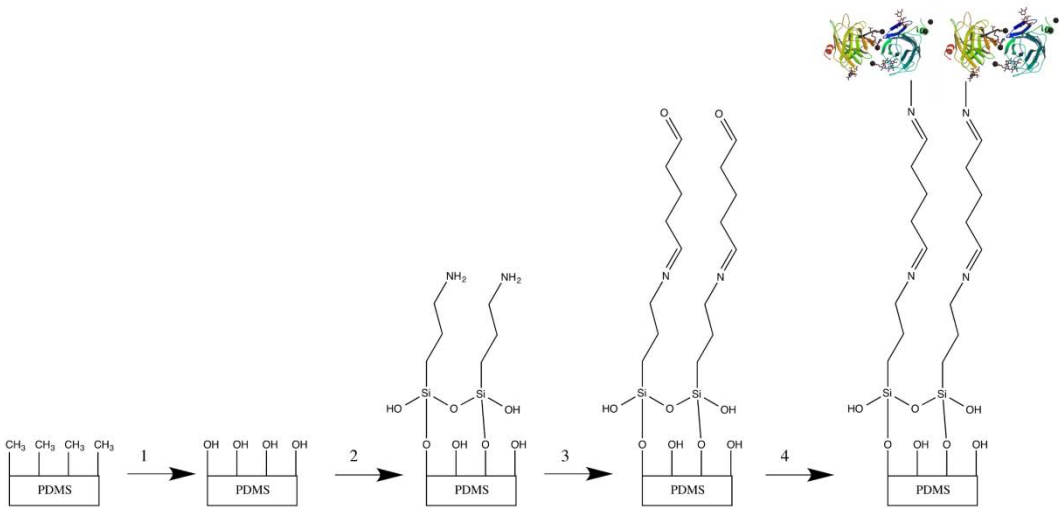

Figure 19: Stepwise immobilization of CDH onto PDMS surfaces; (1) plasma treatment, (2) grafting APTES,(3) grafting glutaraldehyde and (4) grafting the enzyme

Immediately after the plasma treatment the films were transferred into a solution containing aminopropyltriethoxysilane (APTES), 0.1 M acetic acid and isopropanol in the ratio 1 : 5 : 5. The silanization (step 2) was carried out during 24 h under steady mixing at 30 rpm on a rotator (Wise mix RT 10, Wisd Laboratory Instruments, Korea). Thereafter, the APTES-modified PDMS films were dried for 1 h at 100 °C. The presence of amino groups on the surface was confirmed by dipping the films in a 2% (w/v) ninhydrin solution followed by incubation at 100 °C until a color change was visible [12]. In step 3, the amino-functionalized films were incubated in a solution containing 10% (w/v) glutaraldehyde in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer pH 7.3 for 1 h at 40 rpm [13]. Thereafter, the silicone films were washed twice with buffer and once with dH_2O to remove unbound glutaraldehyde. CDH immobilization (step 4) was carried out overnight under constant stirring in different buffers (0.05 M citrate pH 5.5; 0.1 M potassium phosphate (K_2HPO_4/KH_2PO_4) pH 6.0; 0.1 M sodium phosphate (Na_2HPO_4/NaH_2PO_4) pH 7.3; 0.1 M sodium phosphate (Na_2HPO_4/NaH_2PO_4 pH 8) containing from 0.5 to 7 mg mL^{-1} enzyme at either 4 °C or room temperature. The CDH grafted films were washed thoroughly with 30 % EtOH and dH_2O to remove any unbound enzyme. The washed off enzyme in solution was quantified using BCA Protein Assay Kit (Pierce, Rockford, USA). A calibration curve was created using bovine serum albumin (BSA) in concentrations ranging from 0 – 2 mg/mL as a standard. The modification of PDMS was further confirmed by Fourier transformed infrared spectroscopy in attenuated reflection mode (ATR – FTIR) using a Spectrum 100 Perkin Elmer FT-IR spectrometer (Massachusetts, USA). In order to visualize the covalently bound enzyme on the surface, CDH was labeled with fluorescein isothiocyanate (FITC) prior to immobilization [14] and was visualized using the Bio-Rad ChemiDoc Universal Hood III (California, USA).

4.2.4 Effect of synthetic urine on the activity and stability of CDH

CDH-functionalized PDMS samples were incubated in artificial urine medium (AUM) prepared as describe by Brooks et al. [15] at 37 °C for 16 days. The H_2O_2 production capacity of the immobilized enzyme was assessed every 2 days. Untreated PDMS films and films coated only with the immobilization reagents served as controls. In order to detect possible leaching of the enzyme into the solution, protein concentration and enzyme activity were measured as described above.

4.2.5 Antimicrobial and antibiofilm activity of immobilized CDH

4.2.5.1 Static antimicrobial assay

Washed CDH-PDMS films (7 mm diameter) were placed on the bottom of a 96-well plate. After adding 163 µL of AUM pH 6.5 supplemented with 1% glucose (AUM +) and 17 µl of 20 mM cellobiose the wells were inoculated using 20 µl of a *S. aureus* culture with a concentration of $3*10^5$ CFU/mL. Untreated PDMS films served as negative controls. After 3 h of incubation at 37°C the supernatant was removed and each well washed twice with 0.9% NaCl in order to remove non-adherent cells. Viable cells were quantified by adding 100 µL of a 10 µM solution of resazurin in 0.9% NaCl. Development of fluorescence was monitored at 37°C for one hour (Ex/Em 550/590 nm) using a microplate reader. The total biomass formed on the coated PDMS films was quantified by drying them at 60°C for one hour. The PDMS films were stained with 200 µL crystal violet (0.1% w/v) for 30 min, washed with dH_2O and the bound crystal violet was re-dissolved in 150 µL acetic acid (30% v/v). Absorbance was measured at 595 nm and all measurements were performed in triplicates.

4.2.5.2 Dynamic biofilm assay using an artificial bladder system

Dynamic biofilm tests were performed in an artificial bladder described by Stickler et. al. [16] with modifications [17] (Figure 20). The CDH-coated Foley catheter was inserted and the catheter balloon inflated with 10 mL dH_2O. Then, the bladder was filled with 60 mL sterile artificial urine (UNE EN 1616) containing 1 mg/mL tryptic soy broth (TSB) and 20 mM cellobiose followed by inoculation with *S. aureus* (OD_{600}=0.01). The catheterized bladder was left for 1 h without urine supply to allow the organisms to adapt to the model and then the supply with 1 mL/min flow rate was started. After 7 days the catheter was removed and washed with dH_2O. Then, different sections of the CDH-functionalized catheter - balloon (1 cm x 1 cm) and urethra (catheter shaft, 1 cm) were cut and subjected to analysis for biofilm formation using crystal violet and Live/Dead staining followed by fluorescence microscopy. The same parts of pristine silicone Foley catheter were used as controls.

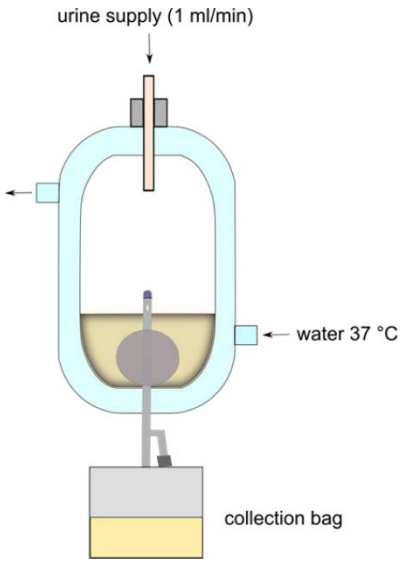

Figure 20: Schematic illustration of the artificial bladder catheterized with a functionalized silicone catheter

4.2.6 Atomic Force Microscopy of PDMS surfaces

High-resolution three-dimensional reconstructions of pristine and enzyme-coated PDMS films were acquired using Atomic Force Microscopy (Solver Pro AFM endowed with a closed-loop scanner from NT-MDT, Moscow, Russia). The measurements were carried out in air at room temperature working in dynamic mode. Cantilevers, characterized by a resonant frequency of about 174 kHz (NSG03 series, resonant frequency 47-150 kHz, force constant 0.35-6.1 nN/nm) were used working at low oscillation amplitudes with half free-amplitude set-point. High resolution images were 512×512 pixels frames acquired at 1-line/second scan speed. All AFM data were analysed using Gwyddion (Nečas and Klapetek, 2012). After obtaining the surface roughness of the CDH-coated films, the bacterial adhesion and biofilm formation on these surfaces was studied. The films (7 mm diameter) were washed with TSB, mQ water and subsequently transferred into 24-well plates (Sarstedt, Germany). Two mL of TSB supplemented with 1% (w/v) glucose and 20 mM cellobiose were added to each well followed by the addition of *S. aureus* with a concentration of $5*10^6$ CFU/mL in order to mimic a high bacterial load. The plates were incubated at 37°C for 3 h with gentle shaking to guarantee even bacterial growth. The supernatant was discarded and the films washed once with 0.9% NaCl and twice with dH_2O in order to remove unbound cells before letting them to air dry. Cells were fixed with methanol by immersing them in 2 mL of a sequence of water solutions with increasing methanol content (0, 30, 60 and 100%). Films were air-dried and stored in nitrogen until AFM image acquisition.

4.2.7 **Biocompatibility studies of CDH in free and immobilized form**

The biocompatibility of CDH in free and immobilized form was assessed against human embryonic kidney cells (HEK 239) and mouse macrophages (RAW 264,7) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT-assay was performed in 96-well plates [18]. The cell lines were grown in different concentrations of CDH (5 – 500 µg mL^{-1}) and cellobiose (0.02 – 50 mM). Thereafter, 10^4 HEK 293 cells were seeded into *Minimal Essential Medium* supplemented with 10 % fetal calf serum (FCS) and a mixture of P/S (final concentration of 100 µg/mL penicillin and 100 µg/mL streptomycin) and 10^4 RAW 264,7 cells were seeded into a 1 : 1 mixture of Dulbecco´s Modified Eagle Medium with Roswell Park Memorial Institute medium supplemented with 5 % FCS and P/S. After 24 h incubation at 37°C, the volume of medium in each well was adjusted to 80 µL, and 20 µL MTT-solution (5 mg/mL in PBS) was added. The plates were then incubated for one hour at 37°C, protected from light, to allow the viable cells to metabolize the MTT and produce formazan. After incubation the supernatant of each well was discarded, 100 µL of lysis buffer containing 90% isopropyl alcohol, 0.5% sodium dodecyl sulfate and 25 mM hydrochloric acid in water were added and the absorption measured at 540 nm. In the case of CDH immobilized on PDMS films, the films were incubated with (0.5, 2 and 5 mM) and without cellobiose as described above and analyzed using a light microscope (Olympus IX 50).

4.3 Results

4.3.1 Chemical activation of PDMS films using plasma

Preliminary PDMS activation studies with piranha solution did not lead to a significant change in the water contact angle (WCA) but to a brittle and opaque surface, hence the oxygen plasma treatment technique was adopted. Two minutes exposure time and 15 sccm of oxygen flow was enough to convert the surface methyl groups of PDMS films to hydroxyl groups resulting in the decrease of the WCA from 104° to 37.4° (Figure 21A). Increasing the exposure time to 5 min and the oxygen flow to 20 sccm further increased the hydrophilicity of the surface (WCA 24.5°). As the lowest angle and highest stability over time was reached with 5 min exposure time and 20 sccm of gas these settings were chosen as the best conditions (Figure 21B).

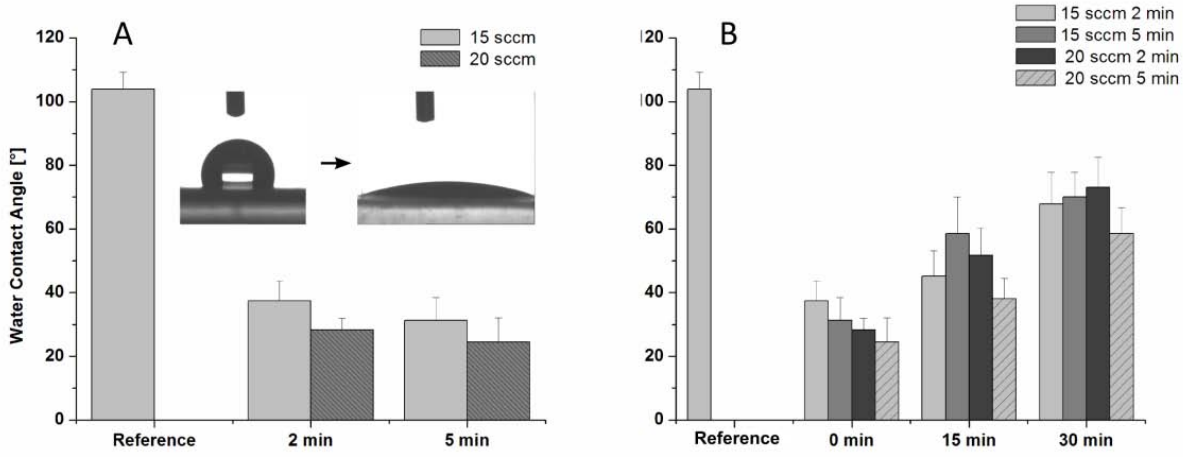

Figure 21: Oxygen plasma activation of PDMS films for enzyme immobilization (A) Change of water contact angle with exposure time and oxygen flow (inlet: photograph of a water drop on the PDMS surface before and after plasma treatment for 5 min at 20 sccm), (B) Increase of water contact angle over time after plasma treatment.

4.3.2 Silanization reaction followed by immobilization of CDH

The hydroxyl group enriched films were further treated with APTES to introduce free amino groups before grafting CDH with the help of glutaraldehyde. Figure 22 shows the changes in the FTIR spectra after APTES treatment and after the immobilization of CDH. APTES treatment lead to changes in the area of 1400-1700 cm^{-1} and 2700–3500 cm^{-1} which are both associated to amines. The ninhydrin assay performed after the APTES treatment showed the presence of amino groups on the PDMS surface. In addition, FTIR spectra showed new peaks in the areas of hydrogen bonds and amide bonds between 3000-3700 cm^{-1} and 1400-1600 cm^{-1}, respectively. FITC labelling also further confirmed successful CDH immobilization Figure 22.

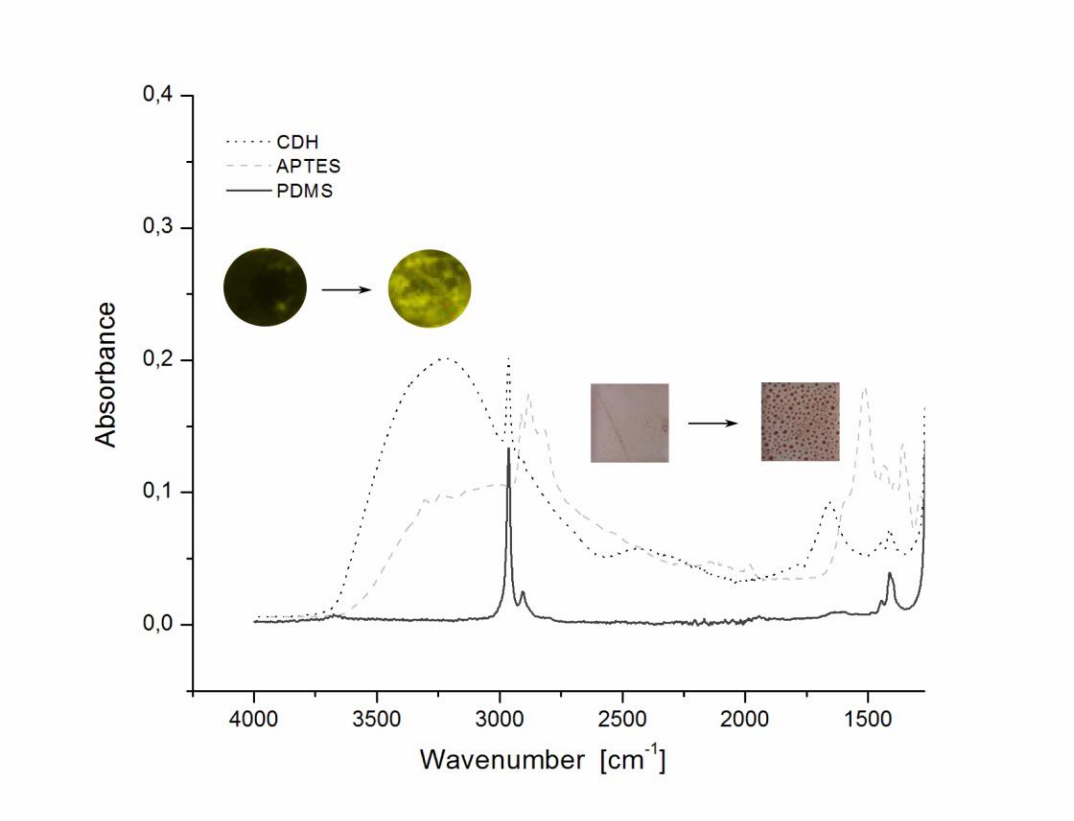

Figure 22: Immobilization of CDH on PDMS films: Changes in ATR-FTIR spectra of PDMS films after APTES treatment (light grey dashed line) and PDMS films after immobilization of CDH (dark grey dotted line) compared to untreated PDMS (black line). Inlet square shaped photographs show PDMS films before and after APTES treatment and circle shaped photographs show PDMS films before and after immobilization of FITC-CDH.

4.3.3 H_2O_2 production by immobilized CDH

The influence of different parameters on the immobilization of CDH was evaluated by measuring the H_2O_2 produced by the immobilized CDH. Results indicated that the enzyme activity was not affected by the immobilization conditions as CDH produced 0.03 µmol H_2O_2/min*mg enzyme in the presence of 200 mM cellobiose. Table 5 shows the amount of enzyme bound on the catheters and its specific activity.

Table 5: Specific activities of CDH before and after immobilization on PDMS films

	Enzyme activity [µmol H_2O_2/min]	Protein concentration [mg/4 mL]	Specific activity [U/mg]
Before immobilization t0	16.6	3.0	0.02
After immobilization t1	13.9	2.5	0.02
Immobilized Enzyme	3.9	0.125 mg per sheet	0.03

Out of all parameters tested, the pH value of the buffer used had the strongest influence on the final H_2O_2 production capacity of the surface, with pH 8 yielding the highest amount 5.9 ± 0.3 µM H_2O_2 (Table 6).

Table 6: Amount of H_2O_2 produced using different immobilization buffers with varying pH

pH of buffer used	µM H2O2 produced
pH 5.5	3.3 ± 1.4
pH 6.0	2.3 ± 0.4
pH 7.3	2.3 ± 1.1
pH 8.0	5.9 ± 0.3

As neither the increase of enzyme concentration from 0.5 to 7 mg/mL nor carrying out the immobilization at 4°C did show any differences in the amount of H_2O_2 produced by the surface the lowest enzyme concentration (0.5 mg/mL) and the simplest reaction condition (RT) was selected.

4.3.4 Effect of urine on CDH activity and stability

The long-term stability of the immobilized CDH was studied in AUM at 37°C. As shown in Figure 23 residual activity CDH on PDMS measured over 16 days shows a significant drop (~40 %) during the first two days followed by a slow decrease during further incubation retaining 35% and 20% of activity on day 7 and after 16 days, respectively. Protein concentration and enzyme activity measurements in the solution did not demonstrate enzyme leaching, indicating that the enzyme activity loss was due to inactivation of CDH over time.

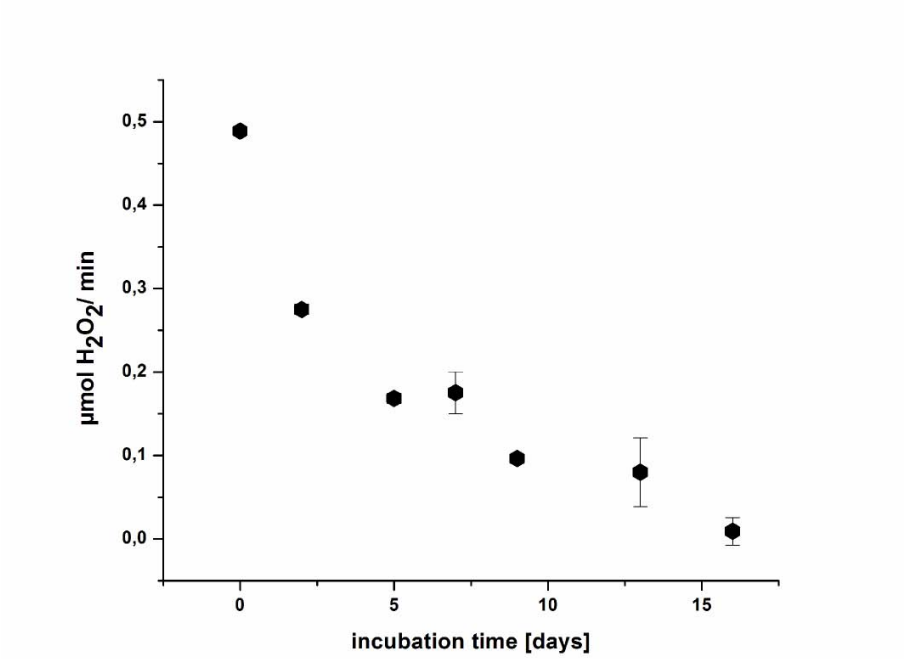

Figure 23: Residual enzyme activity [µmol H_2O_2/min] of CDH immobilized on PDMS films incubated at 37 °C in artificial urine for 16 days

4.3.5 Antimicrobial and antibiofilm activity of immobilized CDH

The potential of the immobilized enzyme to inhibit bacterial biofilm formation was assessed under static and dynamic conditions. Under static conditions CDH functionalized PDMS films reduced the amount of viable bacteria by 60 % and the total biomass deposited on the surface by 30 % as compared to pristine PDMS films (Figure 24).

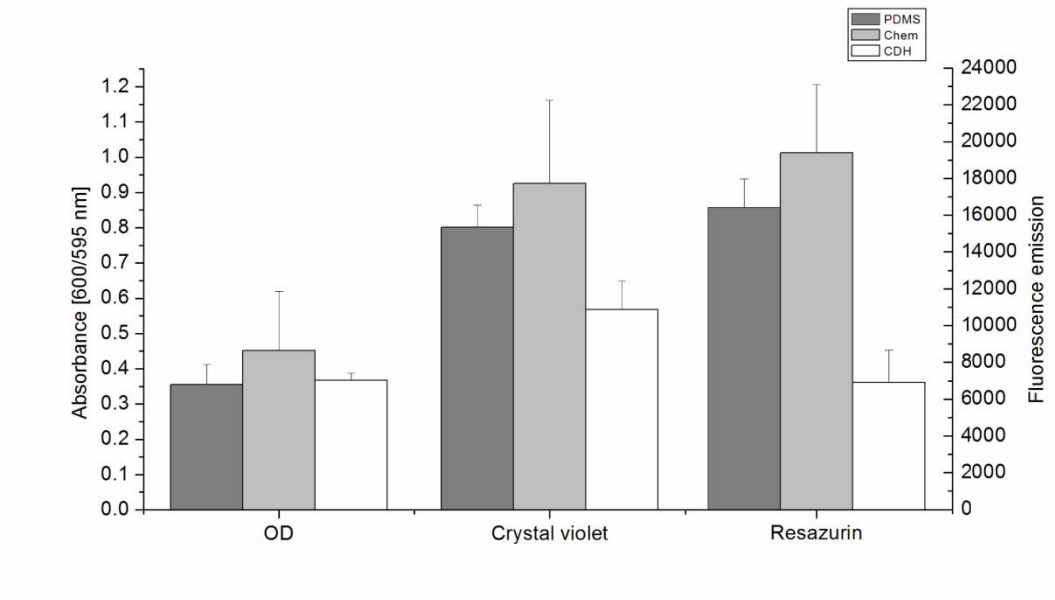

Figure 24: Influence of surface modification of PDMS films (PDMS – dark grey, PDMS treated with immobilization reagents – light grey, PDMS with immobilized CDH – white) on planktonic bacteria growth (OD), amount of viable cells present on the surface (Resazurin) and total biomass deposited on the surface (crystal violet) after 3 h of incubation at 37°C in presence of *S. aureus*

The ability of CDH coated PDMS films to inhibit *S. aureus* biofilm formation were further investigated under dynamic conditions using an *in vitro* catheterized bladder model. The total biomass on CDH coated silicone catheters was reduced by 70% on the catheter balloon (Figure 25A). The fluorescence images in Figure 25B show the distribution of live and dead cells on the surface of the balloon. Pristine PDMS was completely covered by the *S. aureus* biofilm whereas the CDH-coated PDMS was only populated by few *S. aureus* colonies of which the majority were red fluorescence stained, indicating that they were dead.

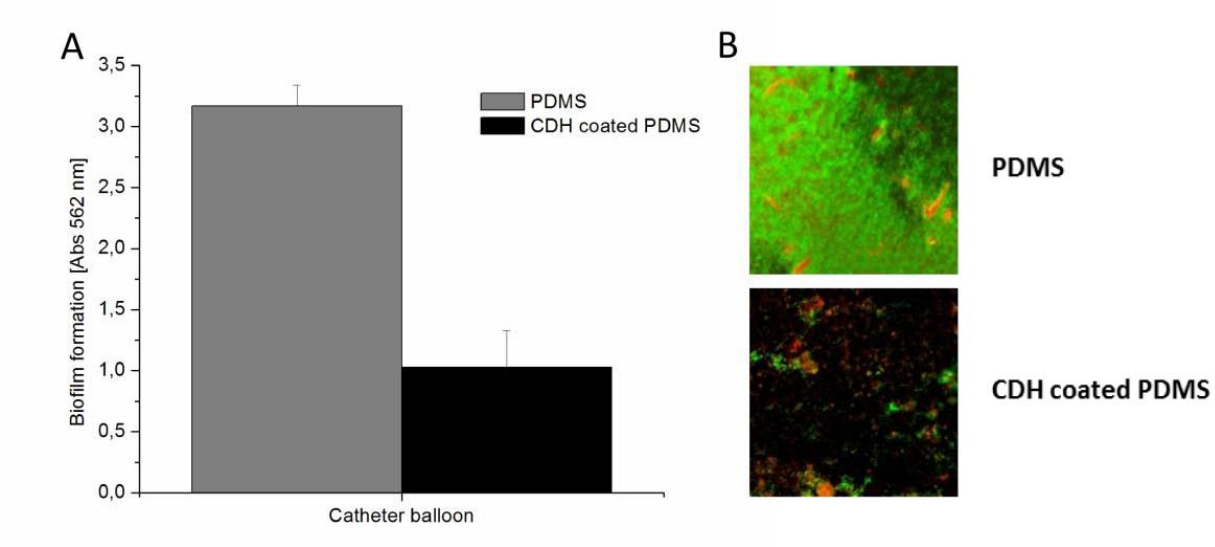

Figure 25: *S. aureus* biofilm inhibition on CDH-embedded urinary catheters incubated under dynamic conditions in artificial urine. (A) total biomass assessed using crystal violet and (B) live/dead bacteria visualized using the LIVE/DEAD cell viability kit.

AFM image analysis of the CDH-coated samples revealed a uniform coating with the exception of irregular furrows with edges decorated by globular spots presumably being enzyme accumulation sites (Figure 25B). The amount of *S. aureu*s bacteria deposited on the surface was reduced on CDH coated PDMS (25D) as compared to uncoated PDMS (25C). Interestingly, the few bacterial cells growing on CDH coated PDMS were smaller than those growing on untreated PDMS (Figure 26D).

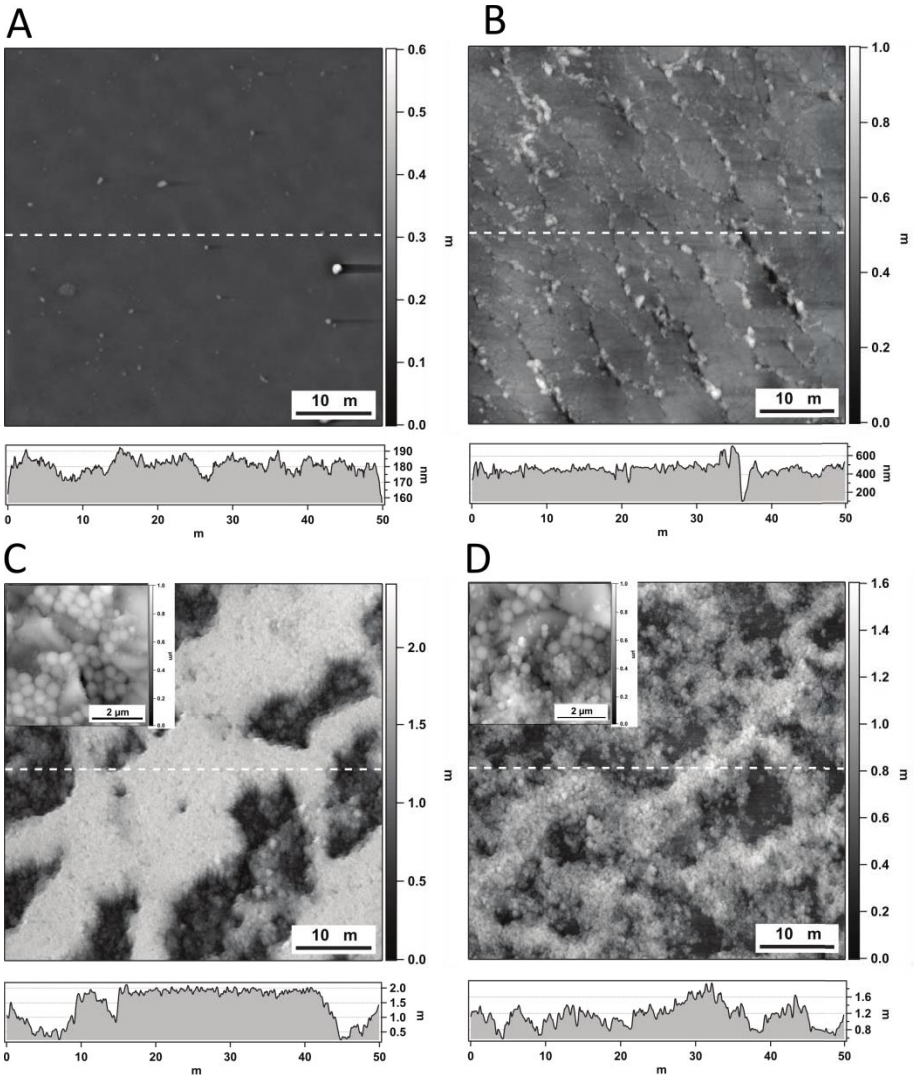

Figure 26: Three dimensional AFM reconstruction with roughness analysis of pristine PDMS film surfaces (A) untreated, (B) CDH modified and the same surfaces after incubation with 5*10^6 CFU/mL *S. aureus* for 3 h (C) and (D), respectively. Representative hight profiles corresponding to the dotted line in the middle of every image are shown below the images. Images C and D contain inlets of higher magnification showing the morphology of the bacteria present on the surface.

4.3.6 **Biocompatibility of free and immobilized CDH in presence of different cellobiose concentrations**

The effect of CDH-functionalized catheters cell viability was investigated using HEK 239 and RAW 264,7 mammalian cell lines. In Figure 27, the cellobiose dependent growth inhibition of *S. aureus* [6] was compared with the cellobiose dependent toxicity effect on the mammalian cell lines. The free enzyme completely inhibits the growth of *S. aureus* at a cellobiose concentration lower than 1 mM, concentrations which did not affect the growth of mammalian cells. Even preliminary incubation of the free CDH in the presence of 2 mM cellobiose did not affect the growth of the cells and their physiological processes as evidenced by the absorption values of the MTT assay being greater than 0.4. Incubation of

PDMS films coated with CDH showed that the coating itself was biocompatible as no change in the growth behavior of mammalian cells was detected.

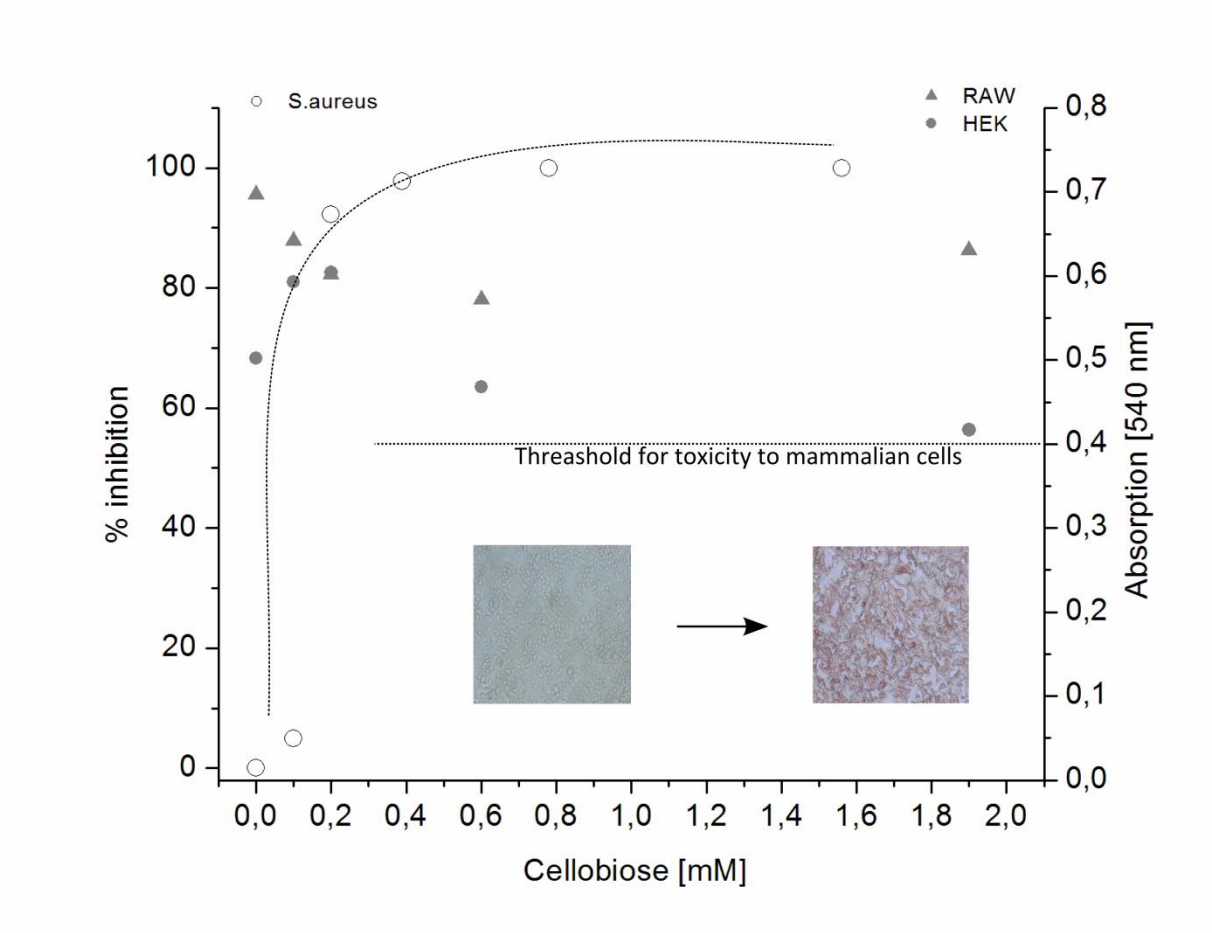

Figure 27: Comparison of cellobiose dependent inhibition of *S. aureus* and cellobiose dependent toxicity of CDH against the mammalian cell lines RAW 264,7 and HEK 239

4.4 Discussion

In this study, a novel enzyme based antimicrobial system for urinary catheters was investigated. Short time plasma treatments were able to effectively convert methyl groups on PDMS surfaces into hydroxyl groups as confirmed by the increasing hydrophilicity and decrease in water contact angle from 104° to 24.5°. Treatment of PDMS surfaces with oxygen plasma is known to produce a reactive surface. The loss of the reactive surface due to the migration of molecules inside the polymer was prevented by extensive washing before exposure to plasma [19, 20]. The generated hydroxyl groups were used to form Si-O-Si bonds with APTES thereby yielding an amino rich surface for grafting glutaraldehyde [21] and subsequent binding of CDH. Immobilization at pH 8 lead to the highest H_2O_2 production capacity (5.9 µM) by immobilized CDH. This is probably due to the fact that amino groups on the surface of CDH were not protonated at this pH leading to a high immobilization efficiency [22]. In our previous studies, *in situ* production of H_2O_2 by CDH, using both cellobiose and bacterial exPS as substrate, had a broad spectrum antimicrobial and antibiofilm activity against microorganisms colonizing catheters even in the <700 µM concentration range including the gram negative species *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* and the gram positive bacteria *Enterococcus faecalis and Staphylococcus epidermidis* [6]. PDMS grafted CDH retained its activity during the immobilization process and was able to generate H_2O_2 in the vicinity of the catheter surface thereby preventing the bacterial biofilm formation and killing bacteria on the catheter surface [23]. The fact that CDH retained considerable activity over a long period (16 days) in artificial urine at 37 °C, shows that the enzyme would considerably increase the life span of the catheter in the patient. Further robustness of the system is evident from the ability of CDH-modified PDMS to show considerable antimicrobial activity. In detail it caused >60 % biofilm inhibition and reduced the amount of viable *S. aureus* by 60 %. Moreover the total biomass deposited on the surface was reduced by 30 % during short and long term studies both under static or dynamic conditions. The strong inhibition observed after 3h of incubation with *S. aureus* proved that this novel coating is already effective throughout the early, very critical stages of biofilm formation [24]. The observed smaller bacterial cells on CDH-coated surface PDMS may be attributed to previously reported oxidative stress [25]. Results of the long term studies suggest that the coating is able to maintain its antibacterial and antibiofilm activity over longer periods. Interestingly, under dynamic conditions the antibacterial efficiency of the CDH coated catheter was most pronounced in the area of the balloon, the part which is primarily the target of microbial colonization in the bladder [2]. The positive results of this current study against bacterial biofilms in combination with the broad antimicrobial activity shown of the free CDH in previous studies proves the effectiveness of this novel system against CAUTIs [26]. Unlike Lysozyme and Subtilisin A which rely on directly attacking the

bacterial cell wall [27, 28], the CDH system produces H_2O_2 which can diffuse and kill bacteria in the surrounding environment. This ability makes CDH functionalized catheters an effective antibiofilm and antimicrobial system thereby preventing infection of the patient. The slow release of H_2O_2 within the 0- 2 mM range [6], did not have cytotoxic effects on HEK 239 and RAW 264.7 cells although effective as antibiofilm and antimicrobial agent. This shows that the slow release of H_2O_2 does not produce cytotoxicity effects thereby contradicting reports that H_2O_2 between 15 and 1500 µM are cytotoxic [29]. Mammalian cells have the capacity to partially remove extracellular H_2O_2 from the culture medium [30] suggesting that they are more resistant to H_2O_2 produced over time than being exposed to high doses at once. In summary, this study shows that CDH is an attractive alternative antibiofilm and antibacterial system for urinary catheters as well as many other biomaterials.

4.5 Conclusion

An immobilization strategy for the covalent grafting of CDH on PDMS surfaces, an enzyme able to produce H_2O_2 from cellobiose and bacterial exPS was successfully developed. The CDH grafted PDMS surfaces were effective in reducing viable bacteria cells and biofilm formation under both static and dynamic conditions by 60 and 70 % respectively. The enzyme retained 20% of its original activity without leaching after 16 days of incubation in artificial urine. Interestingly, CDH functionalized catheters did not affect the growth of the HEK 239 and RAW 264.7 mammalian cell lines. Thus, using CDH covalently bonded onto urinary catheters could be a viable alternative for developing an antibacterial and antibiofilm coatings.

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4.6 References

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5

Ultrasound coating of polydimethylsiloxanes with antimicrobial enzymes

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Abstract

There is an urgent need for antimicrobial functionalization of urinary catheters to prevent their colonization by microbes and biofilm formation. Here, the antimicrobial hydrogen peroxide (H_2O_2) producing enzyme cellobiose dehydrogenase (CDH) was for the first time grafted onto polydimethylsiloxanes (PDMS) using an ultrasound assisted coating method. This resulted in the development of an effective *in-situ* continous H_2O_2 producing system able to continually prevent microbial colonization and biofilm formation on catheters. This enzyme has an added advantage that it uses various oligosaccharides including expolysaccharides (an important part of the bioflim produced by the microbes while colonizing biomaterials) as electron donors to produce H_2O_2. Successful immobilization of active CDH nanoparticles on PDMS was confirmed by ESEM and AFM analysis as well as quantification of H_2O_2. Depending on the initial enzyme concentration, CDH-nanoparticles of varying sizes from 65±17nm to 93±17nm were created by the ultrasonic waves and subsequently deposited on the PDMS surface. PDMS sheets treated for 3 min produced 18 µM of H_2O_2 within 2 hours which was sufficient to significantly reduce the amount of viable *S. aureus* cells as well as the total amount of biomass deposited on the surface. The ultrasound assisted coating of antimicrobial enzymes therefore provides an easy approach to immobilize enzymes and create a surface with antimicrobial properties.

5.1 Introduction

Microbial biofilms are a major source of infections accounting for >80% of nosocomial infections in hospitals and are the cause of many indwelling medical device associated infections e.g. in the urinary tract [1]. Catheter associated urinary tract infections (CAUTI) are frequently occurring healthcare associated infections which are caused by biofilm forming pathogens colonizing the catheter surfaces [2, 3]. Enzymes are routinely used in medicine, for example for the treatment of lysosomal storage diseases [4],in addition some enzymes are increasingly being exploited for developing antibiofilm and antimicrobial agents[5]. Compared to free enzymes, immobilized enzymes are more stable and enhance their effect in-situ [6, 7]. Consequently, grafting of enzymes to surfaces facilititates their application (continuous processes with enzyme recycling, rapid termination of reactions, and greater variety of bioreactor designs) [8-11]. Moreover, immobilization of enzymes opens new frontiers in their therapeutic applications. Recently, a novel subcutaneous glucose sensor was suggested employing an immobilized enzyme on an electrode [12].There are several techniques for enzyme immobilization including the well-established methods physical adsorption of enzymes, covalent binding, and entrapment of enzymes in a growing polymer [13-15]. The sonochemical process was found to be a very efficient method of synthesizing and simultaneously depositing inorganic nanoparticles (NPs) on a wide variety of surfaces such as fabrics [16], fibers and polymers [17, 18]. Only recently, the sonochemical technique was found to be very efficient in formation of enzymes nanoparticles from their solution [19, 20]. Cellobiose dehydrogenase from *Myriococcum thermophilum* is an oxidoreductase oxidizing cellobiose and a wide variety of structurally different cellooligosaccharides with oxygen as electron acceptor leading to the generation of hydrogen peroxide (H_2O_2) [21, 22]. The strong oxidative properties of H_2O_2 lead to bleaching applications of fibers [23] while the antimicrobial effect was recently exploited for the develpoment of wound dressing hydrogels [5, 24, 25]. H_2O_2 in low concentrations is widely used as antimicrobial agent and disinfectant in both medical and food related sectors [26, 27]. One big advantages of using CDH over pure H_2O_2 is the *in-situ* /on-demand production of the oxidative agent which is otherwise known to have a low stability when kept in solution [28]. Immobilization and consequently the possibility of reusing the enzyme and/ or avoid leaching to the process or human body would make it even more interesting for industrial applications. CDH is very temperature stable, with no measurable decrease in enzyme activity up to 70°C. Therefore, CDH is a very suitable enzyme for immobilization using a one-step sonochemical process, as elevation of temperatures is possible during this treatment. The aim of the present study was to investigate antimicrobial functionalisation of PDMS urinary catheters with NPs of CDH grafted with ultrasound.

5.2 Experimental

Media components and cellobiose were purchased from Carl ROTH (Karlsruhe, Germany). All other chemicals used were of analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany). The recombinant *Myriococcum thermophilum* cellobiose dehydrogenase (rMtCDH) was optimized for increased oxygen reactivity by a single mutation (N748G) and was produced in *Pichia pastoris* as previously described [21]. Catheters and films were provided by Degania Silicone Ltd (Israel). *Staphylococcus aureus* ATCC 25923 was obtained from the culture collection of the Institute of Environmental Biotechnology (Graz, Austria).

5.2.1 **Immobilization of CDH on PDMS surface**

A typical reaction for the preparation of CDH coated PDMS sheets was carried out as follows: Silicone sheet (1x1cm^2) was placed in a beaker containing the enzyme solution (30ml, 1.2mg/ml). The beaker was placed in an ice bath in order to prevent the overheating of the enzyme solution during the sonication process. The solution was irradiated for 3, 10, 15 or 30 minutes using a high intensity ultrasonic Ti-horn (Sonics and Materials, VC-600, 20 kHz, acoustic power 100 W/ cm^2, 750 W at 22% amplitude). The tip of the horn was immersed in the solution to a depth of about 10 mm. Several parameters were tested for their effect on immobilization efficiency including the initial enzyme concentration.

5.2.2 **Characterization**

The sonochemically-immobilized CDH - NPs on PDMS were characterized using environmental-scanning electron microscopy (ESEM, Quanta FEG, FEI), and Atomic Force Microscopy (AFM) with a Nanoscope V Multimode scanning probe microscope (Digital instruments, Santa Barbara, CA).

5.2.3 **Water Contact angle (WCA) measurements**

The changes in water contact angle of the ultrasound coated silicone sheets as compared to the untreated silicone sheets was assessed as described by Gindl- Altmutter et al. [29]. Briefly, the WCA measurements were based on the sessile drop method (Scheikl and Dunky 1998) and determined by means of a 2/3″ CCD camera device (Sony 93D model XC-77CE, Tokyo, Japan), which was adjusted against background light. Specimens were fixed on a moveable table and drops applied using a manual dosage system (canula, 1 ml syringe, µm screw driven dosage). Drop shape analysis was performed by the Krüss DSA 1 (Krüss

GmbH, Hamburg, Germany) software 20 s after drop application. WCA values mean contact angle of 10 measurements per surface.

5.2.4 **Quantification and determination of enzyme activity of the immobilized enzyme**

The enzyme in solution and the enzyme deposited on the silicone surface were quantified using BCA Protein Assay Kit (Pierce, Rockford, USA) with slight modifications. The coated silicone sheets were incubated in 2 ml of working reagent for 30 minutes at 37°C to allow color formation. Then, 200 µl were transferred into 96-well plates and absorbance measured at 562 nm. A calibration curve was recorded using bovine serum albumin (BSA) in concentrations ranging from 0 – 2 mg/mL as a standard. The obtained values were calculated as mg/plate protein . H_2O_2 production capacity of the immobilized CDH was assessed by the Amplex Red Assay with some modifications using cellobiose as substrate. The CDH - PDMS films were incubated in a solution containing 425 µL 0.25 M sodium phosphate (Na_2HPO4/NaH_2PO4) buffer pH 7.4 and 75 µL 200 mM cellobiose at 37 °C for 2h at 600 rpm to allow H_2O_2 production. After incubation, 197.5 µL of the sample were transferred into a black 96-well plate (Sarstedt, Germany) and 0.5 µL Amplex Red and 2 µL of horseradish peroxidase added. Fluorescence was monitored on a microplate reader (Tecan Infinite M200 Pro Platereader, Switzerland) with excitation and emission wavelengths set at 550 and at 587 nm (Ex/Em 550/587 nm), respectively. Fluorescence data were converted to H_2O_2 concentrations by using a calibration curve recoreded with various dilutions of a 30 % H_2O_2 stock solution yielding 0 – 15 µM H_2O_2 in the assay. Pristine PDMS films and films treated only with the immobilization reagents served as references. All experiments were performed in triplicates with sheets deriving from three indepent experiments. The measured values were used to calculate the average and the standard deviation.

5.3 Antimicrobial activity of CDH deposited on PDMS

The antimicrobial activity of CDH coated silicone sheets was investigated by incubation with *S. aureus* in the presence of cellobiose (final concentration 25 mM) in a 24-well plate (Sarstedt, Germany). Briefly, the media containing tryptic soy broth supplemented with 0.5 % (w/w) glucose (TSB+) inoculated with actively growing bacteria (final OD600 0.1). The sheets were incubated for 3 hours at 37 °C without shaking. After incubation, non-attached cells were removed by washing them three times with 0.9% NaCl. Untreated PDMS sheets treated in the same way as the samples served as control. The sheets were stained with the LIVE/DEAD cell viability assay (Life technologies, Carlsbad, USA) for one hour and bacteria visualized using a FluoView 1200 Confocal Laser Scanning microscope (CLSM) from Olympus (Pennsylvania, USA).

5.4 Results and Discussion

5.4.1 **Characterization of CDH immobilized on PDMS surfaces**

The immobilization of the CDH-NPs on PDMS silicone sheets was confirmed by environmental scanning electron microscope (E-SEM) investigations. Figure 28b illustrates the morphology of the PDMS surfaces coated from the low concentration solution of 0.13 mg/ml. When the concentration of CDH in the starting solution was increased from 0.13 mg/ml to 0.52 mg/ml, the amount of CDH particles on the surface increased creating a homogenous coating of PDMS with CDH-NPs (Figure 28c). The mean particle size and the distribution of the particle sizes was also affected by changing the initial CDH concentration (Figure 28d, e). Spherical particles of 65±17 nm were formed from 0.13 mg/ml CDH while from 0.52 mg/ml CDH an increase of the particle size to 93±17nm was measured. When 1.2 mg/ml CDH was used and the reaction time reduced from 30 to 3 min to lower the heating effect, particles of 75.2± 10.4 nm were obtained (As seen in the TEM image, Figure 29). These findings are in agreement with previous studies where the size of the nanoparticles formed also depended on the initial enzyme concentration (i.e. higher concentration of enzyme, led to the fomation of larger nanoparticles) [19].

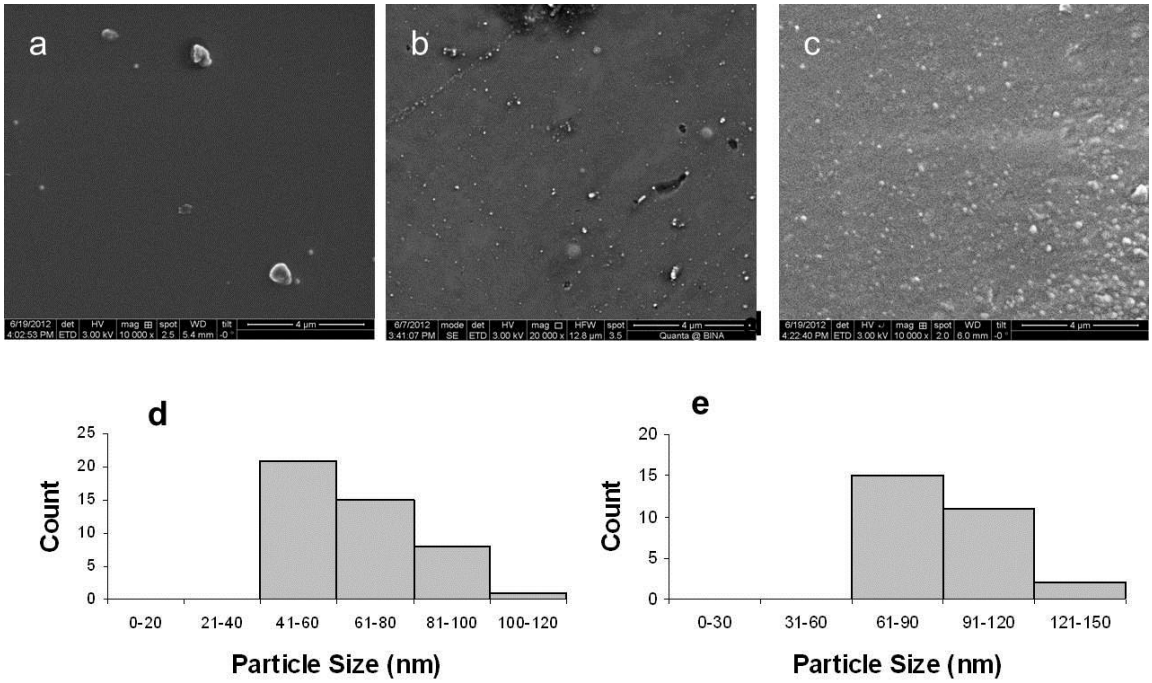


Figure 28: ESEM images of a) pristine PDMS surface, b) PDMS coated with 0.13 mg/ml CDH; c) PDMS coated 0.52 mg/ml CDH; d) and e) histograms of particle size distribution created from 0.13 mg/ml and 0.52 mg/ml CDH, respectively.

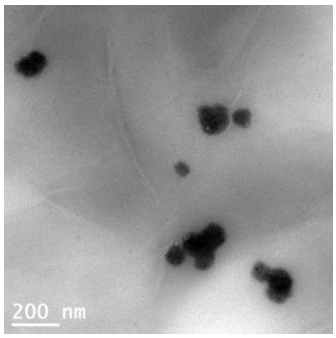
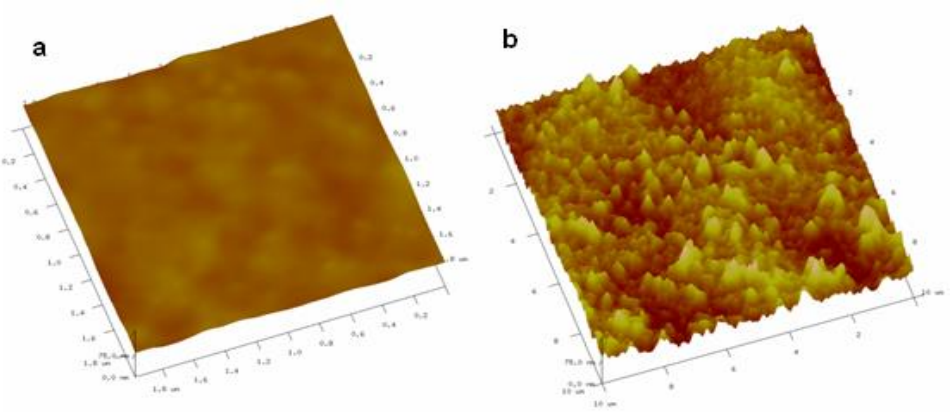


Figure 29: Enzymes NP in solution

AFM inspection of the topography of the PDMS indicates complete coating with enzyme nanoparticles (Figure 30b). The surface roughness at the nanometer scale was significantly altered upon the deposition of enzyme NPs on the PDMS surface. Root Mean Square (RMS) analysis by the nanoscop software revealed a more than 5 fold increase in surface roughness by comparing the pristine and coated surfaces.



RMS (Rq)	Uncoated	CDH Coated
Average	2.03 nm	13.8 nm

Figure 30: Atomic force microscopy (AFM) images and roughness of (a) pristine PDMS silicone sheet and (b) ultrasound coated CDH NPs.

Previously, deposition of a large variety of inorganic nanoparticles on different substrates was achieved using the sonochemical method. The sonochemical mechanism by which the nanoparticles were deposited on the substrates was discussed and is related to the creation of microjets and shock waves as the after effects of cavitation [16]. In the current case, when high-intensity ultrasound is applied to the aqueous solution of CDH, we hypothesize that the molecules of CDH are adsorbed on the formed acoustic bubbles. When the cavity collapses, the absorbed molecules are exposed to extreme, localized conditions of temperature and pressure. As a consequence, these molecules impact on each other and nanoparticles of the

organic compound are produced. Cavitation occurs not only in liquid, but also in a solid-liquid interface, i.e. near polymer surface, due to increased nucleation rate in presence of a solid substrate [30]. After the bubbles collapse, high-speed jets of the liquid transport the generated nanoparticles at high speed toward the PDMS surface where they remain strongly embedded.

5.4.2 Water contact angle measurements

Contact angle measurements of pristine PDMS and PDMS sheets treated with solutions containing 0.13, 0.52 or 1.2 mg/ml CDH were performed. PDMS is a very hydrophobic material with a contact angle of 106.7 ± 2.1°. When treating the PDMS sheet with a solution containing 0.13 mg/ml of CDH the amount of CDH deposited was not high enough to significantly change the WCA, as it measured to be 108,7±3°. A decrease in WCA was noted for the PDMS films treated with higher concentrations of CDH leading to angles of 92.6 ± 3.4° and 88.3 ± 7.3°, respectively (Figure 31).

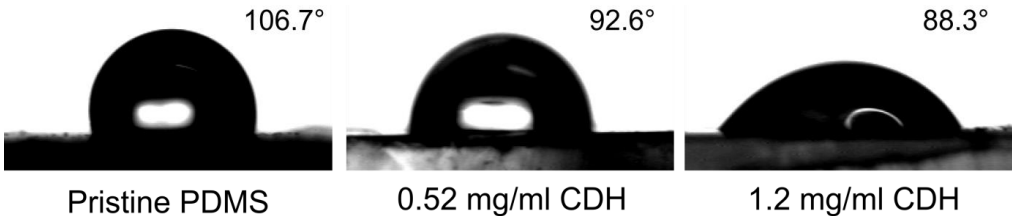


Figure 31: WCA analysis of pristine PDMS films and sonicated in CDH solutions with different concentrations

The WCA measurements demonstrate again that the amount of CDH deposited on PDMS depends on the concentration of the starting solution confirming the findings above and previous data for ultrasound coating of α-amylase nanoparticles on polyethylene films [19]. The biggest advantage of this one step immobilization strategy over conventional immobilization methods is the short amount of time needed to achieve a coated surface as well as the unnecessity of activation of the immobilizeation support and toxic coupling reagents.

5.4.3 Analysis of immobilized enzyme: Influence of initial concentration and sonication duration on immobilization

Two parameters were found to have an effect on the immobilization efficiency, namely initial protein concentration and sonication duration. It can be stated, that longer sonication times have two consequences: on the one hand, the amount of enzyme deposited on the PDMS surface increases, while on the other hand the enzyme activity of the deposited CDH decreases. In detail, the immobilization efficiency calculated as fraction of the amount of protein intially in solution deposited on the PDSM film increases from 1.4 % after 3 min of sonication to 2.4% after 30 min of sonication, respectively. Interestingly, increasing the

sonication time tenfold only lead to about 50% more protein deposited while the enzyme activity almost decreased ten-fold (Figure 32). It is worth mentioning that an attempt was made to follow the leaching of the anzyme off the PDMS surface into water and saline solution. However, we could not detect any measureable amount. First because the coated amount was small and second the leaching was minimal.

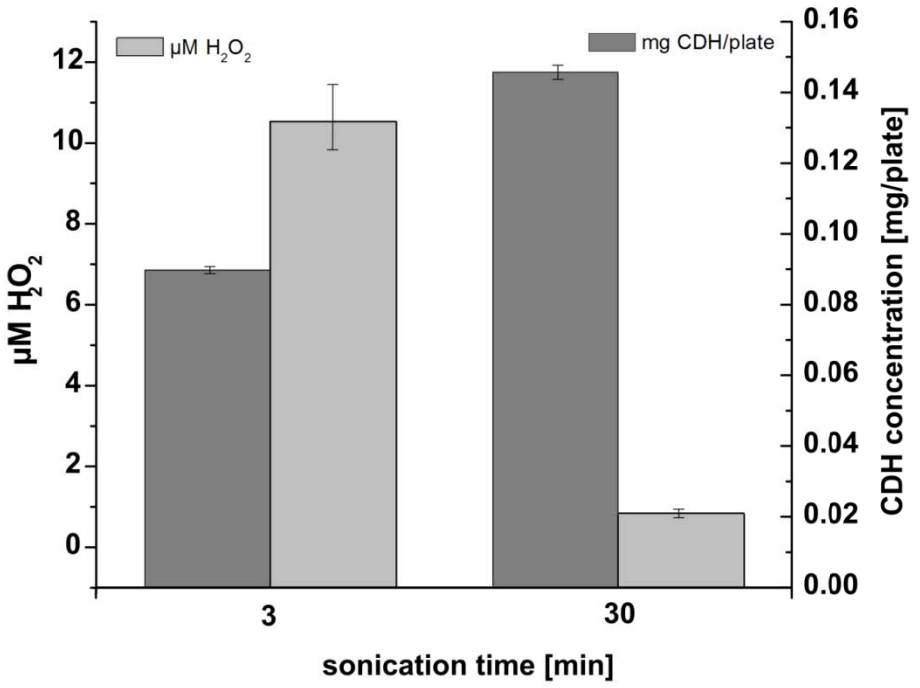


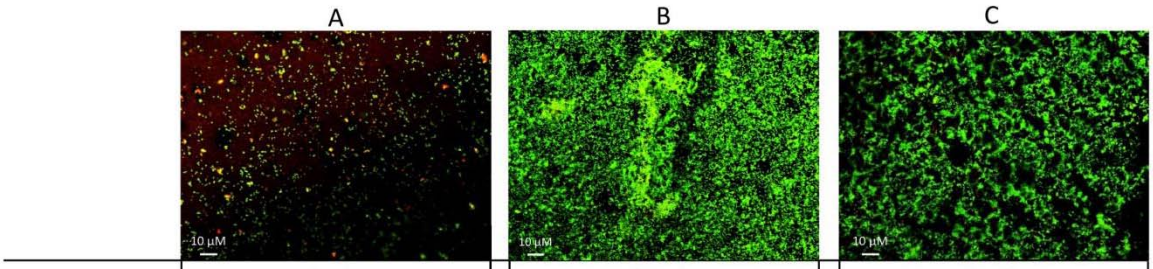
Figure 32: Effect of duration of sonication on amount of protein deposited (dark grey) and resulting enzyme activity given as H_2O_2 production (light grey) when using an initial enzyme concentration of 0.6 mg/ml

These two parameters developing in a contrary fashion over time can have multiple reasons. As temperature in the enzyme-buffer solution increase with time of the sonication treatment it was expected that CDH would lose a certain amount of its activity. Literature also reports that enzymes can undergo conformational changes when exposed to sonochemical treatment thereby decreasing the enzyme activity [31].

In order to yield a surface producing higher amounts of H_2O_2 the effect of the initial enzyme concentration was assessed. Therefore the CDH concentration was increased to 1.2 mg/ml of CDH. Indeed, after 3 minutes of sonication this led to an increase of the enzyme activity from 10.0 to 18.4 µM H_2O_2 produced within 2 hours. Again, an increase of the sonication time from 3 to 15 minutes reduced the specific enzyme activity on the plate from 21.1 to 4.4 nmol H_2O_2/min mg protein. In contrast, 3 minutes of sonication did not signficantly change the enzyme activity when compared to the native enzyme (21.2 nmol H_2O_2/min mg protein). Based on the characterization results, the optimal conditions were determined as 3 min of sonication and an initial concentration of 1.2 mg/ml.

5.4.4 Antimicrobial activity of CDH deposited on PDMS

CDH deposited on PDMS was able to produce H_2O_2 in the µM range which, in previous studies, proved to be very effective against a wide range of clinical isolates commonly found in biofilms colonizing urinary catheters[32]. Consequently, LIVE/DEAD staining of *S. aureus* colonies attached to the surface of PDMS was performed and indeed demonstrated that deposited CDH did not only exert its antimicrobial activity but was also able to reduce the amount of cells deposited on the surface (Figure 33).



Sonication time	3 min	15 min	0 min
Specific activity	21.1	4.4	-

Figure 33: CLSM pictures of LIVE/DEAD stained *S. aureus* colonies attached to silicone sheets after sonochemical grafting of CDH for (A) 3 min, (B) 15 min and (C) positive control – untreated PDMS sheet and the corresponding specific activity of the immobilized enzyme [nmol H_2O_2/min mg protein]

Expectedly, the amount of alive bacteria (green fluorescence) increases with increasing sonication time which is concomitant to the decrease of enzyme activity and H_2O_2 formation (Figure 32 and Figure 33). However, the sample treated for 15 min does not show any difference to the control despite the fact that some H_2O_2 was measured for this sample. A possible reason, for the decreasing antimicrobial activity even though the same amount of H_2O_2 is produced by the samples sonicated for 3 min and 15 min, could be attributed to the inactive/ or only partly active enzyme deposited on the surface. This could help the bacteria with the initial adhesion and therefore cancel out the antimicrobial effect of the H_2O_2 produced.

5.5 Conclusion

An enzyme immobilization strategy for the deposition of CDH nanoparticles on PDMS surfaces using a sonochemical approach was successfully developed. ESEM analysis proved the formation of CDH nanoparticles with an average size of 93±17 nm and their deposition on PDMS sheets. Initial enzyme concentration and time of sonication had a strong influence on the amount of enzyme being deposited on the surface and its activity. Longer sonication time lead to higher amounts of enzyme deposited which, however, was partially denatured as reflected by decreasing activity. The amount of H_2O_2 produced by immobilized CDH (18 µM) was sufficient to significantly reduce the amount of viable cells and the total biomass deposited on the surface when tested against *S. aureus*. The method presented here is a fast approach to immobilize enzymes and subsequently produce antimicrobial surfaces.

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6

GENERAL CONCLUSION

The work of this thesis was embedded in the NOVO project of the seventh framework program of the European Union, which aimed at developing novel antimicrobial coatings for urinary catheters in order to prevent the formation of pathogenic bacteria biofilms on this indwelling medical device. Three approaches were pursued in the European project including coating catheters with antimicrobial active metal oxides, coating with enzymatically modified polyphenolic compounds and the immobilization of the antimicrobial enzyme cellobiose dehydrogenase (CDH) investigated in this thesis.

The recombinant version of CDH from the thermophile organism *Myriococcum thermophilum* genetically engineered to increase its affinity for oxygen and to produce high concentrations of hydrogen peroxide (H_2O_2) was chosen as a potential candidate with both antimicrobial and antibiofilm activities. As a first step, the optimal substrate concentrations (cellobiose) for CDH to exert maximum antimicrobial activity were established both in liquid medium and on agar plates. Gram positive bacteria (*Staphylococcus aureus*) proved to be more susceptible towards H_2O_2 as the apparent inhibitory concentration 50 (IC_{50}) was found to be 0.19 mM of cellobiose combined with 0.33 U of CDH in liquid growth medium. A 50 % growth reduction of the gram negative bacterium *Escherichia coli* was achieved with 0.25 mM of cellobiose whereas 0.8 mM were able to completely inhibit the growth of both organisms. On agar plates on the other hand, where the diffusion of both the substrate and the product are limited 2 mM of cellobiose were necessary to completely inhibit the growth of *E. coli* and *S. aureus*. Comparison of the effect of H_2O_2 produced *in situ* with the same

concentrations of already formed H_2O_2 on the growth of *S. aureus* biofilms proved that the antimicrobial effect of H_2O_2 was not reduced when produced by the enzyme over time.

The ability of CDH to kill or prevent the formation of biofilms was further tested with many different clinical isolates commonly colonizing catheters. The strongest antimicrobial effects (100% inhibition) were measured for *A. baumanii* and the multidrug resistant *S. aureus* (MRSA) in the presence of 10 mM cellobiose while *S. epidermidis*, *P. mirabilis* and *S. maltophilia* were also significantly inhibited at this cellobiose concentration. Only *E. faecalis* and *K. pneumoniae* did not show any signs of inhibition when confronted with the CDH/cellobiose system.

Even though the preferred substrate of CDH, cellobiose, does not enhance the growth of microorganisms, a self-sustaining system not requiring the addition of substrate would be desirable. As CDH possesses a wide substrate range, the exopolysaccharides (EPS) present in the matrix surrounding the biofilm were tested concerning their oxidation by CDH. EPS extracted from *E. coli* and *S. aureus* only led to very small quantities of H_2O_2 produced. However, when pretreated with enzymes (i.e. alpha Amylase, Endoglucanase and Mannanase) to release short-chain oligisaccharides or monosaccharides the H_2O_2 production could be boosted to levels sufficiently high to exert antimicrobial activity.

One possible approach to tackle the infection problem associated with urinary catheters is to incorporate the CDH/Amylase system in lubricant, which is commonly used to facilitate the insertion of catheters. Therefore 0.66 U of both CDH and Amylase were mixed with catheter lubricant, low concentrations of substrate added and applied to already formed *S. aureus* biofilms. Analysis of the LIVE/DEAD stained biofilms with the help of confocal laser scanning microscopy (CLSM) revealed that the enzymes incorporated into lubricant possess strong antibiofilm activity and are a promising approach for expanding the life span of urinary catheters.

In a second step, two immobilization protocols were developed in order to attach CDH to the polydimethylsiloxane urinary catheter surface. Different strategies based on covalent linkages and ultrasonic treatment were applied as they each have advantages and disadvantages compared to the other.

A 5-step immobilization protocol using the bi-functional linker glutaraldehyde was developed. Low-pressure oxygen plasma was used to introduce hydroxyl groups onto the surface, which were consequently converted to amino groups using aminopropyltriethoxysilane. Glutaraldehyde was then used to covalently link the enzyme to the polydimethylsiloxane surface. Each step was optimized and evaluated by measuring the H_2O_2 production capacity of the surface using a fluorescent assay and by using the appropriate method to detect the novel functional groups introduced (water contact angle

measurements, Fourier transformed infrared spectroscopy). In order to visualize the enzyme on the surface it was fused with fluorescein isothiocyanate prior to the immobilization process and then photographed using a fluorescent camera.

The advantage of using a covalent immobilization technique is the irreversibility of the attached enzyme even though the methods are usually more time consuming and the enzyme is at risk of losing activity throughout the procedure. In the case of CDH, it could retain its original activity as the specific activity measured to be 0.02 and 0.03 U/mg before and after immobilization, respectively. A non-leaching surface is of special interest when developing surfaces for indwelling devices, as it is not desired for the coating to end up in the human body. The novel surface proved to be very stable as no leaching could be detected when incubated in synthetic urine at 37°C for 16 days. Considerable enzymatic activity could be measured over the entire time span as the enzyme retained 20% of its original activity until the end of the experiment.

When working with the prevention of biofilm formation on surfaces there are two critical stages that need to be inhibited: the initial adhesion of bacteria to the surface and the long term antimicrobial activity of the surface/enzyme. The effect against initial adhesion was tested by incubating the surface with *S. aureus* for 3 hours under static conditions, which showed that the amount of viable cells could be reduced by 60% and the total biomass deposited was reduced by 30 % as compared to the control. A catheterized bladder model was used to assess the long-term effect (7 days) of the coating against *S. aureus* and confirmed the results of the short-term experiments, as biofilm formation was reduced by 70%.

Apart from antimicrobial activity and longevity of the coating the biocompatibility is a third very important factor to consider. No signs of toxicity could be detected when incubating HEK 239 and RAW 264.7 mammalian cell lines with the surface coating in the absence of substrate. Additionally the cell lines were incubated with a defined amount of free CDH and increasing cellobiose concentrations, which showed that substrate concentrations below 2 mM, which translates to the same amount of H_2O_2, do not adversely affect the growth. The developed coating containing CDH therefore fulfills all requirements for an antimicrobial coating that can be tested *in vitro*.

As the covalent immobilization requires a three-day procedure, a second approach was employed, which is based on the ultrasonic deposition of enzyme nanoparticles on the polydimethylsiloxane surface. This one-step procedure only requires a buffer solution containing CDH and a 3-minute high power ultrasound treatment. The thermophilic character of the enzyme enabled it to retain its original activity to a high extend despite the elevated temperatures occurring during the procedure. The successful formation of CDH-

nanoparticles with an average size of 93±17 nm and the consequent deposition on PDMS was proven using environmental scanning electron microscopy as well as atomic force microscopy. An initial CDH concentration of 1.2 mg/ml enzyme lead to PDMS films producing 21.1 nmol H_2O_2/min mg protein showing a strong antimicrobial activity against *S. aureus* proven by LIVE/DEAD staining and CLSM analysis. This approach could therefore be an interesting alternative for the creation of antimicrobial surfaces.

Cellobiose Dehydrogenase is an enzyme with a broad spectrum antimicrobial activity that can be used both for the prevention of biofilm formation as well as for the treatment of already formed biofilms. It is suitable for different types of immobilization therefore highly applicable for the creation of novel antimicrobial coatings for urinary catheters. Due to the high occurrence of CAUTI and the problems associated with antimicrobial coatings already applied in the hospital setting the novel system presented here offers a promising alternative to systems like antimicrobial silver or antibiotics.

7

APPENDIX

7.1 Publications

7.1.1 **Papers**

1. **Thallinger B.,** Brandauer B., Burger P., Sygmund C., Ludwig R., Ivanova K., Scaini D., Kun J., Burnet M., Tzanov T., Nyanhongo G.S., Guebitz G.M., 2015 Cellobiose dehydrogenase functionalized urinary catheter as novel antibofilm system. J. Biomed. Mater. Res. Part B Appl. Biomater, accepted

2. Lipovsky A**., Thallinger B.**, Perelshtein I., Nyanhongo G., Guebitz GM., Gedanken A., 2015 Ultrasound assisted coating of PDMS with H_2O_2 producing enzymes. J Mat Chem B, accepted and available online, **DOI:** 10.1039/C5TB00671F

3. **Thallinger B**., Argirova M., Lesseva M., Ludwig R., Sygmund C., Schlick A., Nyanhongo G.S., Guebitz G.M. 2014. Preventing microbial colonisation of catheters: Antimicrobial and antibiofilm activities of cellobiose dehydrogenase. Int J Antimicrob Agents, 44:402–408. **DOI:** 10.1016/j.ijantimicag.2014.06.016.

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6. Elward J.E., **Thallinger B.**, Chakraborty A., Calculation of electron-hole recombination probability using explicitly correlated Hartree-Fock method. J Chem Phys, 2012; 136(12):124105. **DOI:** 10.1063/1.3693765

7.1.2 Oral Presentations as presenting author

EMRS - Warsaw - Poland

B. Thallinger, A. Flitsch, C. Sygmund, R. Ludwig, G. S. Nyanhongo and G. M. Guebitz Novel antimicrobial and anti-biofilm system based on cellobiose dehydrogenase, EMRS Fall Meeting 2012, Warsaw, Poland

EPF – Pisa - Italy

B. Thallinger, M. Brandauer, K. Bleymaier, C. Sygmund, R. Ludwig, G.S. Nyanhongo, G.M. Guebitz Generating a new antimicrobial PDMS surface using cellobiose dehydrogenase, European Polymer Federation 2013, Pisa, Italy

IPTB - Braga – Portugal

B. Thallinger, M. Brandauer, A. Schlick, R. Ludwig, C. Sygmund, G. Nyanhongo, G. Gübitz Cellobiose Dehydrogenase – antimicrobial functionalization of polydimethylsiloxane, International Conference on Polymer and Fiber Technology 2014, Braga, Portugal

EMRS – Lille - France

B. Thallinger, M. Brandauer, A. Schlick, R. Ludwig, C. Sygmund, G. Nyanhongo, G. Gübitz Covalently bound Cellobiose Dehydrogenase creates PDMS surface with antimicrobial properties, EMRS 2015, Lille, France

7.1.3 Poster Presentations (most relevant)

ÖGMBT – Graz - Austria

Thallinger B., Schlick A., Nugroho Prasetyo E., Sygmund C., Ludwig R., Nyanhongo G.S. and Guebitz G.M. Novel anti-biofilm system based on cellobiose dehydrogenase, ÖGMBT 2012, Graz, Austria

Eurobiofilms – Ghent - Belgien

Thallinger, B., Sygmund, C., Brandauer, M., Schlick, A., Ludwig, R., Nyanhongo, G. and Gübitz, G., Immobilisation of Cellobiose Dehydrogenase as an antibiofilm agent on silicon catheters , Eurobiofilms 2013, Ghent, Belgium

FEMS – Leipzig – Germany

Thallinger, B., Sygmund, C., Ludwig, R.,Brandauer, M., Schlick, A., Nyanhongo, G. and Gübitz, G., Novel anti-biofilm system based on the degradation of Exopolysaccharides, FEMS 2013, Leipzig, Germany

ÖGMBT– Vienna - Austria

Thallinger, B., Brandauer, M., Schlick, A., Burger, P., Ludwig, R., Sygmund, C, Nyanhongo, G,S., Guebitz, G.M, Modification of PDMS with Cellobiose Dehydrogenase yielding an antimicrobial surface, ÖGMBT 2014, Vienna, Austria

ICAAR– Madrid - Spain

Thallinger, B., Schlick, A., Ludwig, R., Sygmund, C., Nyanhongo, G. and Gübitz, G., Novel enzymatic antimicrobial and anti-biofilm system, ICAAR 2014, Madrid, Spain

FEMS – Maastricht – The Netherlands

Thallinger, B., Brandauer, M., Schlick, A., Burger, P., Ludwig, R., Sygmund, C, Nyanhongo, G,S., Guebitz, G.M, Functionalization of PDMS with Cellobiose Dehydrogenase yields antibiofilm surface, FEMS 2015, Maastricht, The Netherlands

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----------THE END ----------

7.5 Statutory declaration

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

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date signature