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BIORESPONSIVE POLYMER SYSTEMS FOR MEDICAL AND TECHNICAL APPLICATIONS

DISSERTATION

zur Erlangung des akademischen Grades eines

Doktors der technischen Wissenschaften

erreicht an der

Technischen Universität Graz

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Institut für Umweltbiotechnologie

Technische Universität Graz

2012

Grundlagenforschung betreibe ich dann, wenn ich nicht weiß was ich tue!

Wernher Freiherr von Braun
(23.03.1912 - 16.06.1977)

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2 ABSTRACT

Serious problems with bacterial and fungal contamination of sensible and essential products like food packaging or devices for medical applications become more and more important. It is not possible or acceptable for our industrial society to sell spoiled goods. Whether low price products like foods or high price products, *e.g.* transfusions are controlled via best-before-dates. This simple statistical method warrants the quality of these products, provided that they are handled in the right and supposed way.

Bioresponsive polymer systems (BRPs) are designed to indicate if a product or equipment is contaminated with microorganisms or ready to use. Microorganisms, whether fungi or bacteria, produce extracellular enzymes to acquire nutrients or to be resistant against other microorganisms. These extracellular (trigger) enzymes, *e.g.* cellulases, pectinases, amylases and proteases were investigated in this work as possible markers for detection of contamination within a BRP. An extracellular enzyme is able to cause a biochemical reaction within this system on specially designed enzyme substrates. This reaction can either lead to the release of functional molecules or lead to a colour change indicating the presence of microorganisms.

A novel concept termed COMB (**C**arrier – **O**perating **M**atrix – **B**arrier) has been developed for the design of BRP based devices involving three layers. First a carrier layer, secondly an operating matrix, consisting in this work of a natural polymer like carboxymethylcellulose (CMC) or polygalacturonic acid (PGA) and finally a barrier layer to protect the system from exsiccating or physical damages. To optimise both sensitivity and stability of the operating matrix, a strategy for chemical modification was developed. Therefore methacrylic groups were successfully covalently coupled to the different polysaccharides followed by cross-linking by radical polymerisation.

To elevate the sensitivity and specificity towards an increased number of enzymes and microorganisms, a blend of at least two methacrylated biopolymers were used (CMC and PGA). The greater the variety of polymers used, the greater is the variety of trigger enzymes/microorganisms which are able to interact with the system. Therefore, the number and nature of different pathogenic or contaminating microorganisms which can be detected by a BRP device can be specifically tuned.

In these BRP based devices small molecules (dyes) and proteins (enzymes) were incorporated and controlled release upon incubation with trigger enzymes or microorganisms was shown. Incorporated enzymes were additionally modified either by coupling with methacrylic groups or

polyethylene glycol (PEG) groups. The increased size after coupling to PEG reduced the diffusion of the enzyme through the operating matrix remarkably. As a second strategy the enzymes were modified with methacrylic groups and cross-linked covalently into the polymer matrix by radical polymerisation.

“Enhanzymes” incorporated into BRPs can be activated by triggers and catalyse biotransformation of immobilised substrates within the device leading to dramatic enhancement in sensitivity for signalling. For example, this chain reaction enhances a signal up to 800 times compared to the direct release of a dye.

3 ZUSAMMENFASSUNG

Bakterielle oder pilzliche Kontaminationen von sensiblen und lebenswichtigen Produkten werden ein immer wichtigeres Thema. In der heutigen technisch hochgerüsteten Zeit kann man es sich nicht mehr leisten verdorbene Lebensmittel oder Medikamente in Umlauf zu bringen. Verfallsdaten sind sowohl bei Niedrigpreisprodukten wie Lebensmitteln als auch bei Hochpreisprodukten wie Infusionslösungen, oft die einzige Möglichkeit die Lebensspanne eines Produktes bei richtiger Lagerung anzugeben, egal ob das Produkt tatsächlich verdorben ist oder nicht.

Sensoren sollen nun mit Hilfe von Bioresponsiven Polymeren (BRP) anzeigen ob ein Produkt, womöglich schon vor dem Verfallsdatum, verdorben ist oder nicht. Mikroorganismen, wie Bakterien oder Pilze, produzieren Enzyme, die sie in ihre Umgebung abgeben, um sich mit Nährstoffen zu versorgen, oder damit mögliche Konkurrenten abzuwehren. Diese extrazellulären Enzyme werden in BRPs verwendet, um die Anwesenheit von Mikroorganismen indirekt nachzuweisen. Treffen extrazelluläre Enzyme, sogenannte Triggerenzyme, auf BRPs, beginnen sie diese zu zersetzen und damit Farbstoffe oder funktionelle Moleküle, wie Antibiotika oder modifizierte Enzyme, freizusetzen. Diese Farbstoffe können nun direkt optisch nachgewiesen werden und zeigen somit an, ob das Produkt durch Bakterien oder Pilze verdorben ist.

Im Rahmen dieser Dissertation wurden bioresponsive Polymere in einem speziellen Schichtprinzip („COMB“) assembliert und bestehen aus einer Trägerschicht (Carrier), einer operativen bioresponsiven Matrix und einer Barriereschicht die das System vor mechanischen Schäden schützt. Um die physikalischen Eigenschaften der bioresponsiven Matrix beeinflussen zu können, wurde sie chemisch modifiziert. Auf natürliche Biopolymere, wie Pektin oder Carboxymethylcellulose, wurden kovalent Methacrylatseitenketten gebunden. Diese wurden erfolgreich chemisch quervernetzt wodurch sowohl die Sensitivität (gegenüber Referenzexperimenten) wie auch Stabilität verbessert werden konnte. Um die Sensitivität gegenüber mehreren Trigger-Enzymen zu erhöhen wurden Polymergemische, bestehend aus mindestens zwei methacrylierten Biopolymeren, nämlich Carboxymethylcellulose und Polygalacturonsäure, verwendet. Dadurch ist eine Interaktion mit mehreren Enzymklassen möglich und damit auch eine Detektion von mehreren verschiedenen pathogenen Mikroorganismen in einem System.

Werden Enzyme als funktionelle Moleküle in die Matrix eingebaut, können sie ebenfalls durch Triggerenzyme mobilisiert und freigesetzt werden. Diese freigesetzten Enzyme können nun als sog. „Enhanzymes“ ihrerseits Farbreaktionen auslösen indem sie immobilisierte Substrate, z.B. direkt auf

der Trägerschicht, umsetzten. Durch diese Kettenreaktion kann die Sensitivität bis zu 800 fach verstärkt werden.

Um diese eingeschlossenen Verstärkerenzyme („Enhanzymes“) fest in der Matrix verankern zu können wurden sie ebenfalls chemisch modifiziert. Durch die Modifikation mit Polyethylenglykol konnte ihre Größe verändert und damit ihr Diffusionsverhalten beeinflusst werden. Als zweite Modifikation wurden sie wie die verwendeten Biopolymere mit Methacrylseitenketten gekoppelt. Durch radikalische Polymerisation konnten diese Enzyme nun mit der abbaubaren Matrix quervernetzt und damit kovalent gebunden werden. Diese Enzym- wie auch Polymermodifikationen verbessern die Stabilität und Sensitivität der BRPs deutlich.

4 PREAMBLE

The present doctoral thesis was written within the MacroFun Project. This project was founded as a K-project by the Comet program of the Austrian FFG and the Styrian SFG. This thesis was carried out in 2 project parts of MacroFun. P1 was focused on the investigation of enzymatic interactions and degradation of natural materials. Most of the bioresponsive materials were investigated in this project part. The second part, P2, is focused on the enzymatic interactions of biosynthetic materials, which means the enzymatic degradation process of chemically synthetic non biobased polymers, *e.g.* polyethylene terephthalates (PET). This doctoral thesis combines the knowledge and resources of P1 and P2 to investigate bioresponsive polymer systems for bacterial and fungal contaminations based on the detection of extracellular hydrolytical enzymes. These enzymes are able to diffuse trough a barrier layer into the bioresponsive system, hydrolyse the polymer matrix and induce a controlled release of functional molecules, which range from simple dyes, *e.g.* alizarin up to complex molecules like chemically modified oxidoreductases, *e.g.* methacrylated laccases from *Trametes hirsuta*.

This doctoral thesis is divided into five main chapters and based scientifically on three reviewed publications. A general summary is given after the scientific part which consists of the mentioned publications.

- **General Introduction**
- **Publication 1**
  Schneider KP, Rollett A, Wehrschuetz-Sigl E, Hasmann A, Zankel A, Muehlebach A, Guebitz GM. Bioresponsive systems based on polygalacturonate containing hydrogels.
  Enzyme and Microbial Technology 2011; 48(4-5): 312-318.
- **Publication 2**
  Schneider KP, Wehrschuetz-Sigl E, Eichhorn SJ, Hasmann A, Flock TC, Kaufmann F, Yat-Tarng, Guebitz GM. Bioresponsive systems based on cross-linked polysaccharide hydrogels.
  Process Biochemistry 2012; 47: 305-311.
- **Publication 3**
  Schneider KP, Gewessler U, Flock TC, Heinzle A, Schenk V, Sigl E, Guebitz GM. Signal enhancement in polysaccharide based sensors by incorporation of chemically modified laccase
  Accepted: New Biotechnology 2012
- **General summery and Conclusion**

Paper 1 is focused on the establishment of a methacrylated form of polygalacturonic acid (PGA) compared to the common way of hydrogel production, which means dropping a polysaccharide blend into a calcium chloride solution. Both types of hydrogels (common calcium chloride based hydrogels and novel methacrylated hydrogels) were tested in terms of hydrolysis with a commercial pectinase from *Aspergillus niger* and with a purified polygalacturonase from *Bacillus pumilus*. After successful tests with the mentioned pure enzymes, the hydrogel based bioresponsive system was investigated with microorganisms.

Paper 2 focuses on the establishment of a second bioresponsive polymer. Carboxymethyl cellulose (CMA) was modified with methacrylic groups, which showed two major benefits compared to common hydrogels. By variation of the methacrylic groups the physical density can be adjusted and optimised. An increased number of methacrylic side groups increases the stability of the hydrogel but reduces the degradability of the polymeric system. An increased density complicates the interactions between enzymes and hydrogel, therefore a good balance between stability and degradability is necessary to design a sensitive and stable BRP system. The second benefit is that methacrylated polysaccharides can be mixed in various ratios. The incorporation of a further enzymatic degradable polymer allows the usage of a second trigger enzyme. A second or third trigger enzyme increases the sensitivity against different microorganisms remarkably. Within the work of paper 2, a hydrogel composite which can be degraded by pectinases as well as cellulases could be established.

Paper 3 deals mainly with enhancement systems of enzyme based detection systems, where an immobilised laccase substrate plays a major role. A trigger enzyme releases the so-called “Enhanzymes” laccase from a bioresponsive polymer which leads to an oxidation of its immobilised substrate. A combination of immobilised substrate and operating matrix (modified CMC or PGA) in one device system allows the enhancement of a detection system up to 800 times compared to a detection device based on the controlled release of a dye molecule, like alizarin.

5 GENERAL INTRODUCTION

5.1 Background of the Project

Bacterial contamination is a major problem in a huge range of applications from packaging of food products, *e.g.* milk bottles or more expensive and complex systems like blood transfusion systems [1]. Nowadays the “best-before-date” is the most common way to avoid bacterial contamination in food products. This “best-before-date” bases on statistically data, but is not able to reflect the status of the product in an online way. Bioresponsive systems are established systems which can be implemented into packaging systems [2, 3].

Bioresponsive systems are designed to react in the presence of bacteria or fungi. This means that if bacterial contamination takes place and a critical level of microorganisms is reached, the extracellular enzymes derived from these bacteria start to react with the polymer. As a consequence, a signal indicates bacterial or fungal contamination and therefore the product must not be used any more. A large number of pathogenic organisms release extracellular enzymes to their environment to acquire nutrients [4-6]. These enzymes can be used for detection systems. So the major goal of this thesis is the development of a biosensor which is able to detect the presence of bacterial or fungal contamination in an online way.

Common and established methods to detect bacterial contamination are for example PCR based methods [7-10] with the disadvantage of being time consuming and/or expensive.

The developed bioresponsive polymer system consists mainly of well-established biopolymers like polygalacturonic acid (pectin), carboxymethyl cellulose, gum arabicum, dextran or alginates. These natural polymers are well known in food industry, where they are used as food additives. Furthermore they are non-toxic, cheap and very well characterised in literature.

5.1.1 COMB System

To realise the bioresponsive polymers, the so-called COMB system (**C**arrier layer, **O**perating **M**atrix, **B**arrier layer) was established. As drawn in Figure 1 the bioresponsive polymer system consists of three main layers.

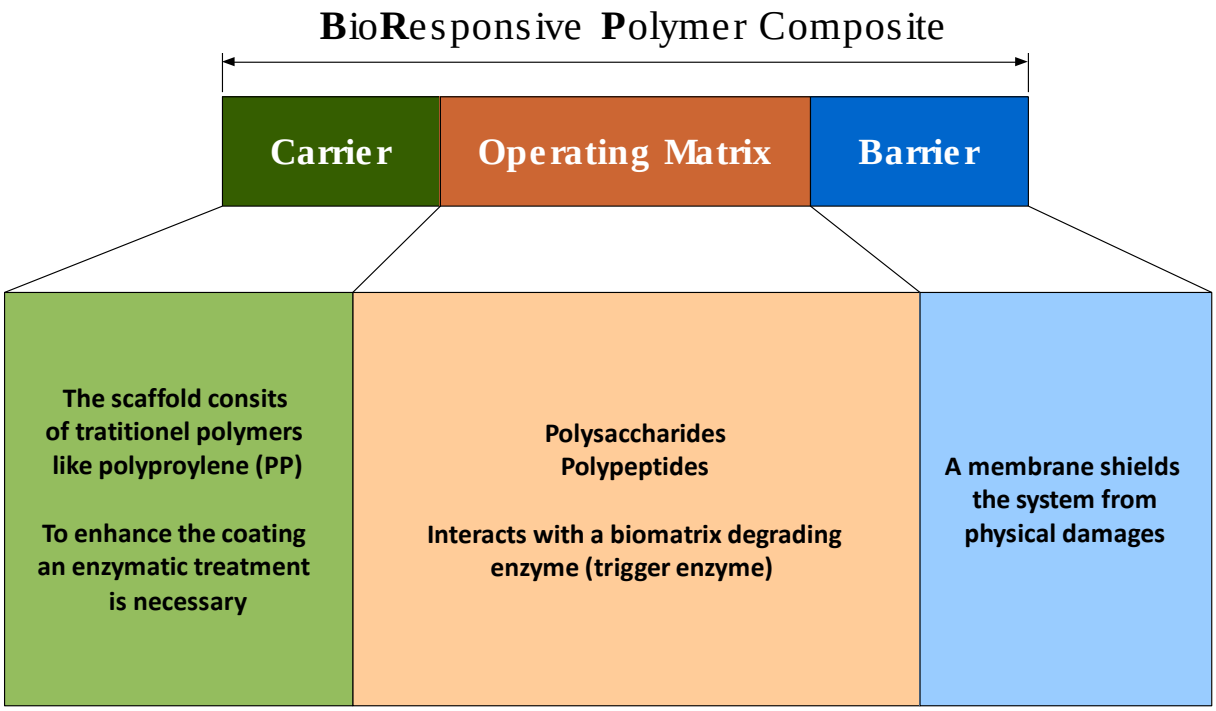

Figure 1 Schematic presentation of a bioresponsive polymer system (COMB)

The carrier consists of functionalised and stable, not enzymatic degradable, synthetic polymers, like polypropylene, polyethylene terephthalates, polyamides or silica gel coated aluminium foils. The main function is the physical stability and the possibility to immobilise dyes covalently to the surface of the carrier layer. To improve the contact between carrier layer and immobilised dye as well as the operating matrix the surface of the synthetic polymers were modified by enzymatic processes. Based on the work of Anita Eberl, Sonja Heumann, Stefan Liebminger and Eva Almansa [11-17] the number of hydroxyl groups on the surface of synthetic polymers (*e.g.* polyamide) was increased. To improve the system, enzyme substrates or dyes were bound covalently to the carrier layer. Triethoxysilane based linkers (Triethoxysilane propylamine) were used to immobilise molecules *e.g.* dyes to silica gel or glass surfaces. These materials were used as carrier layer beside enzymatic activated synthetic polymers [18].

The operating matrix consists of modified polysaccharides and is able to interact with enzymes. As mentioned before it is based on natural enzymatic degradable biopolymers. The polysaccharides were assembled as hydrogels with approximately 5% solid mass and 95% water. Hydrogels are well known in literature and can be used for enzyme substrate reactions such as gelatine, alginate, hyaluronic acid or chitosan hydrogels for wound healing [19-35].

To protect the fragile and soft operating matrix the bioresponsive polymer system was covered with an enzymatically modified PET membrane. The modification was performed similar to the

modification of the carrier layer. Beside protection of the system a membrane with defined pore size can be used to entrap dyes or enzymes within the system. This is important to protect the product, *e.g.* milk in a bottle from contaminations with dyes or enzymes.

5.1.2 COMB system for sensoring applications

Two different strategies were used as bioresponsive polymer systems. The first application, a sensoring system was established to release dyes from a modified biomatrix (Figure 2).

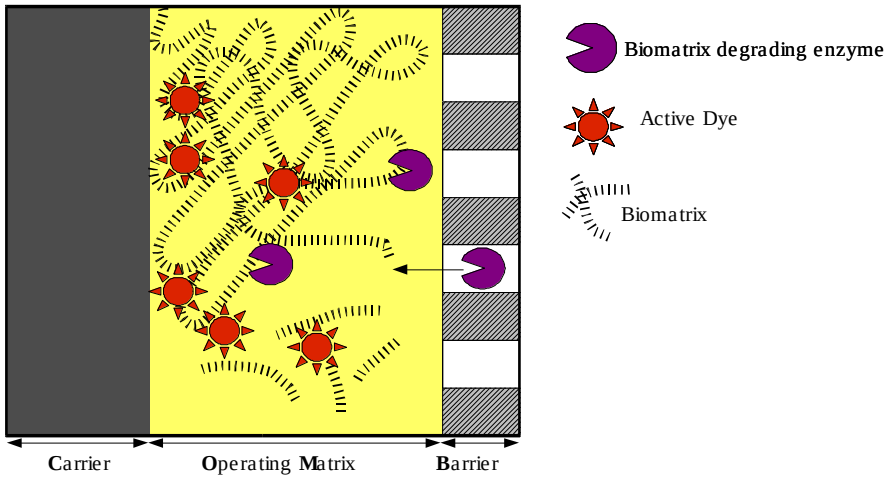

Figure 2 Bioresponsive polymer systems for sensoring by release of a dye from a bioresponsive matrix consisting of carboxymethylcellulose (CMC) or polygalacturonic acid (PG)

Extracellular enzymes are able to diffuse through the barrier layer and hydrolyse the operating matrix so that the active dye can be released.

Additionally to the release of a dye a second system was established to release complex, chemically modified molecules like methacrylated enzymes (Figure 3).

5.1.3 COMB system for the release of cross-linked modified enzymes

Compared to the release of active dye molecules the release of modified signal enhancing enzymes ("Enhanzymes") shows several pros. A schematic assembling of an Enhanzyme released by an enzyme system is shown in Figure 3.

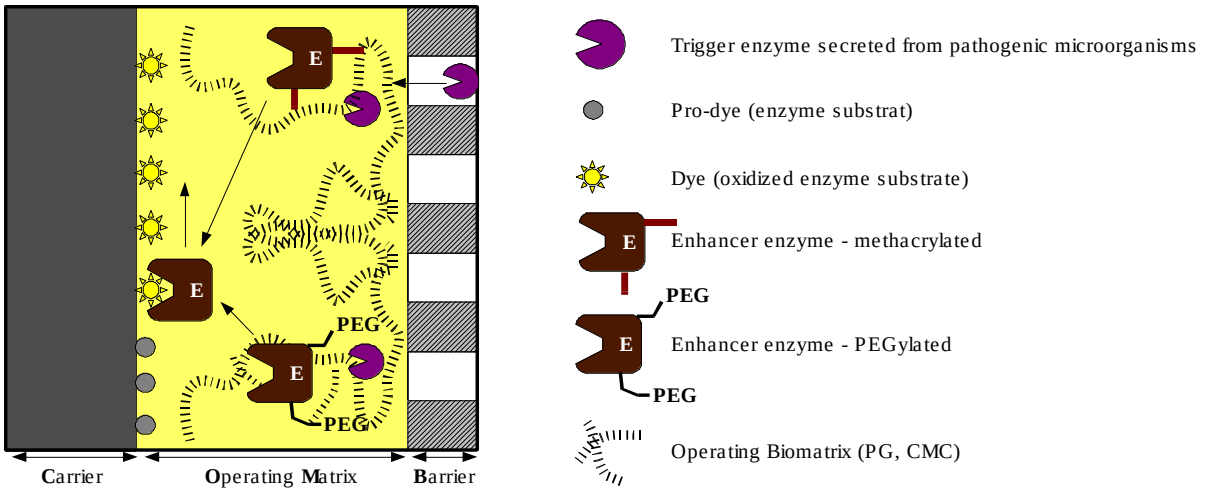

Figure 3 Controlled release of a signal-enhancing enzyme ("Enhanzyme") by an extracellular enzyme from a bioresponsive matrix consisting of carboxymethylcellulose (CMC) or polygalacturonic acid (PG)

The major benefit of a system in which an enzyme is able to release another enzyme instead of dye is the fact that an enzyme is able to catalyse a chemical reaction and is able to react with a pre dye to give a bright colour.

An extracellular enzyme is able to release several immobilised enzymes and these former immobilised enzymes are able to react several times with immobilised dyes, *e.g.* substrates for laccases. This gives an approximately 800 times higher signal compared with a simple dye release. To optimise this reaction, enzymes were modified with methacrylic linker groups which allow a covalent bond to the degradable matrix.

5.2 Used Biopolymers

Four biopolymers were used as polymeric source for bioresponsive polymer systems. Polygalacturonic acid, alginic acid and carboxymethyl cellulose are polysaccharides, the fourth used biopolymer, peptidoglycan, is a major part of bacterial cell walls.

5.2.1 Polygalacturonic acid (PGA)

The crude version of this biopolymer is also known as pectin and often used in food industry. Polygalacturonic acid is a biopolymer, found in natural sources like citrus or apple peels (approximately 20-40% in citrus and 10-20% in apple peels). Pectin can be extracted ad pH 1.5 to 3 at 60 -100 °C.

Natural PGA is a complex mixture of monomers with approximately 65% galacturonic acid subunits. Three types of monomers are important to build the pectin structure. A [1,4] bound α-D galacturonic acid (Figure 4), galacturonans with different sidechains consisting of apiose, fucose, arabinose and xylose and finally rhamanogalacturonans with a backbone of [1,4] linked subunits of α-D-galacturonic acid and [1,2] α-L rhamanogalacturonans. The rhamanose parts are linked to arabinan- and galactan-chains.

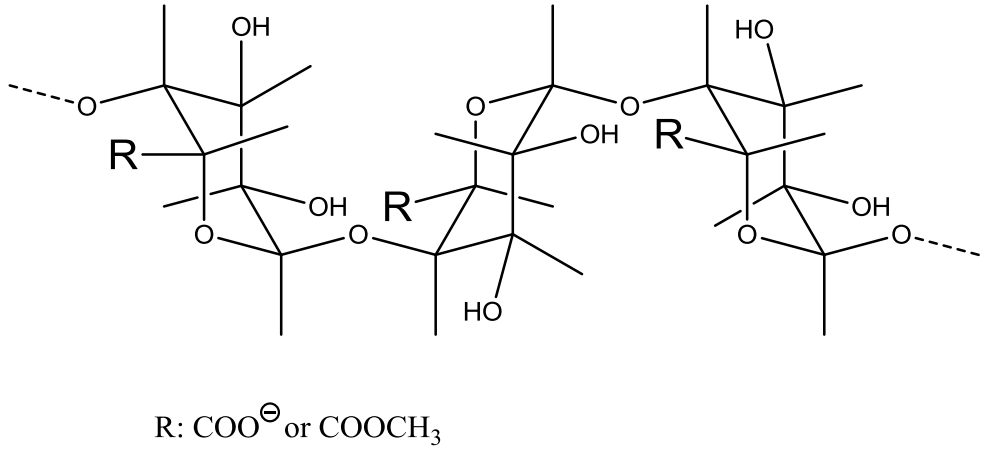

Figure 4 Chemical structure of pectin (modified from [36, 37])

The viscosity of carboxymethyl cellulose is related to the degree of esterification of the carboxylic groups of the galacturonic acid.

Beside other natural polysaccharide molecules (alginic acid or carboxymethyl cellulose) the carboxylic group of PGA can be used for gelation processes by dropping a polysaccharide solution into an aqueous calcium chloride solution. This method leads to the formation of egg-box shaped structures of the anionic polysaccharide around the cations [38-40] and is an easy way to assemble hydrogels. Unfortunately the stability of theses hydrogels is insufficient especially in aqueous environments.

To compensate the reduced stability the polysaccharide based hydrogels were chemically modified by esterification with chemical cross linkers which can be cross-linked by radical polymerisation (Figure 5). The modification based on work of J.F.A.S. Maior [41]. Glycidylmethacrylate is esterified to the free carboxylic groups of the galacturonic acid units under acidic conditions at 40°C.

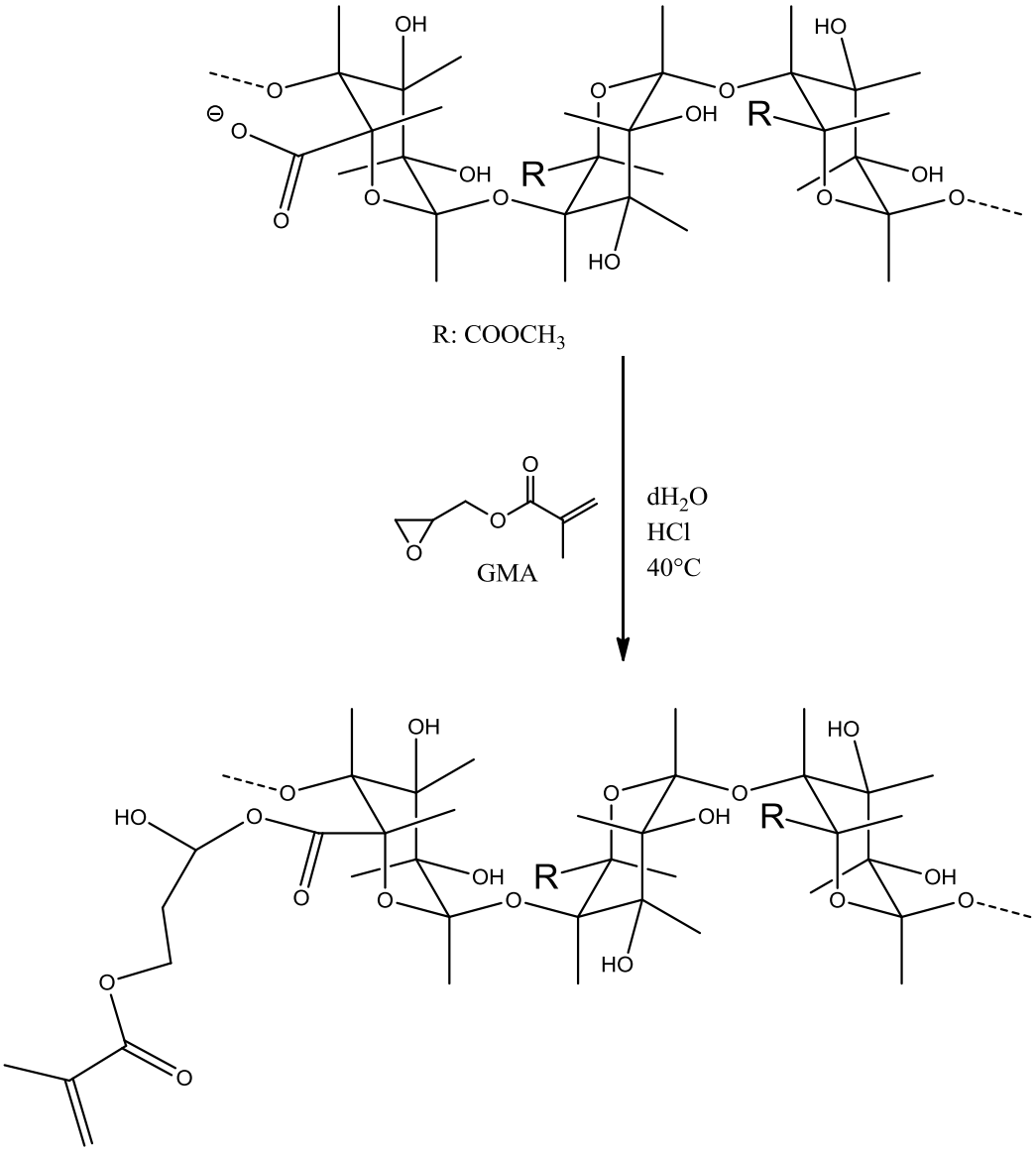

Figure 5 Chemical modification of polygalacturonic acid (Adapted from J.F.A.S. Maior et al 2008 [41])

The so formed stable hydrogel was loaded with dyes or functional molecules and was polymerised by elevated temperature or UV light irradiation.

As mentioned before, polygalacturonic acid and modified, methacrylated polygalacturonic acid are enzymatic degradable biopolymers. A range of enzymes are able to hydrolyse the polysaccharide backbone (Table 1). These enzymes can be used for selective degradation of a polygalacturonic acid containing matrix and therefore to perform a controlled release. These, via inserted methacrylic groups cross-linked polymers show similar degradability compared to gellated ones, but the stability without enzymatic activity increases remarkable.

Beside enzymatic controlled release polygalacturonic acid is often used in food industry. PGA is used for example as emulsifying agent in chocolate milk, or as thickening agent in creams, pudding or jams [42]. For the reason of its non-toxicity, cheapness and its high degree of characterisation, PGA is a good source to design bioresponsive polymer systems for application in food packages or medical devices.

5.2.2 Alginic acid

Beside pectin, alginic acid was used as a source of bioresponsive polymer systems. Compared to polygalacturonic acid, alginate shows slightly different physical properties. After blending alginic acid with pectin, the bioresponsive polymer showed an increased physical density and stability [43].

The structure of alginate is related to the structure of pectin. The basic unit is β-D-mannuronic acid linked [1,4] to α-L guluronic acid (Figure 6)

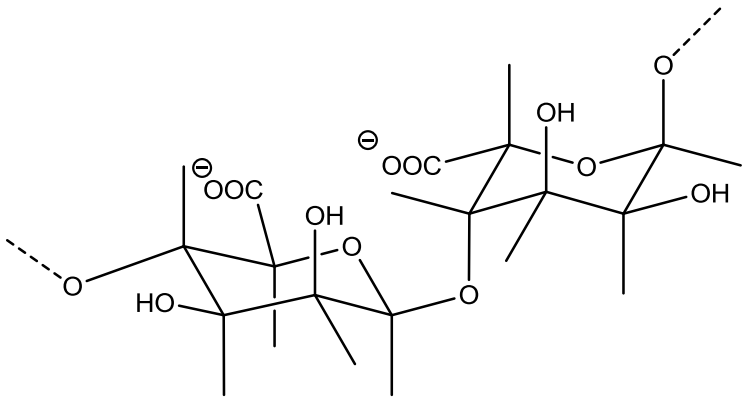

Figure 6 Chemical structure of alginate [44, 45]

Normally, the ratio between mannuric acid to guluronic acid is around 1:5. As a consequence of the carboxylic groups, alginate can build hydrogels with salt ions like Ca^{2+} [38, 39, 45]. Additionally alginate can be modified by covalent esterification similar to pectin with glycidylmethacrylate groups [41]. This reaction based on the publication of J.F.A.S. Maior et al is drawn in Figure 7 [41].

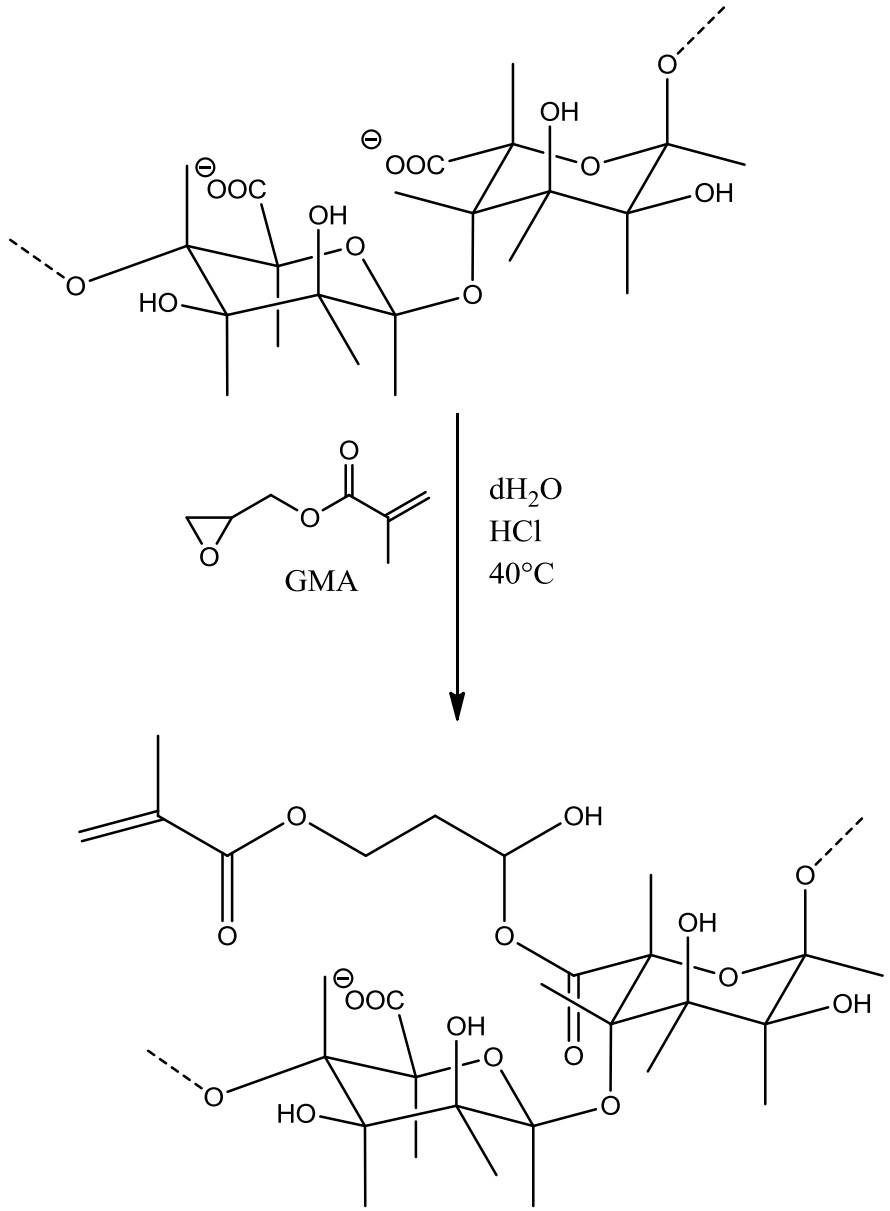

Figure 7 Chemical modification of alginate by glycidylmethacrylate (Based on J.F.A.S. Maior et al 2008 [41])

The reason of methacrylation is the possibility to blend modified alginate in any ratio with modified pectin, providing the possibility to optimise stability and sensitivity of a bioresponsive polymer system. An increased contingent of pectin elevates the sensitivity against pectin degrading enzymes, but decreases the physical stability. In contrast an increased percentage of alginate increases the stability of the system.

Alginate is non-toxic, cheap and used in food industry for example as thickening agent or stabiliser, *e.g.* in chocolate milk [44].

In this study alginate is not used as degradable substrate for enzymes; it is just used as stabiliser and agent for optimisation properties.

5.2.3 Carboxymethylcellulose

Carboxymethyl cellulose is a derivate of common cellulose. To produce carboxymethyl cellulose, cellulose was modified by treatment with sodium hydroxide and afterwards etherified with the sodium salt of chloracetic acid [46, 47].

Carboxymethyl cellulose (Figure 8) has carboxyl groups, similar to pectin and alginate. These groups can be modified by esterification.

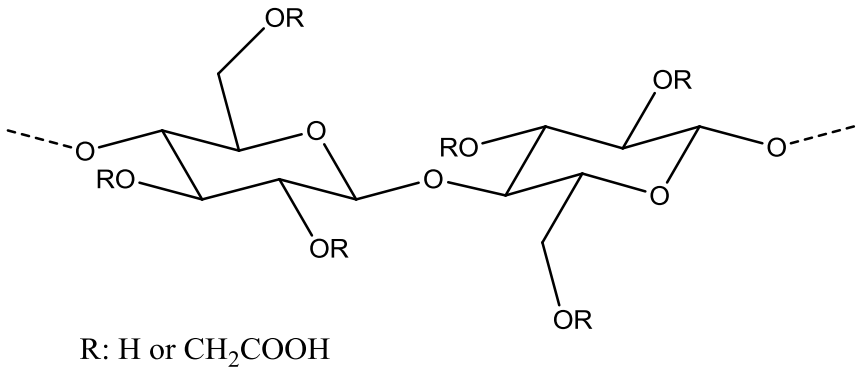

Figure 8 Chemical structure of carboxymethylcellulose [46, 48]

Part of this thesis was the chemical modification of carboxymethyl cellulose by esterification with glycidylmethacrylate (Figure 9).

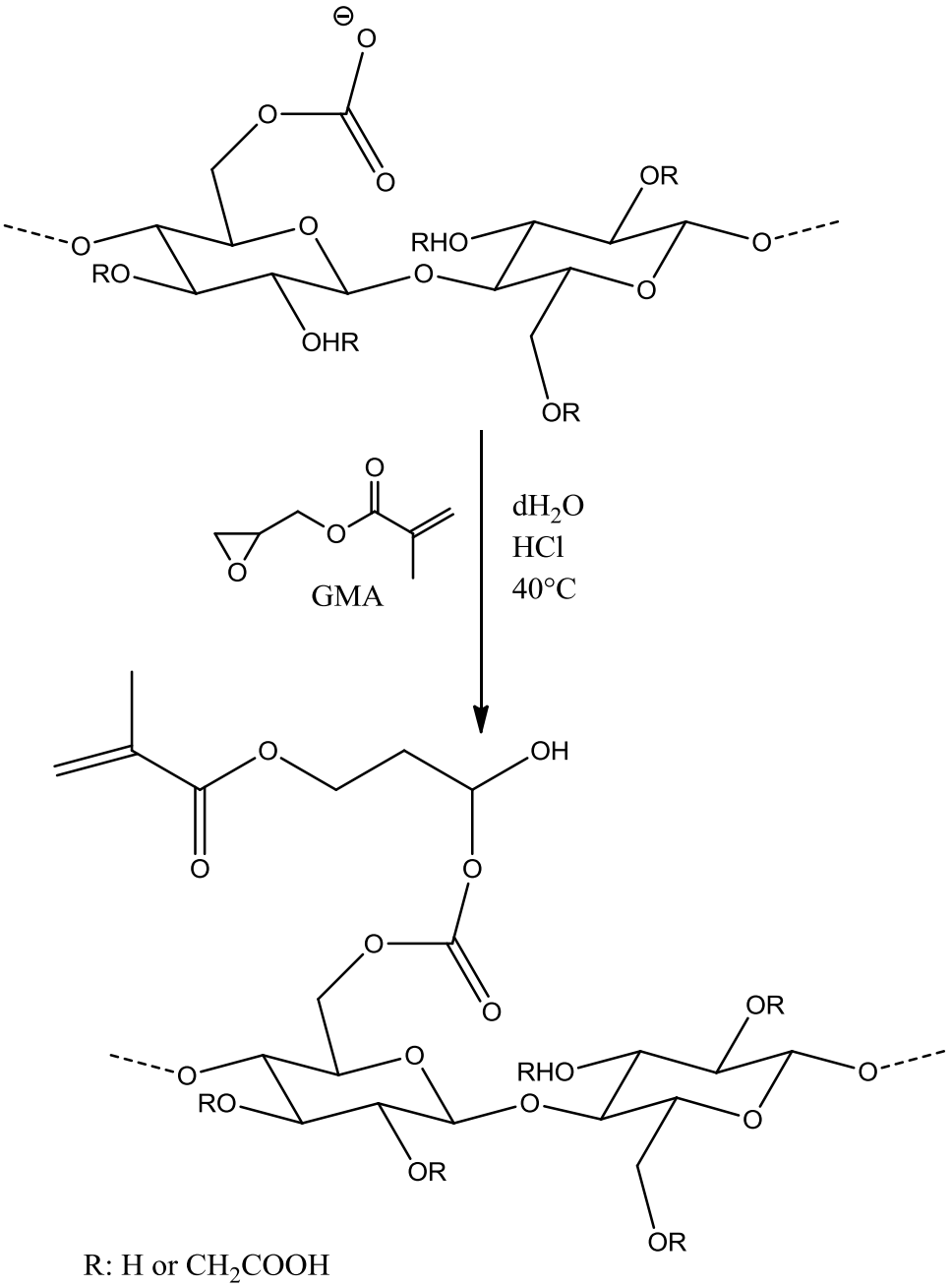

Figure 9 Chemical modification of carboxymethylcellulose with glycidylmethacrylate (Based on J.F.A.S. Maior et al 2008 [41])

Carboxymethylcellulose is insoluble in water; hence for any modification or degradation reactions the sodium salt was used. Carboxymethyl cellulose is used in food technology as thickening agent [36]. Cellulose and derivates of cellulose can be degraded by enzymatic activities. It is well known and described in literature that cellulases are able to degrade cellulose and cellulose derivates, *e.g.* carboxymethylcellulose [49-53].

5.2.4 Peptidoglycan from *Micrococcus lysodeiktikus*

As mentioned before peptidoglycan is part of the bacterial cell wall. The structure consists of two major sugar molecules (N-acetylglucosamine and N-acetylmuramic acid) and peptide based bridges (L-alanine, Isoglutamate; L-lysin and D-alanine). The L-lysin is connected to a D-alanine of the next building block by a pentaglycin bridge (Figure 10) [54-60].

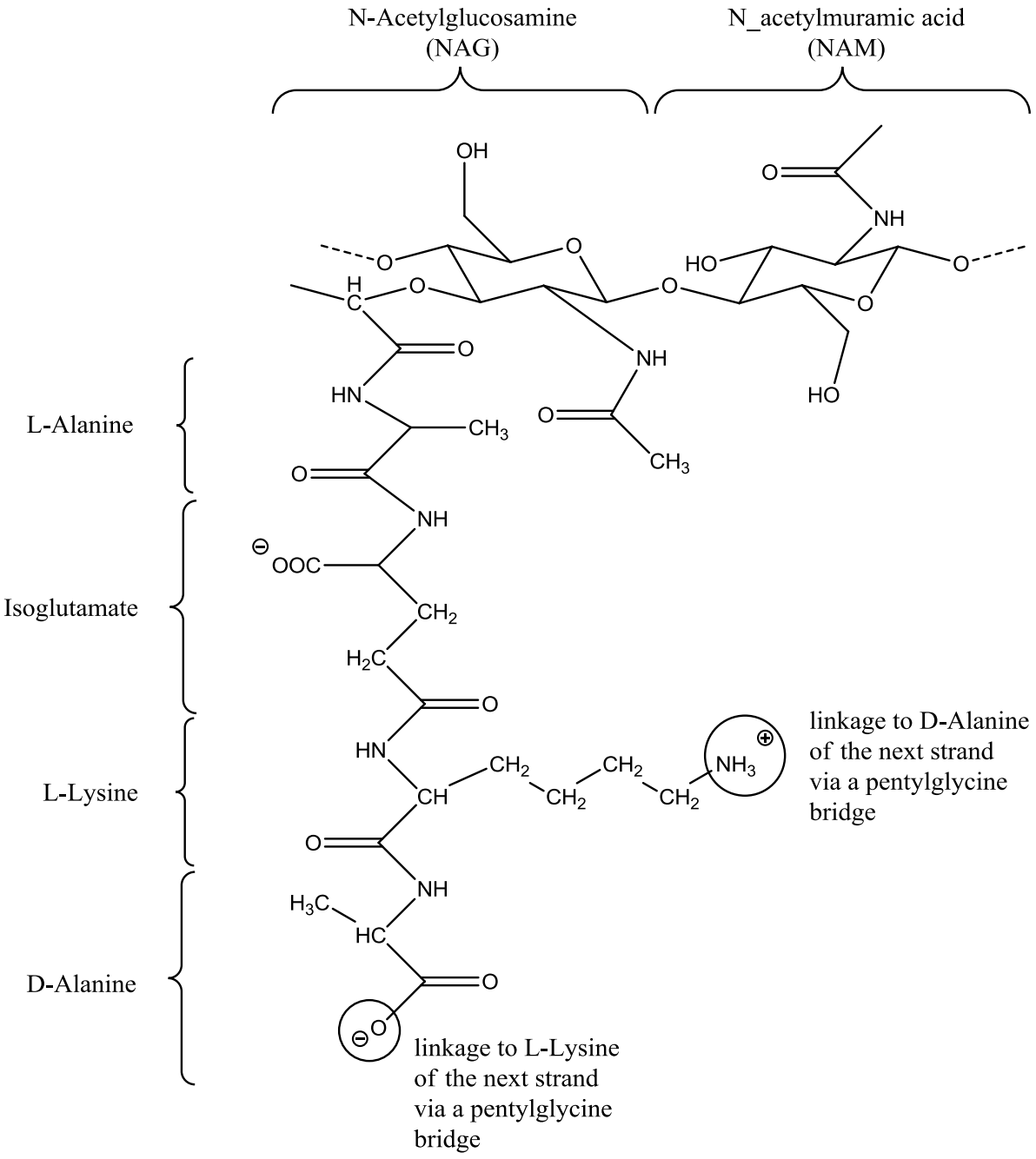

Figure 10 Structure of peptidoglycan [54, 58-60]

To sum up, peptidoglycan is build up of two types of linkages, the polysaccharide part, consisting of N-acetylglucosamine and N-acetylmuramic acid cross-linked with polypeptide parts. In this study peptidoglycan was used as substrate for lysozyme as trigger enzyme to perform a controlled release of model dyes, alizarin and modified laccase. To establish a bioresponsive polymer system, it is useful to mix several different biopolymers. Each used biopolymer is a substrate for a more or less specific hydrolytical enzyme. Pectin and carboxymethylcellulose show a broad specificity to enzymes compared to peptidoglycan which is a specific substrate and can only be degraded by lysozyme or proteases [57, 61-63].

5.3 Trigger enzymes

A range of different enzymes were used to realize a biodegradable and bioresponsive polymer system. To test the bioresponsive polymer system against extracellular enzymes, pectinases, pectat lyases, cellulases and lysozyme were used in this study.

5.3.1 Polygalacturonases or pectinases

A number of fungi secrete high amounts of polygalacturonases or pectinases [64, 65], in contrast pectat lyases are mainly produced by bacteria [66-68]. Pectinases and pectat lyases are able to degrade pectic substances, by hydrolysation (polygalacturonases or pectinesterases) or by an elimination of the glycosidic bound (pectin lyases) [68]. Some general pectic substances degrading enzymes are listed in Table 1.

Table 1 Extract of important pectin or polygalacturonic acid degrading enzymes [64-68]

Enzyme	Action mechanism	EC-number	substrate
Endo-Polygalacturonase	Hydrolysis of the glycosidic bound	3.2.1.15	pectic acid
Exo-polygalacturonase		3.2.1.67	pectic acid
pectin methyl esterase		3.1.1.11	pectin
Exo-polygalacturonase lyase	Trans-elimination of the glycosidic bound	4.2.2.9	polygalacturonic acid
Endo-polymethyl-D-galactosiduronat lyase		4.2.2.10	Unsaturated poly-(methyl-D-digalacturonates)
Endo-polygalacturonase lyase		4.2.2.2	pectic acid

Figure 11 gives an overview of common regions of PGA which can be hydrolysed by enzymatic activities.

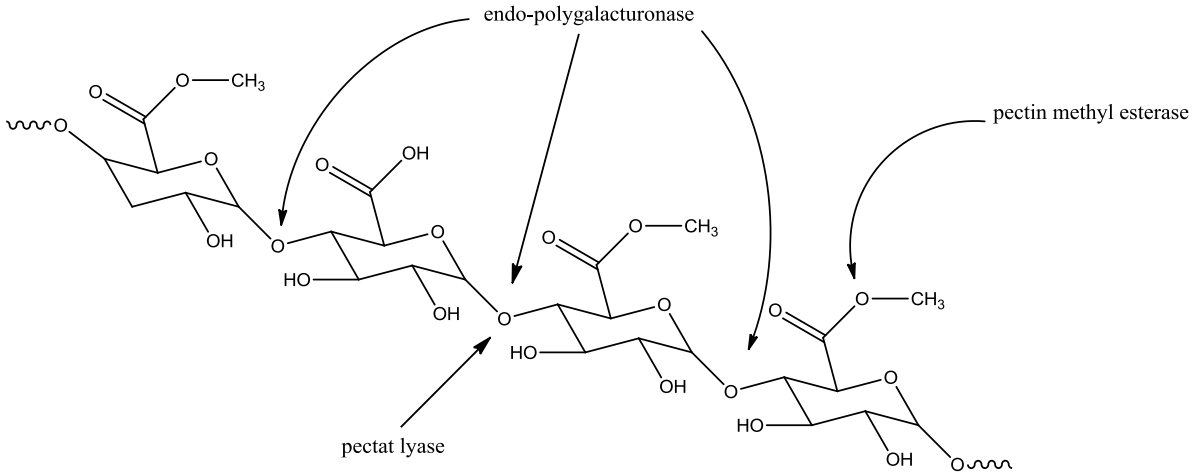

Figure 11 Positions of attack of pectat lyases, pectin methyl esterases and endo-polygalacturonases to hydrolyse a polygalacturonic backbone (pectat lyases and endo-polygalacturonases) and pectin methyl esterase to cleave the esters [68-71]

The following parts illustrate the hydrolytical reaction of the polygalacturonases and pectinesterases as well as the elimination reaction of the pectat in detail.

5.3.1.1 Elimination of the glycosidic bound catalysed by lyases

Endo-polygalacturonase lyase (EC 4.2.2.2: [1,4]-α-D-galacturonan lyase) or Endo-polymethyl-D-galactosiduronat lyase (EC 4.2.2.10: [1,4]-6-*O*-methyl-α-D-galacturonan lyase) are able to degrade polygalacturonic acid or crude pectin by cleaving the bond between two sugar monomers. Endo-polygalacturonase lyase shows a requirement of Ca^{2+}-ions to place the galacturonate groups in the right position in the active site. The pH optimum of pectat lyase is approximately around 8.5 to 9.5 while in contrast pectin lyases are much more active at lower pH values [69].

The proposed reaction of pectat lyases and pectin lyases is shown in Figure 12. The ARG_{218} stabilises the substrate while the Asp activates the cleavage of the [1,4] bond by establishing a double bond in the monomeric unit [69, 70, 72, 73].

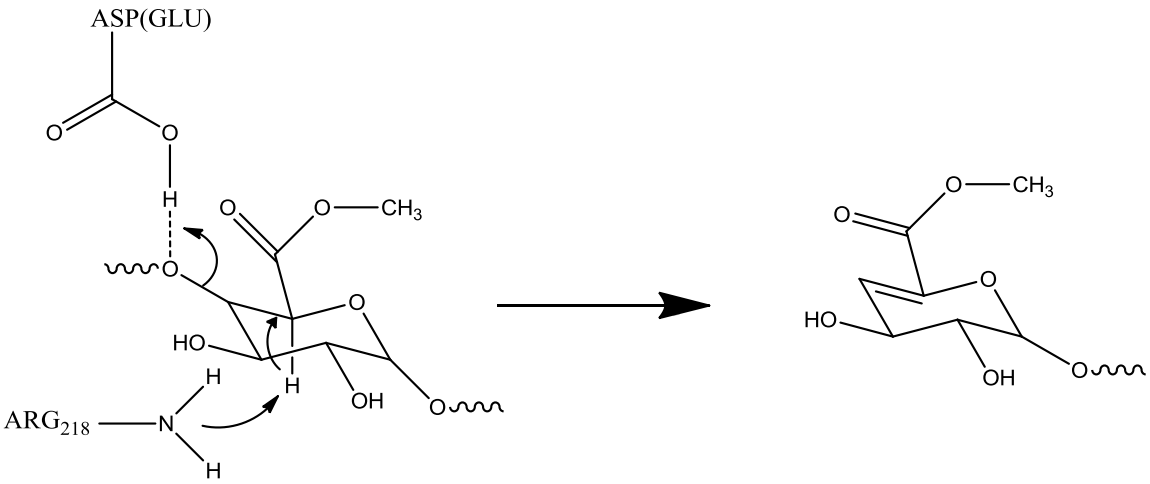

Figure 12 Proposed hydrolytical reaction of pectat lyase and pectin lyase [69, 70, 72, 73]

5.3.1.2 Pectin methyl esterases

In contrast to the former mentioned pectat or pectin lyase, the pectin methyl esterases (PME) are not hydrolysing the backbone of pectin but they attack the ester of the galacturonic acid monomeric unit.

Pectin methyl esterases (PME), (EC 3.1.1.11: pectin pectylhydrolase) are well characterised enzymes from fungal sources. This type of enzyme is stable from pH 2 up to pH 10 and in a temperature range from approximately 40 to 70 °C [69]. Similar to lipases or serine proteases the cleavage mechanisms of PME includes a triade of asparagine, histidine and serine. Two asparagine and one arginine residues (position 178, 199 and 267) are typical for pectin methyl esterases [69]. One asparagine is not protonated and helps the attack of the nucleophile water molecule to the carbonyl carbon. The second asparagine is acetic and protonates the oxygen of the carboxylic group. An intermediate is formed and collapse to yield the free acid and methanol (Figure 13) [69, 74, 75]

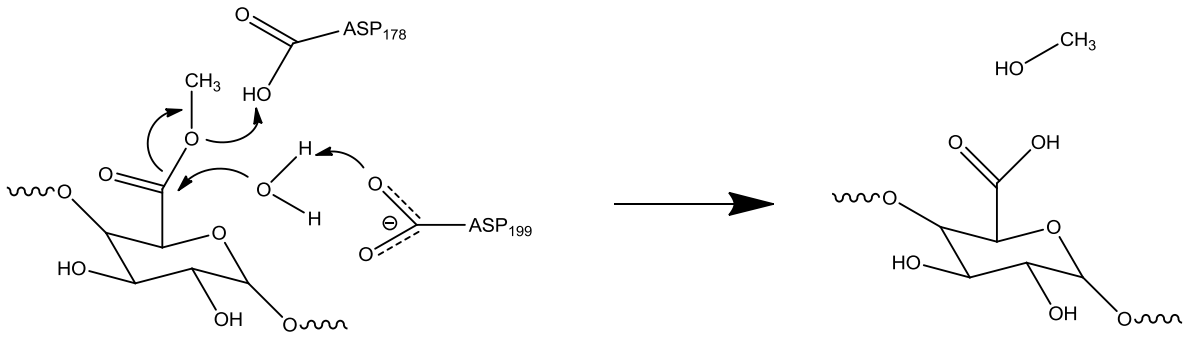

Figure 13 Reaction mechanism of a pectin methyl esterase to cleave a the ester of a pectin to methanol and unesterified pectin [69, 74, 75]

5.3.1.3 Endo-polygalacturonase

The last used pectin degrading enzyme is the endo-polygalacturonase. The polygalacturonase or galacturonide [1,4]-α-galacturonidase (EC 3.2.1.15) is a member of the glycosyl hydrolase family. The polygalacturonases from *Aspergillus niger* (Figure 14) show three important asparagine residues in the active site. Asp_{180} and Asp_{202} activate the water molecule to attack the glycosidic bond as a nucleophile. Asp_{201} protonates the leaving group, supported by His_{223} and a TYR_{209} [69].

Polygalacturonases are stable enzymes. The optimal pH value is from 3.5 up to 6.0, while the ideal working temperature is located in the field of 40 to 55°C. The typical molecular weight of polygalacturonase is between 30 and 75 kDa [69].

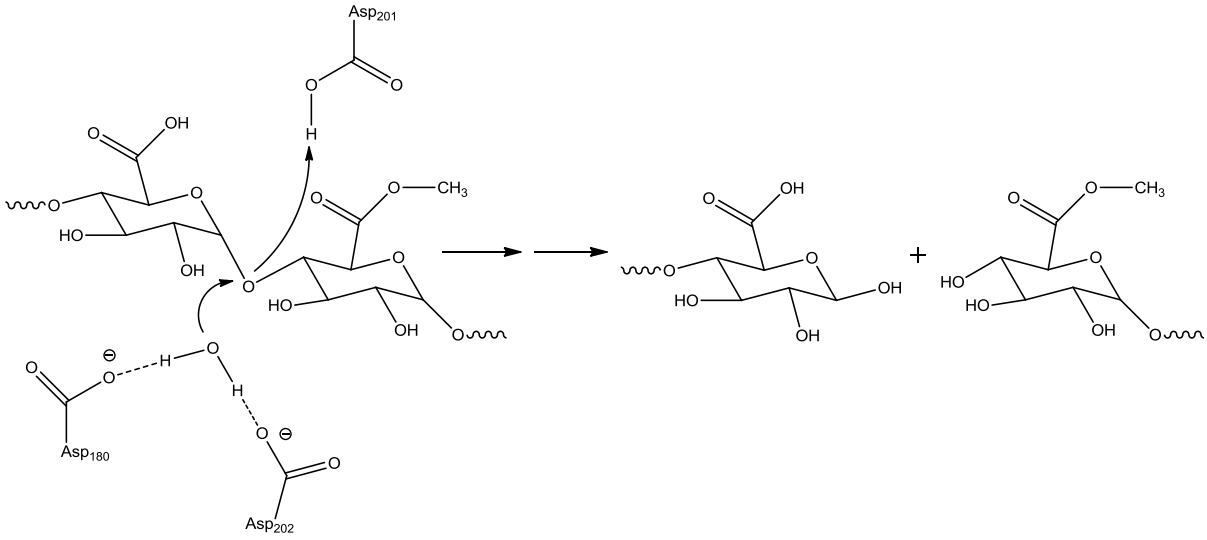

Figure 14 Reaction mechanism of an endo-polygalacturonase from *A. niger* [69, 76]

5.3.2 Cellulases

This work is not focused on getting soluble sugars out of natural resources. Cellulases as well as polygalacturonases are used as trigger enzymes in this work. These extracellular enzymes are produced by a huge range of microorganisms with partly pathogenic potential. *Aspergillus* is a worldwide present microorganism [77] whereof 19 species of it cause human diseases [78]. Common relevant species are *Aspergillus fumigates*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus terreus* and are frequently responsible for human infection [49-53, 79].

The topic cellulase and related enzymes is under research since the early 1950s [80]. Cellulase is a generic term of all cellulose degrading enzymes. Cellulases can be divided into three enzyme classes:

endoglucanases, exoglucanases (cellobiohydrolases) and β-glucosidases [51, 81]. An overview of enzymatic attack of a cellulose stand is shown in Figure 15.

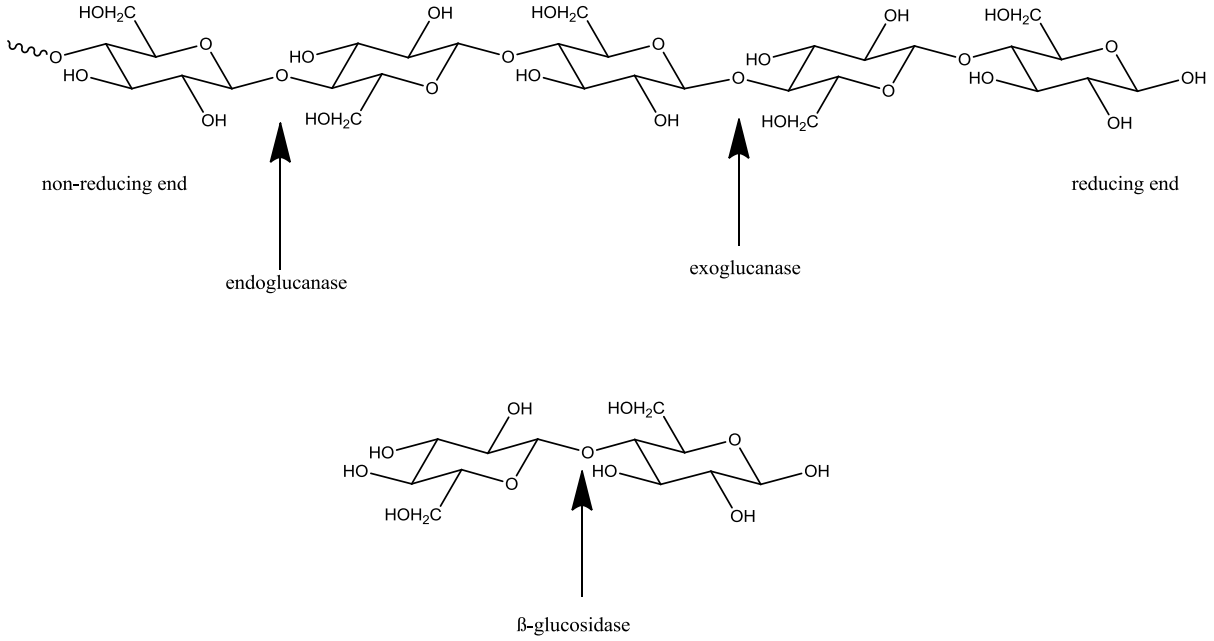

Figure 15 Overview of the enzymatic attack of cellulases [81, 82]

Endoglucanases (EC 3.2.1.4) are hydrolases which cuts the polysaccharide backbone of the cellulose randomly. Exoglucanases in contrast acts on microcrystalline cellulose by cutting cellobiose units, two glucose monomers, from the non-reducing end of the polysaccharide strand [81, 83]. To degrade cellulose completely to the glucose monomers, a cellobiose degrading enzyme, *e.g.* β-glycosidase, is needed.

As example for a cellulase the reaction mechanism (Figure 16) of an exoglucanases is well described in literature [84].

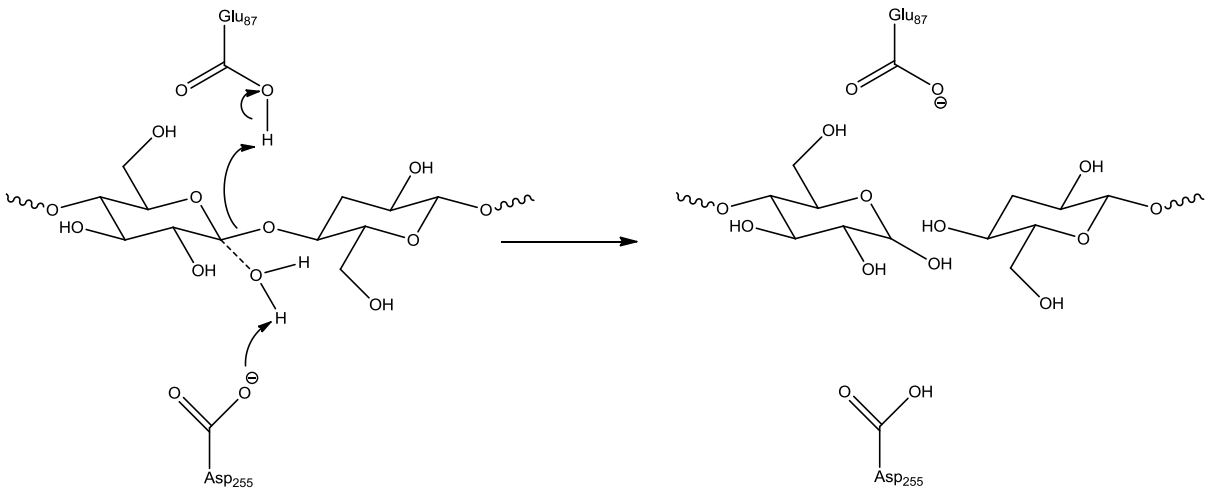

Figure 16 Schematic reaction of a cellobiohydrolase CelS from *Clostridium thermocellum* [84, 85]

The catalytic relevant residues Asp_{255} and Glu_{87} allow the nucleophilic water molecule to attack the glycosidic bond and split the polymeric sugar.

β-Glucosidase (EC 3.2.1.21) is a hydrolytical enzyme which is able to hydrolyse the glycosidic bond of polysaccharides, *e.g.* cellobiose [82].

Beside bioenergy cellulases can be used in food industry for extraction and clarification of fruit and vegetable juices, the production of fruit nectars and purees, the extraction of olive oil and the improvement of bakery products. Cellulases, hemicellulases and pectinases are also relevant in beer brewing and wine production. Industrial applications are further textile industry, where cellulases are involved in bio-stoning of denim garments or biopolishing of non-demin fabrics [80, 81].

Additionally all types of cellulases are well established for recycling processes. Cellulases from *Trichoderma viride* can be used to acquire glucose from wastepaper [86]. The enzymatic generated glucose can be used to produce further biotechnology based products like bioethanol as energy source.

5.3.3 Lysozyme

Additionally to the two polysaccharide degrading enzymes above, lysozyme can degrade cell wall constituent parts of mainly gram-positive bacteria, namely peptidoglycan consisting of N-acetylglucosamine (NAG) and N-acetyl muramic acid (NAM) (chapter 5.2.4).

Lysozyme is a well-established and described enzyme and was characterised for the first time by Alexander Fleming in 1922 [87].

In nature lysozyme can be found in various sources from human immune system to parts of the hen egg. The importance of lysozyme as bacteria killer is known since the early 20th century [88]. The mechanism of bacterial cell wall degradation is shown in Figure 17. In the human body lysozyme was found in various body fluid systems, *e.g.* serum of adults, children or new-borns, gastric juice, mother's milk, sperm or tears [89]. So lysozyme is a wide spread enzyme which is used to destroy bacterial cell wall to avoid bacteria presence. The immune system is using lysozyme to protect the body from bacteria colonisation, *e.g.* as part of tear fluid, while fungi species, *e.g. penicillium sp.* are using lysozyme to protect themselves from bacterial attacks [57, 87].

Lysozyme is a small enzyme and consists of 129 amino acids. The hen egg white lysozyme is structurally similar to the human produced lysozyme [56, 90, 91]. The hydrolysation process of peptidoglycan catalysed by lysozyme is shown in Figure 17.

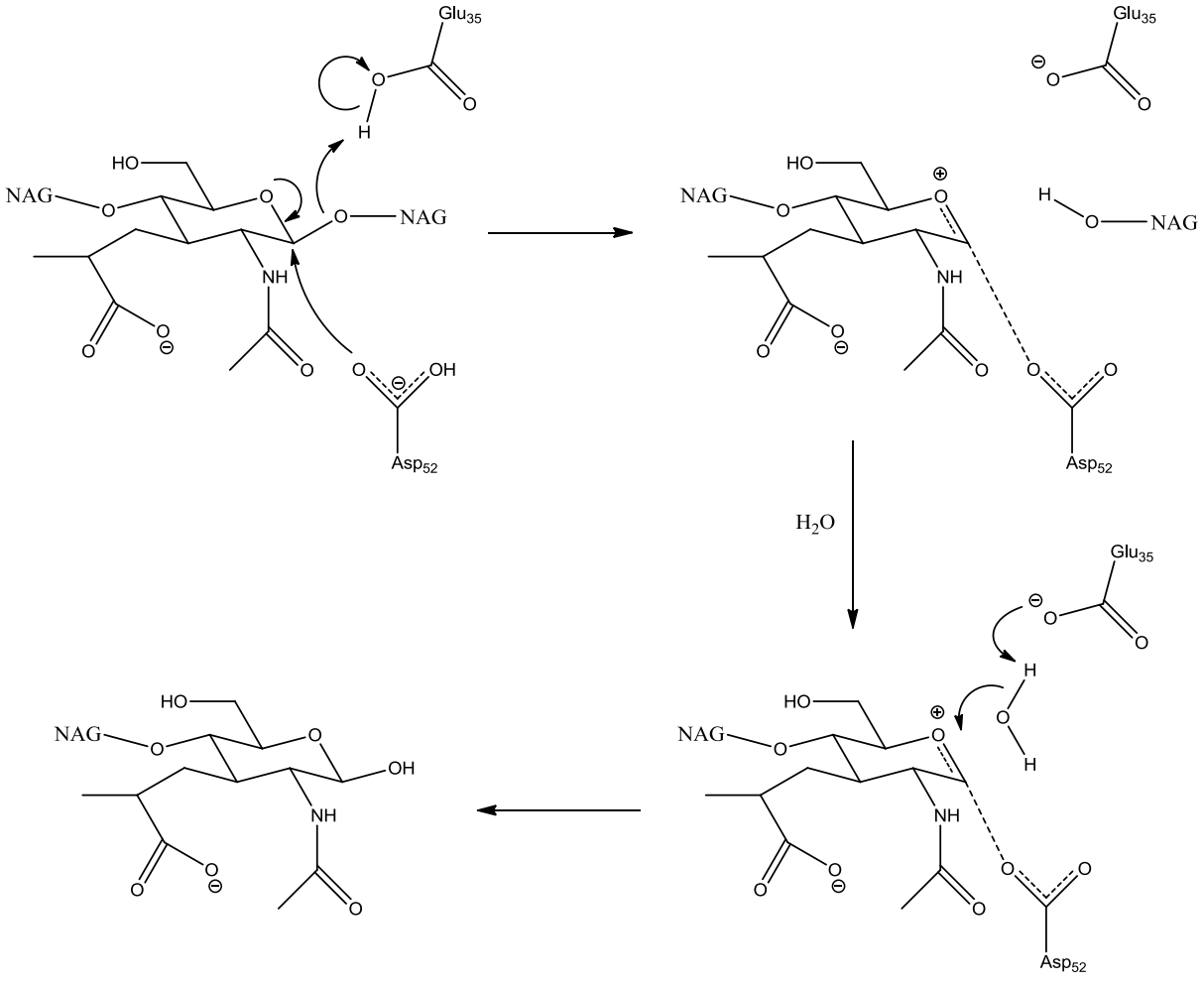

Figure 17 Hydrolysation of peptidoglycan catalysed by lysozyme [57, 91].

The relevant residues Glu_{35} and Asp_{52} catalyse the degradation of peptidoglycan. Asp_{52} is in the first step of the reaction the nucleophile to form a glycosyl-enzyme complex while the NAG part can

leave. In the second step a water molecule is activated by the Glu_{35} residue and attacks the positive charged acetal bond, stabilised by Asp_{52} muramic acid [57, 91].

It is known from previous work from Andrea Hasmann, that lysozyme plays an important role during the wound healing process of infected wounds. This is the reason to use lysozyme as trigger for bioresponsive polymers. Elevated levels of lysozyme in wound fluids can be used for detection of wound infection [61].

5.4 Released Substances

To realise a bioresponsive polymer, test substances were needed which can be released by trigger enzymes. Two systems were established during this PhD thesis, alizarin and chemically modified laccase from *Trametes hirsuta*.

5.4.1 Alizarin

In a first step, alizarin (Figure 18) was incorporated into the bioresponsive system. During the degradation process more and more alizarin was released to the supernatant over the time. After a pH value change to alkaline conditions, the amount of released alizarin can be measured by simple photometric methods (510 nm). The reason for the change of the pH value is a consequence of the fact, that alizarin is a pH indicator and shows improved absorption behaviour at an alkaline pH value [92].

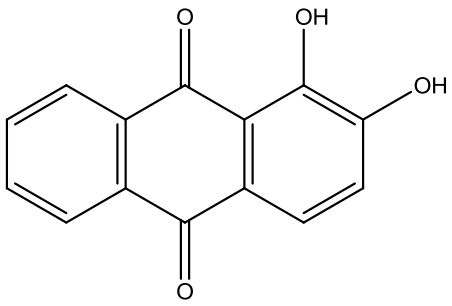

Figure 18 Chemical structure of alizarin [92]

5.4.2 Laccases

After the successful release of alizarin as simple dye [43, 93], the next step of enzyme triggered controlled release was the release of complex molecules, *e.g.* enzymes.

The controlled release of enzymes has considerable advantages. After the release of a dye, the dye is detectable in the supernatant, but is not able to catalyse a reaction. In contrast, enzymes are able to catalyse further reactions with specific substrates.

The main goal of incorporating enzymes into a bioresponsive polymer (BRPs) is the realisation of an enhancement of the signalling reaction. That means that a trigger enzyme can release instead of a certain amount of dye molecules more or less the same amount of active enzymes. These released active enzymes are further able to convert a certain amount of inactive pre-dye molecules into an active dye. So if one trigger enzyme is able to release 100 molecules of dye the reaction is finished, but if one trigger enzyme release 80 molecules of an active enzyme, this enzyme can convert up to thousands of molecules of a pre-dye. This thesis shows that the sensitivity of bioresponsive polymer systems can be enhanced by the integration of enzymes catalysing a secondary colour forming reaction. Laccases from *Trametes hirsuta* were selected to catalyse this secondary signal enhancing reaction and consequently termed “Enhanzymes”.

Laccases (EC 1.10.3.2) are well established and described enzymes in literature. Laccases are multicopper containing oxygen oxidoreductases. They are able to oxidise various substances like phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, hydroxyindols, benzenethiols as well as inorganic/organic metal compounds by reduction of molecular oxygen to water [94-98].

Biochemical or technical applications are well described in literature and were discussed in various reviews [98-105]. A simplified reaction mechanism of laccase with ferulic acid based on the review of Tukayi Kudanga is drawn in Figure 19 [106]. The laccase generates a radical stabilised by the aromatic ring of the ferulic acid. This radical can be used to crosslink two molecules together or changing the colour or absorption of the substrate.

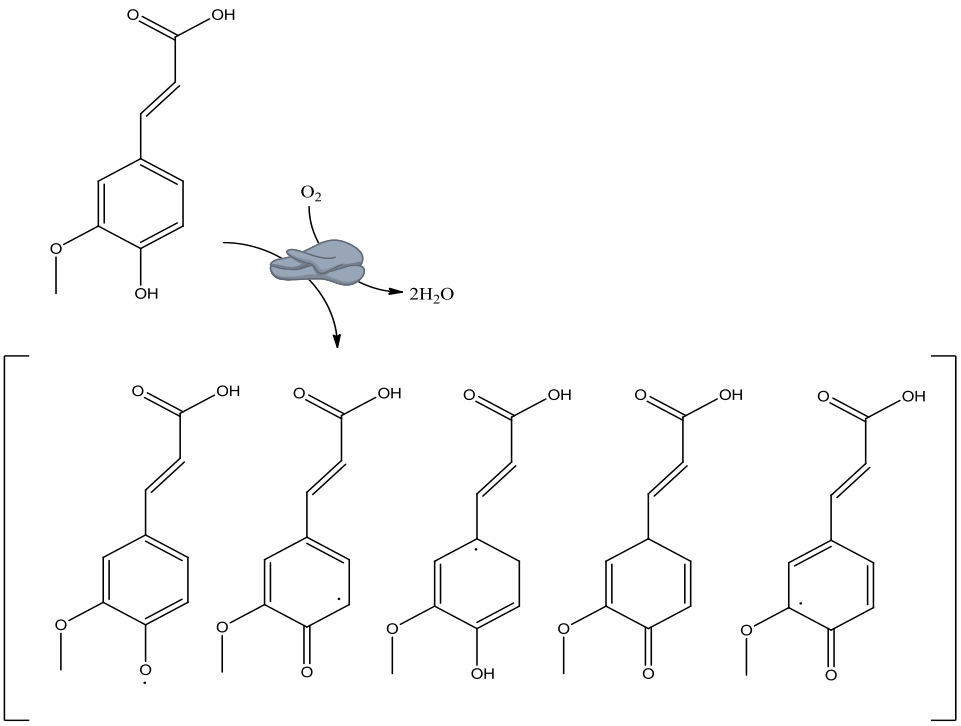

Figure 19 Simplified reaction mechanism of laccase oxidation of ferulic acid as substrate [106]

To minimise leaching, that means auto-release of laccase from the BRPs, the immobilised enzyme was chemically modified. Two major modifications were investigated. The first modification is based on the previous work of Marc Schroeder, where laccases were modified by covalent linkage to polyethylene glycol (PEG) molecules to increase the molecular weight from approximately 62 kDa to 67 kDa. This increased size reduces the auto diffusion. PEG modifications are well described methods to change enzymes for technical applications [107-110].

The modification was done with PEG, activated by cyanuric chloride to prepare 2-*O*-methoxypolyethylene glycol-4,6-dichlorotriazine. The triazine function couples to the amino groups on the surface of the enzymes (Figure 20) [107, 109, 111].

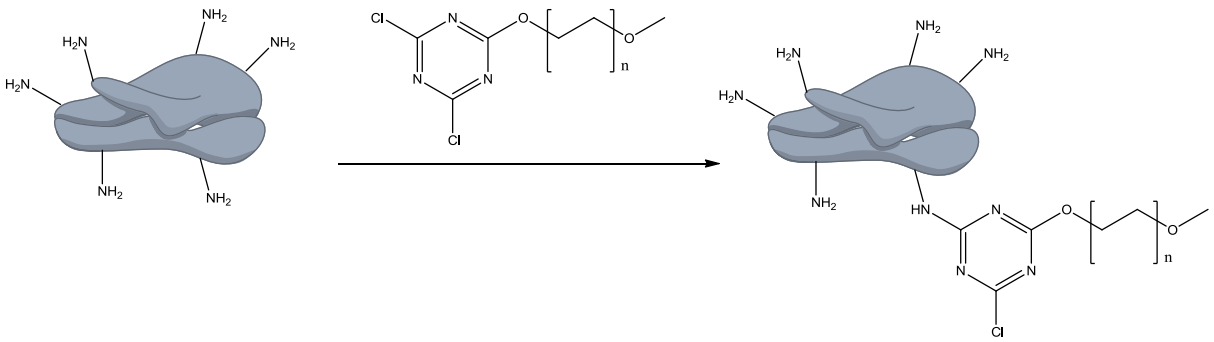

Figure 20 Chemical modification of laccase with activated PEG [107, 109, 111]

To reduce the auto-diffusion of laccase a second modification reaction was established. To get a covalent linkage between the polysaccharide based BRPs (methacrylated CMA or methacrylated PGA) the laccase itself was modified by coupling methacrylic side chains which can build up a covalent network with the methacrylic groups of the biopolymers. The same functional polymeric monomers, in that case methacrylate, can be cross-linked by radical polymerisation.

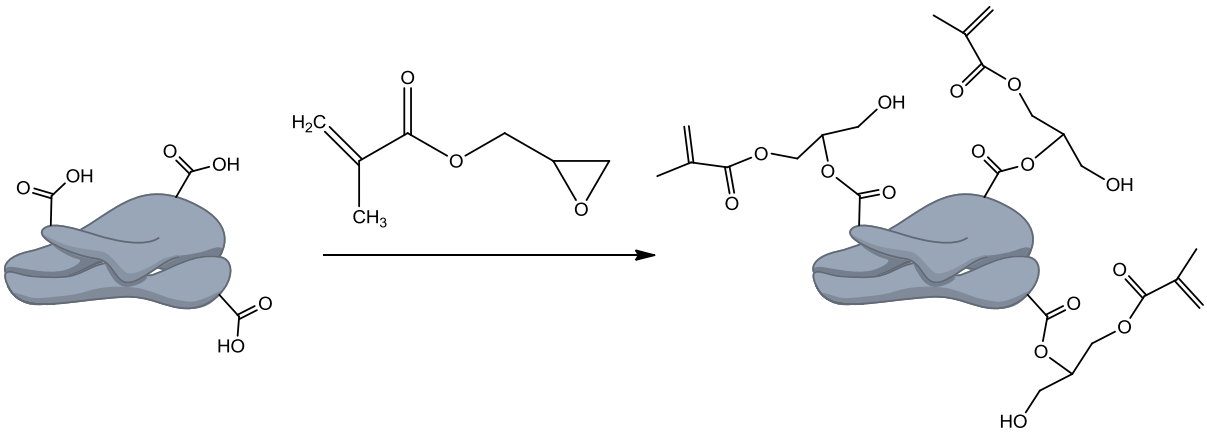

Figure 21 Chemical modification of laccase with glycidylmethacrylate (Based on J.F.A.S. Maior et al 2008 [41])

Figure 21 shows the modification of laccases from *T. hirsuta* with glycidylmethacrylate. The reaction was done in a 50 mM succinic acid buffer at pH 4.5 at room temperature overnight.

Trigger enzymes are able to release laccase and each molecule of laccase is able to oxidise the immobilised substrate to yield a visible colour change. The technique of immobilizing laccase substrates, in that case ferulic acid onto silica gel is well described in literature [18, 112].

5.5 Immobilised Laccase substrate

In a first step silica gel was modified with amino-groups by covalent coupling with (3-Aminopropyl)triethoxysilane (APTS) [18; 112]. Second the amino groups were esterified with ferulic acid by the usage of EDAC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) and HOBT (hydroxybenzotriazol) as laccase substrates [18, 113].

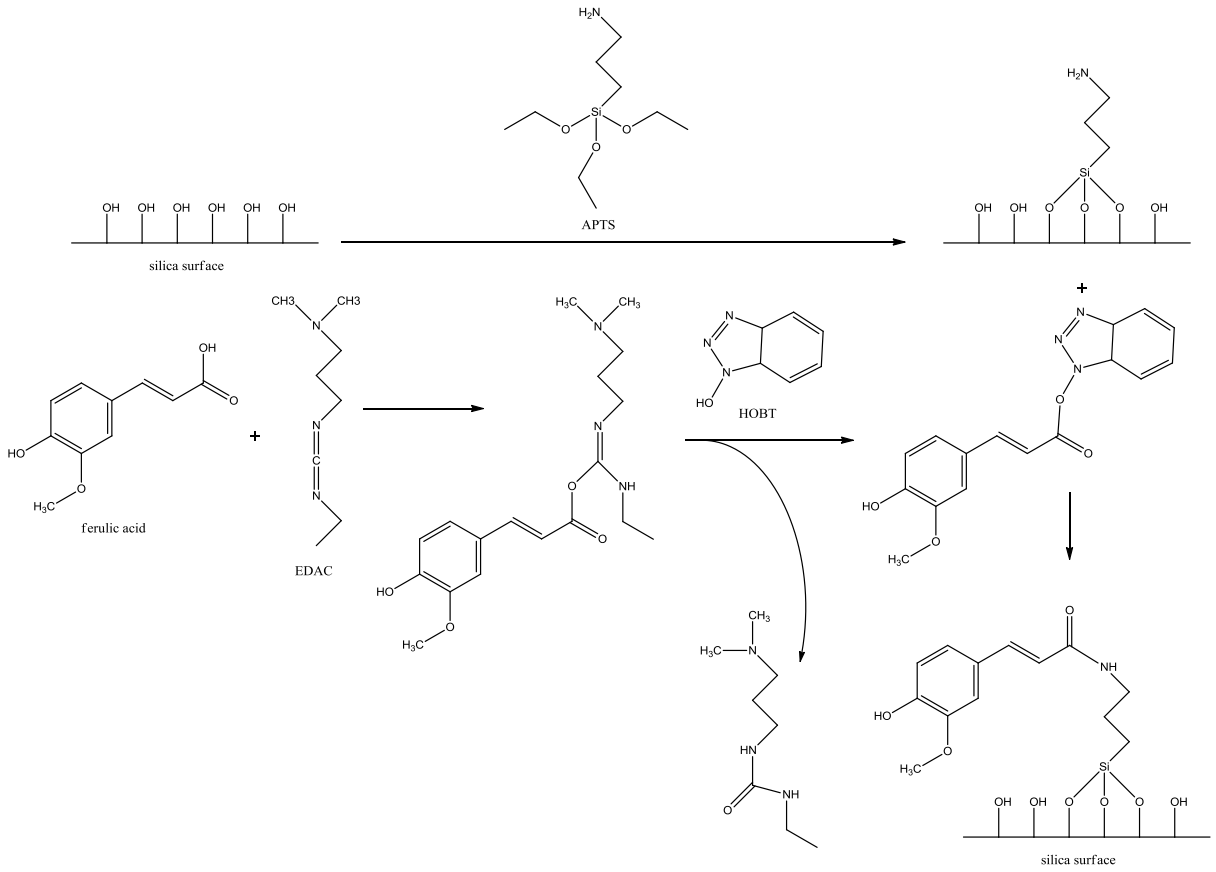

Figure 22 Reaction of ferulic acid coupling onto silica gel surfaces by EDAC and HOBT (adapted from Rollett et al. 2010 and Chan et al. 2007 [18, 113])

EDAC activates the carboxylic group of the ferulic acid by esterification to an *O*-arylisourea group. Afterwards HOBT attracts the ester and forms a HOBT-ester which is a good leaving group for esterification with a less active amino group.

As mentioned before the usage of immobilised laccase substrates shows clear benefits compared to free soluble substrates. It is not possible that substrate molecules can diffuse to the environment and contaminate and reduce the quality of the product (*e.g.* meat packed into a food packaging system). Immobilised substrates were combined with the controlled release of modified laccases to realise an enhancement of a visible detection system (Paper 3)

5.6 References

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6 BIORESPONSIVE SYSTEMS BASED ON POLYGALACTURONATE CONTAINING HYDROGELS

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6.1 Abstract

Polysaccharide acid (PSA) based devices (consisting of alginic acid and polygalacturonic acid) were investigated for the detection of contaminating microorganisms. PSA-$CaCl_2$ hydrogel systems were compared to systems involving covalent cross-linking of PSA with glycidylmethacrylate (GSA-GMA) which was confirmed with Fourier Transformed Infrared (FTIR) analysis. Incubation of PSA-$CaCl_2$ and PSA-GMA beads loaded with alizarin as a model ingredient with trigger enzymes (polygalacturonases or pectat lyases) or bacteria lead to a smoothening of the surface and exposure of alizarin according to Environmental Scanning Electron Microscopy (ESEM) analysis. Enzyme triggered release of alizarin was demonstrated for a commercial enzyme preparation from *Aspergillus niger* and with purified polygalacturonase and pectat lyase from *Sclerotium rolfsii* and *Bacillus pumilus*, respectively. In contrast to the PSA-$CaCl_2$ beads, cross-linking (PSA-GMA beads) restricted the release of alizarin in absence of enzymes. There was a linear relation between release of alizarin (5 to 348 µM) and enzyme activity in a range of 0-300 U ml^{-1} dosed. In addition to enzymes, both PSA-$CaCl_2$ and PSA-GMA beads were incubated with *Bacillus subtilis* and *Yersinia entercolitica* as model contaminating microorganism. After 72 hours, a release between 10 µM and 57 µM alizarin was detected. For protection of the hydrogels, an enzymatically modified PET membrane was covalently attached onto the surface. This leads to a slower release and improve long term storage stability based on less than 1% release of dye after 21 days. Additionally, this allows simple detection by visual inspection of the device due to a colour change of the white membrane to orange upon enzyme triggered release of the dye.

Keywords: Controlled release, polygalacturonic acid, modification, Pectinase, Bioresponsive Polymer

6.2 Introduction

In our high-tech society microorganisms find increasingly niches for growth and contamination ranging from filters in air conditioning systems to packaging materials and household devices [1]. A great number of bacteria are pathogenic and/or release toxic metabolites or convert e.g. food into toxic substances [2-4]. Thus, there is an increasing demand for detection and control of contaminating microorganisms. There are many sophisticated methods such as PCR suitable for the detection of the presence and identification of hazardous bacteria [5-8]. However, most methods require special equipment and expertise and are often time consuming. Bioresponsive systems which interact with extracellular microbial enzymes could be easy-to-use and cheap alternatives for the detection and/or control of contaminating microorganisms. Bioresponsive systems can also act as online measurement systems integrated *e.g.* in package systems [11-13]. Bioresponsive devices can be constructed by assembling or coating of biomaterials which can be modified by enzymes [14]. In general, bioresponsive polymers and capsules can simply react to pH or temperature changes in different environments [10, 9]. A more selective delivery is obtained with devices responding to enzymes as triggers. Here, polysaccharide acids (PSA, comprising alginic acid and polygalacturonic acid) were used for the construction of bioresponsive devices since many contaminating microorganisms secrete polygalacturonic acid (PGA) degrading enzymes. Alginic acid (ALC) was used to stabilise the fragile PGA. In the case of PSA-$CaCl_2$ it was necessary to stabilise the PGA. PGA alone was not able to form hydrogels by gellating in a $CaCl_2$ solution.

The backbone of polygalacturonate consists of galacturonic acid units with partly free and esterified carboxylic groups [15]. These carboxylic groups can be used for functionalisation [16] and were used for cross-linking in this study. Polygalacturonates can be blended with related polysaccharides like alginate. The backbone of alginate consist of (1-4) glycosidically linked β-D-mannuronic acid and α-L-guluronic acid monomers. [9]. Guluronic acid can be esterified similar to polygalacturonate.

In this study bioresponsive polygalacturonate based hydrogels were produced by two different methods. A common and well described method of producing polygalacturonate based hydrogels described by Robyt et al. [17] and Aydin et al [18] was compared with an approach involving cross-linking of the polysaccharide by inserting methacrylate groups which can be radically polymerised [16]. Polygalacturonate can be cleaved by enzymes including pectinases, polygalacturonases and pectin lyases [9, 19, 20, 21, 22] which can be used as triggers to detect the presence of microorganisms. Pectin lyases (from *B. pumilus*, [20]) and polygalacturonases (from *S. rolfsii*, [19]) both capable of PGA hydrolysis were assessed in this study for triggering release. These enzymes

cleave polygalacturonate based on two distinct mechanisms namely beta-elimination and hydrolysis, respectively.

Alizarin as a model ingredient was incorporated into polygalacturonate based devices to study enzyme triggered release. Upon pH change from neutral up to pH 10 alizarin changes its colour from lightly orange to dark violet which can be used for signal enhancement [23]. Beside pure enzymes, release of alizarin triggered by two different bacteria, namely *Bacillus subtilis* and *Yersinia entercolitica*, secreting pectin degrading enzymes [24-27], were used.

6.3 Material and Methods

6.3.1 Chemicals and enzymes

Alginic acid (ALC) from brown algae, HPLC grade dichloromethane and calcium chloride hexa-hydrate were purchased from Carl Roth (Karlsruhe, Germany). Technical grade alizarin, glycidylmethacrylate (GMA), sodium persulfate, polygalacturonic acid (PGA), commercial pectinase from *Aspergillus niger*, dextran standards for size exclusion chromatography and cultivation media components and buffers were from Sigma Aldrich (St. Louis). The PG 1 (39.5 kDa) polygalacturonase form *Sclerotium rolfsii* and the pectat lyase from *Bacillus pumilus* were produced and purified as previously described [19, 20]. Polygalacturonase and pectat lyase activity was measured according to the work of Klug Santner et al 2009 and W. Schnitzhofer et al 2007. [19, 20].

Ultrafiltration was performed with Vivaspin 20 centrifugal concentrator MWCO 10000 Da. by Satrorius (Goettingen, Germany)

6.3.2 Polysaccharide based hydrogels (PSA-$CaCL_2$)

The basic principle of gel forming by dropping polysaccharides like alginate or pectin is well described in literature by Robyt et al. [17] and Aydin et al. [18]. Prior to preparation of hydrogels both polysaccharide powders (ALC, PGA) were sterilized under UV-C light for 12 h while the aqueous solution of $CaCl_2x6H_2O$ and distilled water were autoclaved. For the dispersion of alizarin, 9.0 g of polygalacturonic acid from citrus peel, 1.0 g sodium salt of alginic acid and 0.4 g alizarin were dispersed in 50 mL dichloromethane, heated up to 50°C under reflux conditions. After 1 h, the solvent was removed by filtration and the polysaccharide containing alizarin was dried under vacuum. In a second step, 5.0 g of the stained polysaccharide was dissolved in 100 mL distilled water at 45 °C over night. To form homogeneous polysaccharide beads, a peristaltic pump was used. A flow of 1.5 mL min^{-1} of stained polysaccharide solution was dropped by the use of a cannula into 0.3 M $CaCl_2x6H_2O$ solution stirred at 150 rpm. The formed hydrogel-beads were filtrated and washed with distilled water four times and stored at 4°C. To avoid bacterial contamination the whole process was carried out under aseptic conditions.

6.3.3 Cross-linking of polysaccharide hydrogels (PSA-GMA)

The preparation of glycidylmethacrylate (GMA) modified polysaccharide acid was carried out according to a modified procedure previously described [16]. The stained PSA was sterilized by UV irradiation over night while the water was sterilized by ultrafiltration to reduce the risk of further contamination during the following degradation experiments. 5.0 g of PSA was dissolved in 100 mL distilled water. Thereafter, 2.5 mL of GMA solution and 1.0 mL 1 M HCl to lower the pH to 3.5 was added and incubated for 48 h at 50°C. After cooling down to room temperature, 50 mg sodium persulfate as radical source was added. The polysaccharide solution was poured into a heat resistant glass cup with an approximated base area of 10 * 20 cm until a thickness of 5.0 mm of the PGA-GMA was reached. To start the polymerisation process, the closed glass bowl was heated up to approximately 65 - 70°C in a thermo-controlled oven for 20 to 30 minutes. The formed hydrogel was cooled down to room temperature overnight and stored at 4°C under sterile conditions.

6.3.4 Minimal cultivation medium

A salt solution containing 2.5 g L^{-1} KH_2PO_4, 3.0 g L^{-1} K_2HPO_4, 2.0 g L^{-1} $(NH_4)SO_4$, 0.2g L^{-1} $MgSO_4$ x 7 H_2O and 5 mL L^{-1} of a micronutrient solution [28] was separately autoclaved from the C-source solution consisting 3 g L^{-1} glucose and 1.5 g L^{-1} polygalacturonate. The pH value was set to pH value of 7.0. Incubation of microorganisms was started by adding an amount of an overnight culture grown in a standard nutrient broth diluted to give a starting optical density at 600 nm of 0.1.

6.3.5 Enzyme triggered release experiments

1 g wet mass of the hydrogels (PSA-$CaCl_2$ and PSA-GMA) were added to 10 mL 25 mM potassium phosphate buffer pH 7.0. Thereafter, 100 µL of enzyme solutions with activities as indicated below were added. To dilute the enzyme stock solution 25 mM potassium phosphate buffer pH 7.0 was used. The samples were incubated at room temperature for 2.5 hours up to 24. The released alizarin was quantified photometrically using a Tecan Infinite 200 platereader (Tecan, Maennedorf, Switzerland). The absorbance signal was calculated to the corresponding concentration by using a calibration curve of alizarin in 25 mM potassium phosphate buffer pH 7.0. 250 µL of sample was taken from the supernatant and added to 25 µL of a 1M glycine buffer pH 10. After centrifugation at 1200 x g, 200 µL were transferred into a 96 well plate, purchased from Greiner bio-one (Kremsmünster, Austria) and measured at 530 nm.

Bacteria were stored at -72°C and grown on a nutrient agar plate for 24 hours at 30°C. To cultivate the strains an overnight culture in nutrient broth was incubated at 37°C. The overnight culture as well as the incubation with hydrogels was performed in 100 mL baffled Erlenmeyer flask. To start the reaction, 30 mL minimal medium were inoculated with an amount of the overnight culture to give an OD_{600} of 0.1. After reaching an OD_{600} of 1.0, one gram of wet mass of PSA-$CaCl_2$ or PSA-GMA was added under sterile conditions. After 72 h of incubation 500 µL sample were taken and added to 100 µL 1M Glycine buffer adjusted to pH 10. After centrifugation at 1200 x g, 200 µL were transferred into a 96 well plate PS flat transparent and the absorbance was measured at 530 nm (purple) and 480 nm (lightly orange) with a photometric platereader system.

6.3.6 Multilayer system

A multi-layer system consisting of 3 g alizarin dyed PSA-GMA (5 ± 0.5 mm thickness) was covered with a layer consisting of enzymatically modified PET (polyethylene terephthalate) membrane in a glass tube to make sure that the enzyme must pass the membrane to enter the hydrogel and cannot enter from another side. A non-stained PSA-GMA layer was used as adhesive to stick the membrane on the stained hydrogel to prevent a peeling off from the lower hydrogel layer. The polymerisation was started by temperature generated radical polymerisation (65 - 70°C) as described above. To ensure a separation of the dye and the membrane the polymerisation was done step by step. After finishing the polymerisation of the stained PGA-GMA layer the membrane was placed, fixed and polymerised in a second step.

After finishing the preparation step the multi-layer system was washed with distilled H_2O and stored at 4 °C. The used PET membrane produced by InoTex (Dvur Kralove, Czech Republic) was enzymatically modified on the surface to both increase the number of hydroxyl/carboxyl groups as potential points for attachment according to Eberl et al. 2009 [29].

After polymerisation, 0.5 mL of 25 mM sodium phosphate buffer pH 7.0 was added to the system to cover the system with liquid. For triggered release of alizarin enzyme activities as indicated below were added. The reaction was performed for 2 days at room temperature.

6.3.7 Characterization

FT-IR spectra were recorded with a Perkin-Elmer (Waltham, USA) “Spectrum One” instrument (spectral range between 4000 and 450 cm^{-1}). To analyse the solid polymer an ATR (attenuated total reflectance) unit was used. The homogenous samples were dried overnight to

reduce influences from water signals. To remove unbound methacrylic groups the polymer was washed before polymerisation by ultrafiltration. The reduced volume was re-added again. The amount of ester groups was determined by hydrolysing of ca. 3 g of the sample with an excess of ethanolic potassiumhdroxide solution (50 mL, 0.5 M) under inert gas (1 h reflux) and back-titration of the KOH with 1.0 M HCl at room temperature with a glass electrode (Ag-AgCl, electrolyte: 10% LiCl in MeOH). The ester value (mEq./g) was determined from the difference to the blank value (no sample). The assay was done according to DIN No. 53401.

The surface analysis was done with an ESEM Quanta 600 FEG (FEI Company, Eindhoven, The Netherlands), choosing the “low vacuum mode” [34] with water vapour as imaging gas at a pressure of 80 Pa. The micrographs were taken at electron energy of 10 keV using the large field detector (LFD), the appropriate detector for secondary electrons, delivering primarily topographic contrast.

Size exclusion chromatography was carried out with an HPLC (DIONEX P-580 PUMP, Dionex Cooperation, Sunnyvale, USA) equipped with an ASI-100 automated sample injector and a refractive index detector (RID). A YMC-PACK DIOL 6 500*8mm and a YMC-PACK DIOL 12 300x8mm were used in series with deionised water containing 0.1 mM NaN_3 as a mobile phase. The flow rate was 0.5 mL min^{-1}, the sample volume 20 µL and the column temperature 25 °C. The calibration plot was obtained by linear regression of peak-area ratio against concentration. As reference dextran standard were used.

6.4 Results and Discussion

In a first stage, PSA based hydrogels were produced. The hydrogels contained 95% water and 5% solid material (PGA and alizarin) which is in agreement with the literature [30]. Release of alizarin from PSA-$CaCl_2$ by enzyme catalysed hydrolysis was studied.

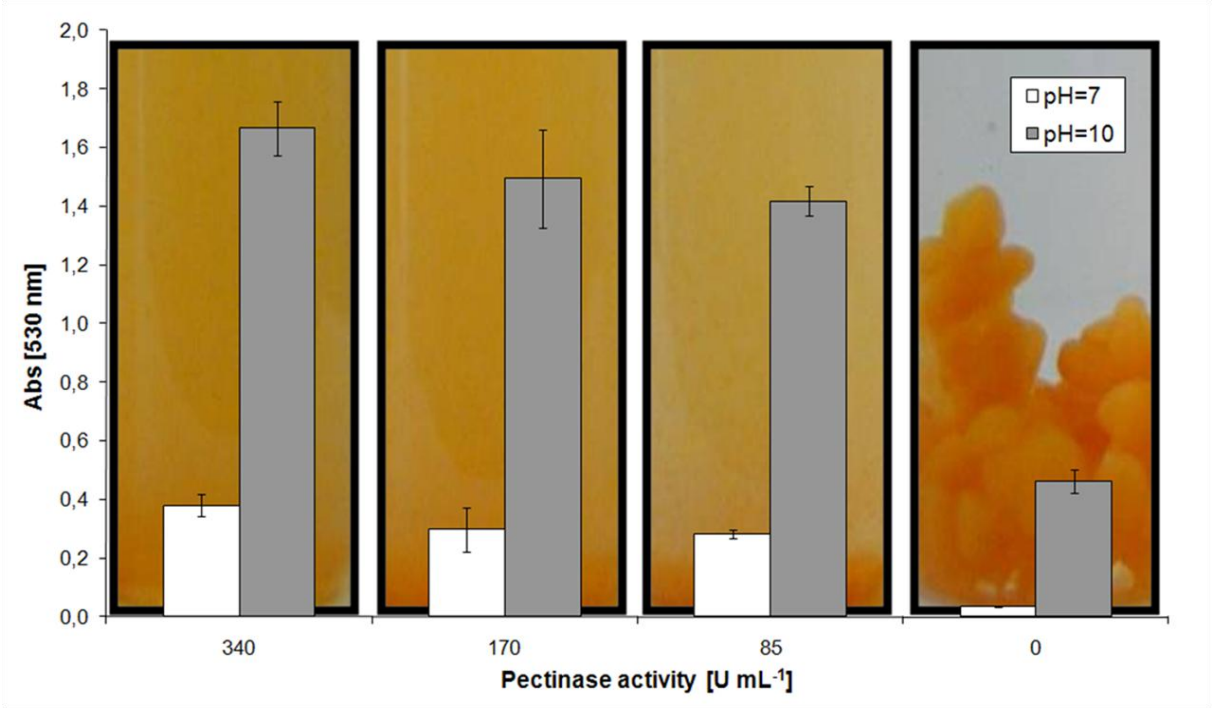

Figure 23 Release of alizarin from PSA-$CaCl_2$ upon incubation with pectinase from *Aspergillus niger* for 24 hours. Supernatants were spectrophometrically analysed at two different pH values while the background images show visible degradation.

Incubation with 85 Uml^{-1} pectinase from *Aspergillus niger* for 24 hours led to a release of 413 ± 24 µM alizarin corresponding to approximately 25 % of the alizarin incorporated. There was a linear relation between release of alizarin (5 to 348 µM) and enzyme activity in a range of 0-300 U ml^{-1} dosed. However, during the same incubation time, some of the alizarin (103 ± 11 µM) was also released from blanks although this was not evident by visual inspection. In the absence of enzymes, a transparent aqueous phase and the well-structured PSA-$CaCl_2$ (Figure 23, background picture) is visible while upon incubation with pectinases PSA-$CaCl_2$ is degraded resulting in a homogenous supernatant with orange colour. To enhance the signal, the pH was elevated before spectrophotometric analysis of the supernatant leading to a colour change from orange to dark purple with a fivefold (i.e. 5.41 ± 0.50– fold) increase of the molar extinction coefficient. Thus, the use of this pH sensitive dye can increase the detection limit. Upon incubation of alizarin dyed PSA-$CaCl_2$ with *Bacillus subtilis* a release of 588 ± 05 µM alizarin compared to 102 ± 24 (reference) was

measured. This indicates that extracellular enzymes from these organisms can act as triggers of this system.

To reduce the release of alizarin in the absence enzymes, cross-linked PSA-GMA based hydrogels were produced and investigated. Using GMA as covalently bound organic linker, the degree of cross-linking and thus the release properties can be tuned. In this study a ratio of 2:1 PSA to GMA was used. GMA based cross-linking has previously been described for polysaccharides including galactomanan or polygalacturonate [16, 31]. These authors have performed the modification either in dimethylsulfoxide (DMSO) with subsequent precipitation of the product in ethanol or simply in water. Here, to avoid a precipitation step the reaction was performed in distilled water. Sodium persulfate was used as radical source at elevated temperature to start the polymerisation process. TEMED (Tetramethylethylendiamin) which was previously employed for this reaction [31] was avoided since it reacts with alizarin.

It's known in literature that polysaccharides like polygalacturonic acid or pectin are sensible to high temperature. G. Li and K. C. Chang [32] showed that the viscosity is reduced after heating a pectin solution up to 100 °C for 5 minutes. Here, polymerisation around 65°C did not change the distribution of the molecular weight of PSA significant. In preliminary experiments the polysaccharide incubated at 30 °C and 70 °C for 45 minutes size exclusion chromatography did not reveal any significant degradation.

Cross-linked polysaccharide hydrogels were analysed with FTIR-ATR and significant peaks of the covalently bound methacrylate groups were seen.

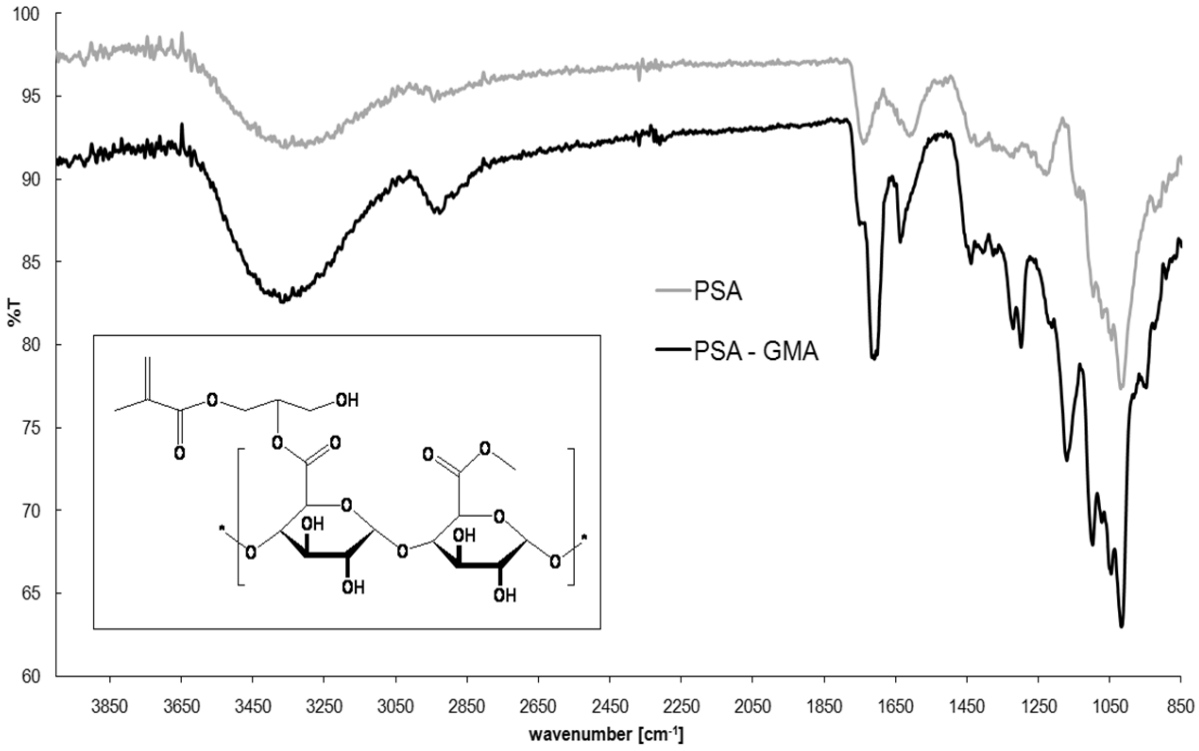

Figure 24 FTIR-ATR analysis of PSA and PSA-GMA

The hydrogel matrix consists of ALC (10% of solid mass) and PGA (90% of solid mass). Both were tested in a mixture as well as pure substances. The occurrence of two peaks is in agreement with previous studies on cross-linking of polysaccharides (Figure 24).

The characteristic shape of the bands (shoulder) at 1750 cm^{-1} represents the C=O bound of the glucuronic acid. The PSA spectra showed a peak according to the wavenumber without that shoulder. This shoulder at 1718 cm^{-1} and a small peak at 1660 cm^{-1} correlates to the carbonylic peak and the C=C double bound of the methacrylate according to literature. [16, 31]

A similar change in the spectrum was previously reported for cross-linked galactomanan, a structural related polysaccharide to PGA, confirming success of cross-linking of PGA in this study [16, 31] with general knowledge on bands of the related functional groups [33] and references to the covalent character of the GMA binding to the PSA surface.

The reaction was also carried out with both individual components (alginic acid and polygalacturonic acid) to prove that signals from each component will not overlay the significant GMA bands.

To further prove the covalent linkage of GMA the degree of esterification was measured and showed that percentage of the occupied esters of the modified PSA (54.0 ± 2.2) was significantly increased compared to the unmodified reference (30.5 ± 1.7).

The cross-linked PSA hydrogel samples were incubated with pectinase from *A. niger* and with *B. subtilis*. The surface of the bioresponsive polymers was characterised using environmental scanning electron microscopy (ESEM), which offers the possibility of imaging without the need of an additional conductive layer (e.g. Carbon or Gold) on the surface of a sample. The analysis was carried out at low vacuum mode (80 Pa) enabling a variety of characterization methods for life science as well as materials science even for the 3D reconstruction of specimens or for *in situ* studies [34-36]. However, here the specimens were fixed on a conventional specimen stub without further preparation using a double-sided carbon tape for mounting.

Enzymatic hydrolysis of cross-linked PSA hydrogel resulted in a smoothening of the surface (Figure 25, images a and b). Similarly to the enzyme induced release of alizarin, incubation with *Bacillus subtilis* shows significant differences when compared to the blank (image d). The needles resulting from hydrolysis are most likely a result of exposure of the incorporated alizarin.

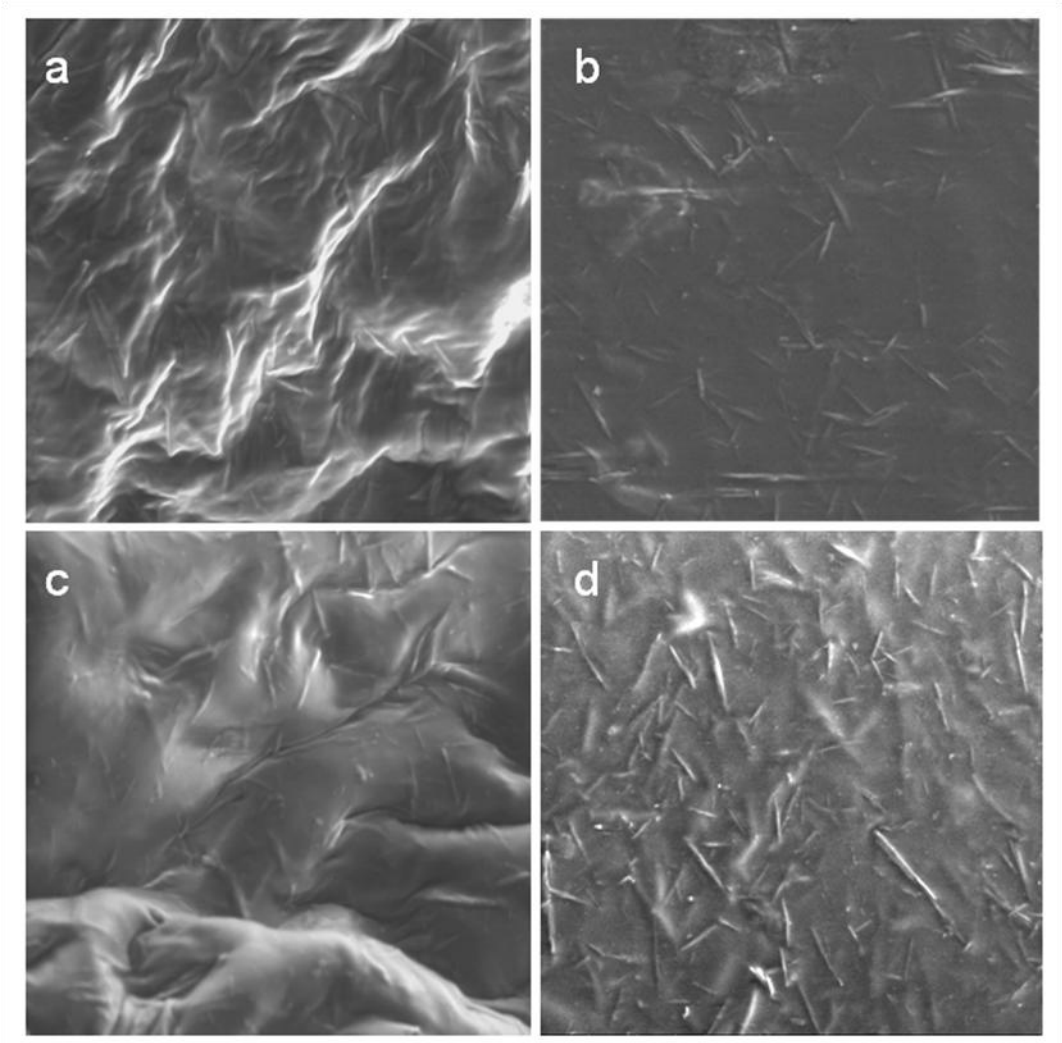

Figure 25 GMA-cross-linked PSA dyed with alizarin after incubation with b) pectinase from *A. niger* (200 U/mL) and d) *Bacillus subtilis* for 12 and 24 hours, respectively, compared to the corresponding blanks (a: incubated in 25 mM potassium phosphate buffer pH 6.5; c: minimal media culture as described for bacteria experiments (image width: 140 µm).

In contrast to the PSA-$CaCl_2$ system, the PSA-GMA shows no significant release of dye from the blank while after 2.5 hours 215 µM alizarin were released upon incubation with 50 U ml^{-1} pectinase. In the absence of enzymes, a transparent aqueous phase and the well-structured PSA-GMA (Figure 4, background picture) is visible while upon incubation with pectinases PSA-GMA is degraded resulting in a homogenous supernatant with orange colour (Figure 26).

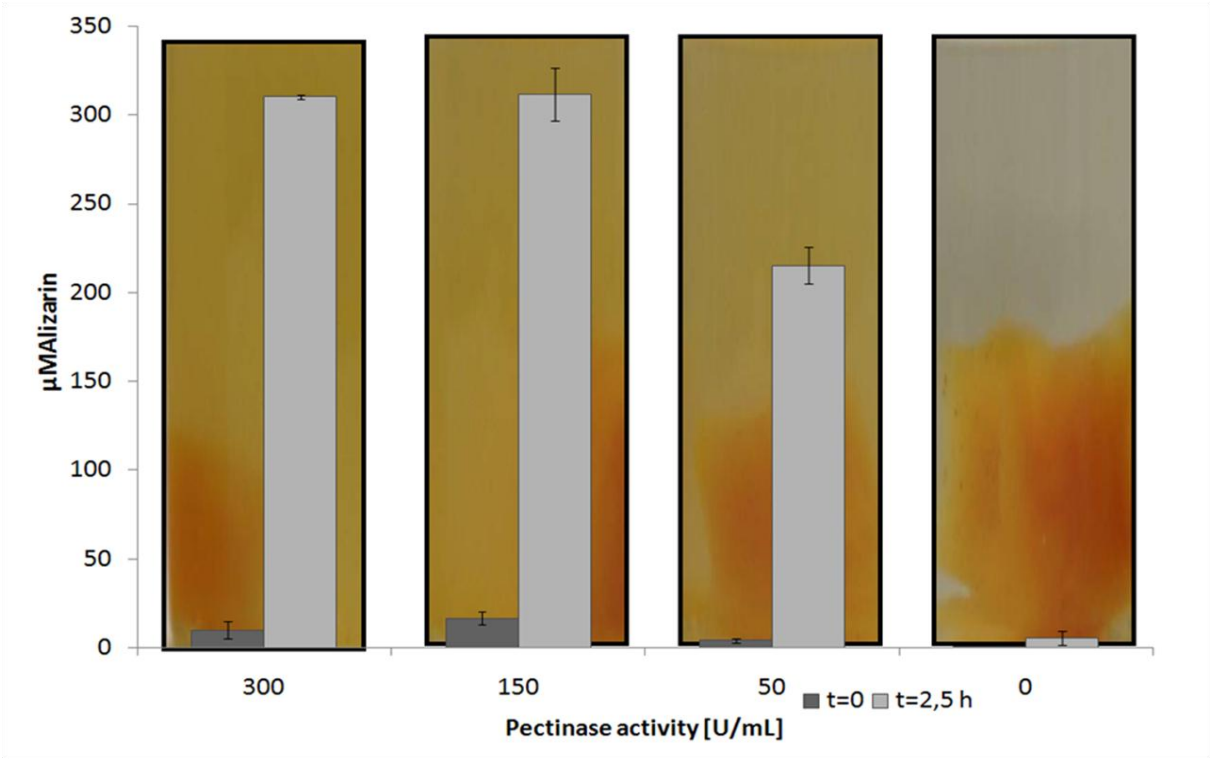

Figure 26 Release of alizarin from PSA-GMA upon incubation with pectinase from *A. niger* after 2.5 hours of incubation and analysis at pH 10. The background images show visible degradation.

Also, the hydrogel was stable for a long time period with less than 5% alizarin release due diffusion within 1 month. Thus, covalent GMA cross-linking has greatly improved the release properties when compared to the PSA-$CaCl_2$ system.

In the latter system, ions can be exchanged as well as washed out due diffusion. Consequently, when the salt concentration in the liquid medium is lower than in the hydrogel network, ions will diffuse to the liquid phase which strongly changes the properties of the hydrogel [17, 18].

In addition to the commercial pectinase from *A. niger*, release of alizarin from PSA-GMA was studied with a purified fungal pectinase from *S. rolfsii* and a purified bacterial pectat lyase from *B. pumilus*. PG1 from *S rolfsii* released 272 µM, while PL from *B. pumilus* released 250 µM which is slightly more than the *A. niger* pectinase preparation when dosed at the same standard activity. *S. rolfsii* produces several polygalacturonases (pectinases). Apart from different substrate specificities on oligomer substrates, they show a different mode of action on polygalacturonate. Based on viscosity reduction measurements we have previously shown that PG1 acts more endo-wise [19]. Consequently, this enzyme should have a larger impact on release of alizarin from the devices studied here. The pectat lyase from *B. pumilus* shows a similar relationship between the specific fluidity of polygalacturonic acid and the released reducing sugars like PG1 from *S. rolfsii* [20]. Consequently these enzymes

released similar amounts of alizarin. Slightly lower release determined for the *A. niger* pectinase preparation could due to the fact that this commercial preparation contains a variety of *exo*-acting glycosidase activities [37] which contribute to the standard activity measurement but have a lower effect in "opening" the devices than the endo-acting enzymes from *S. rolfsii* and *B. pumilus*.

After studying enzyme triggered release of alizarin, the response of the devices towards microorganism were tested. Polygalacturonic acids and its structural related natural polysaccharides can be degraded by different types of enzymes. Many fungi secrete high amounts of polygalacturonase [20, 19] while especially the production of pectat lyase is widely distributed among bacteria [24, 26]. Candidates with a potential degradation activity beside pectinases, pectat lyases and pectin hydrolases are rhamnogalacturonan hydrolase, rhamnogalacturonan lyase, rhamnogalacturonan rhamnohydrolase, rhamnogalacturonan galactohydrolase [38- 41] as well as α-arabinofuranosidase, endoarabinase, β-galactosidase or endogalactanase [42]. Here, *B. subtilis and Y. entercolitica,* were used as models for contaminating organism (Figure 27).

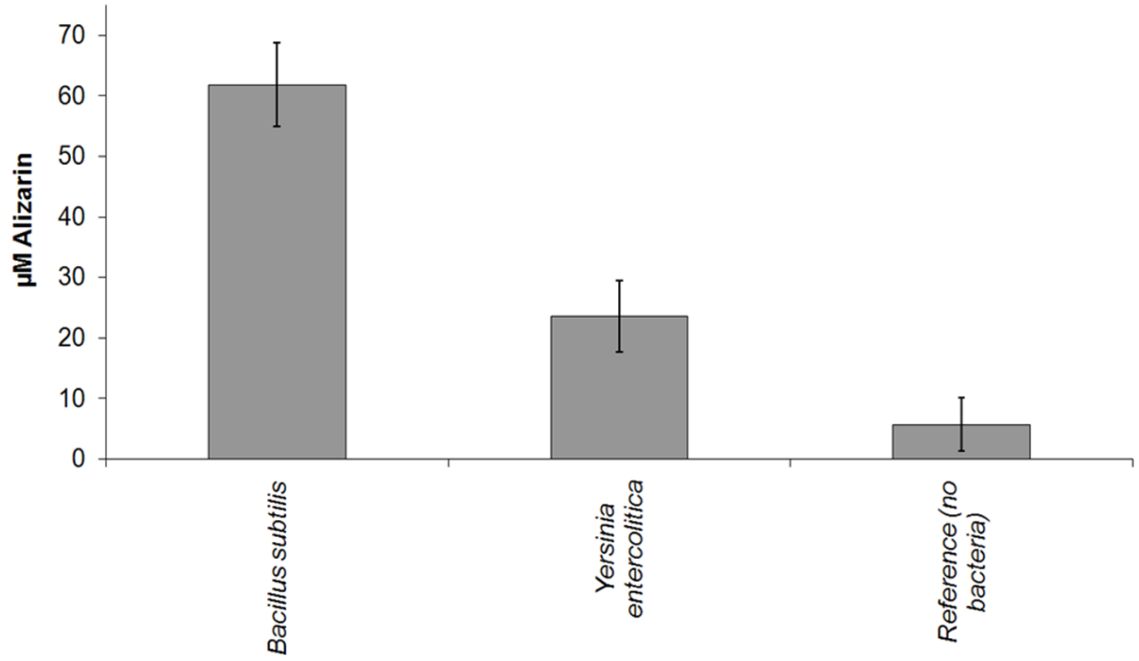

Figure 27 Release of alizarin from PGA-GMA upon incubation with *B. subtilis* and *Y. entercolitica* for two days

Bacillus subtilis was able to degrade both types of hydrogels which accords to the work of Cao et al. and S. Ahlawat et al. [24, 43] based on secreted pectinases [42, 24]. For the PSA-$CaCl_2$, a much higher release of alizarin was seen. For example, B. subtilis released 588 µM from the calcium based hydrogel compared to 62 µm from the covalent cross-linked PSA-GMA (Figure 27). However, for the PSA-$CaCl_2$ the reference was also releasing considerable amounts alizarin (102 µM).

In case of the PSA-$CaCl_2$ based system, the calcium may play an additional role. On the one hand, calcium itself is required by microorganisms as cofactor in cells. On the other hand, it is known and well described that calcium chloride can be toxic for bacteria. For example, the growth of the gram negative aerobic bacterium *Rhizobium meliloti* can be negatively influenced by calcium concentration between 300 to 3000µM [44]. In contrast to the PGA-$CaCl_2$ based system, the release of alizarin from PSA-GMA systems should be mainly due to partial hydrolysis of the PGA by extracellular enzymes.

The ability of *B. subtilis* to produce pectinases is well described in literature [24-26]. Similarly, the foodborne human pathogen *Y. entercolitica* has been described to produce both a 55 kDa pectat lyase and a 66 kDa polygalacturonase. Analysis of oligomers released from PGA indicated that the polygalacturonase is an exo-acting enzyme while the pectat lyase released larger fragment endo-wise [45]. This is in agreement with the fact that *Y. entercolitica* was able to degrade PSA-GMA resulting in the release of a significant amount of alizarin compared to reference (23 µM alizarin to 6 µM alizarin).

To allow visual inspection of alizarin release from PSA-GMA and to improve long-term stability the device was covered with a covalently attached PET-membrane. Therefore, to increase the number of hydroxyl groups for GMA crosslinking with PSA a novel approach based on partial enzymatic hydrolysis of the PET surface was used [29].

Enzymes were able to penetrate through the PET membrane which changed its colour from white to orange due to release of alizarin (Figure 28). The release of alizarin after 24 hours of incubation as quantified in the supernatant (15 µM alizarin when using 150 Uml^{-1} pectinase from *A. niger*.) was significantly lower when compared to PSA-GMA system without PET membrane (312 µM alizarin when using 150 U ml^{-1} without PET coating compared to 15 µM alizarin after 24h using a PET membrane see Figure 26 and Figure 28)

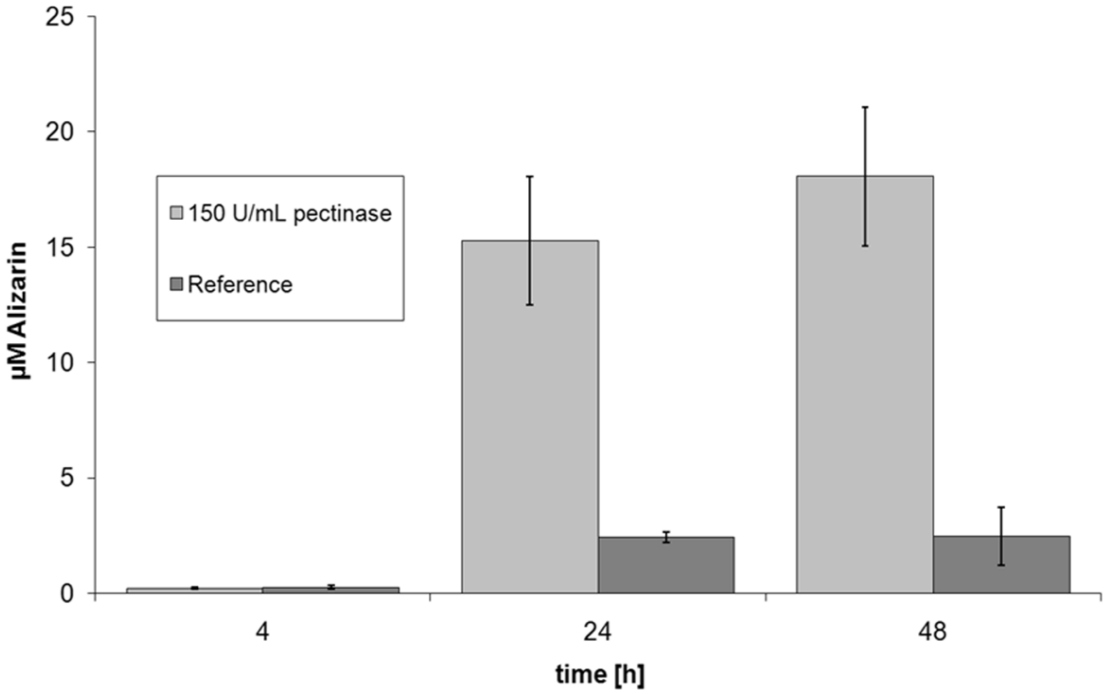

Figure 28 Controlled release of alizarin out of a device consisting of a PET membrane protected PSA_GSA hydrogel

6.5 Conclusion

The potential of two polygalacturonate based hydrogels to act as bioresponsive devices were demonstrated. Therefore, a simple hydrogel made by gelation of PSA in a calcium chloride solution and a more complex chemically modified PSA radically polymerised were constructed. The calcium chloride based polygalacturonic hydrogel (PSA-$CaCl_2$) showed a lower stability in water compared to the cross-linked system. Crosslinking was achieved with glycidylmethacrylate and a controlled release of the test molecule alizarin was demonstrated for purified galacturonase and pectat lyase and for bacteria. For further protection, the hydrogel was covalently bound to an enzymatically activated PET membrane which was despite a slower dye release an enhancement of stability of the system. Future studies should involve testing of the response of these materials towards a wider range of microorganisms. Also, integration of other poly- or oligosaccharides into the system could be a strategy to adapt the system to a desired range of organisms. Application related development should finally involve optimisation of the device properties (e.g. via tuning the degree of cross-linking) for specific applications considering the influence of all components and additives in food (packaging) materials.

6.6 Acknowledgement

This study was performed within the COMET K-Project MacroFun. Financial support from the FFG, the SFG and the Province of Styria is gratefully acknowledged.

Important measurements were done by Annemarie Marold, Teresa Flock, Alexander Schenk and Thomas Rath.

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7 BIORESPONSIVE SYSTEMS BASED ON CROSS-LINKED POLYSACCHARIDE HYDROGELS

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7.1 Abstract

Detection of bacterial and fungal contamination is of extreme importance in the fields of medical products or food packaging. Here a diagnostic tool based on pectinase and cellulase triggered release of a dye from a cross-linked polysaccharide matrix was developed. The hydrogel-based bioresponsive matrix consisted of carboxymethylcellulose (CMC) as a substrate for cellulases and polygalacturonate (PGA) as substrate for pectinases. To improve the stability of the hydrogels, methacrylic groups were inserted as crosslinking molecules. For polymerisation, two different methods were used, namely UV and thermal crosslinking. Controlled release triggered by extracellular enzymes of potentially pathogenic/contaminating microorganisms was investigated by the incorporation of alizarin into the hydrogels. UV polymerised hydrogels turned out to be more suitable than thermal cross-linked polymers. Integration of such polymer based bioresponsive systems in medical surfaces or package systems could therefore act as an in situ monitoring system for detecting the presence of bacteria or fungi like *Aspergillus sp.*

Keywords: Diagnosis, Carboxymethylcellulose, Cellulase, Pectinase

7.2 Introduction

The role of microorganisms as a source of contamination of food products is well known [1-3], while the presence of potentially pathogenic microorganisms in hospitals causing infections and/or diseases is a problem that needs urgent address. A wide range of pathogenic microorganisms, both bacteria and fungi, are able to release toxic metabolites into their environment. Therefore there is a strong need for a cheap and simple, but yet specific systems to reduce this risk of contamination, particularly for special products like medical devices or food packages. Beside bacterial contamination, mainly fungi and fungal spores which can easily be inhaled can cause severe complications for immunosuppressed patients. *Aspergillus*, for example, is a ubiquitous dimorphic fungus with global distribution [4]. More than 300 *Aspergillus* species have been identified, whereof only 19 cause human diseases [5]. *Aspergillus fumigates* represents the most common species, whereas *Aspergillus niger, Aspergillus flavus* and *Aspergillus terreus* are frequently responsible for human infection [6]. Although *Aspergillus spp.* can virtually infect any organ, the lung is by far the most commonly affected part of the body. Within the lung, a spectrum of diseases exists depending on the host`s immune status. While *Aspergillus* can colonise in the airways of immunocompetent patients, a weakened immune defence in severely immunocompromised persons can lead to tissue invasion [7].

A bioresponsive system placed in hospital environments or directly embedded in medical devices or in packaging systems [8], could indicate the presence of microorganisms and thus help to avoid the risk of contamination and infection. Currently sophisticated methods like PCR or standard microbial techniques are used for detection and identification of hazardous microorganisms [9-12]. These methods not only require special expertise and equipment but also cause partially high costs. Furthermore, the integration into devices or products is not possible. Therefore the use of bioresponsive systems provides a cheap alternative to the established techniques. In general, bioresponsive polymers and capsules can use simple environmental triggers like pH or temperature changes [13, 14], where an improved selectivity can be reached by the use of enzymes as triggers to initiate a response. Extracellular microbial enzymes specifically interact with a number of polymers. These biochemical reactions could be transformed into a simple signal, *e.g.* colour change, indicating the presence of microorganisms [13, 14]. Such polymer based bioresponsive systems integrated in medical surfaces or package systems could therefore act as an in situ monitoring system and is schematicly shown in Figure 29 [15-17].

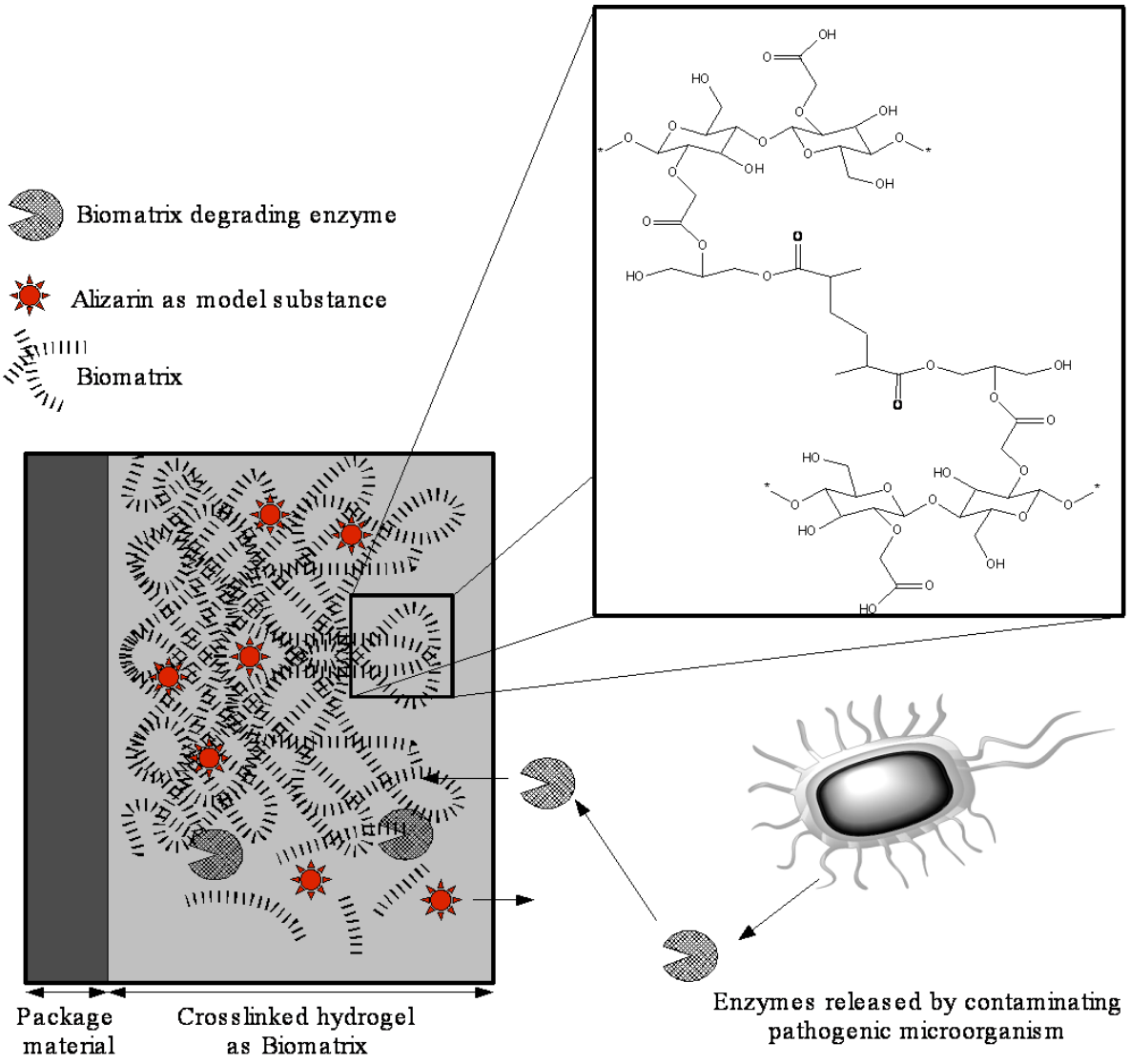

Figure 29 Schematic presentation of a bioresponsive system

In this study, carboxymethylcellulose (CMC), as a substrate for cellulases [18-20] and a combination of CMC with polygalacturonate (PGA) as substrates for pectinases were used for the design of hydrogel-based bioresponsive systems. To improve the stability of the hydrogels, methacrylic groups were inserted as crosslinking molecules. Organic cross linkers can easily be used in different ratios between linker and backbone, providing stability against auto degradation. This method compares favourably with *e.g.* alginate based hydrogels [21, 22]. Hydrogels like this have widely been used for biomedical applications as they can be transformed by different enzymes [23]. The production of extracellular cellulases by different *Aspergillus species*, which are able to hydrolyse CMC besides other cellulose substrates, is well examined [24-26].

Likewise pectinases which catalyse the hydrolysis of PGA are well studied while several *Aspergillus sp*. pectinases are industrially used [27, 28]. In addition to fungi, cellulases as well as pectinases are produced and secreted by different bacterial species like *Bacillus sp.* [29]. In this study, commercial cellulases and pectinases from *Aspergillus sp.* and purified endoglucanases (EGS and EGT) from the respective fungi *Gloeophyllum sepiarium* and *Gloeophyllum trabeum* [30], as well as pectin lyases from *Bacillus pumilus* [31] and polygalacturonases from *Sclerotium rolfsii* [32], were tested with different hydrogels to simulate degradation reactions caused by extracellular enzymes of contaminating bacteria.

Enzyme controlled release was investigated by incorporating alizarin into the hydrogels. Alizarin release was measured after changing the pH from neutral up to pH 10 as a colour change from light orange to dark violet [33].

7.3 Material and Methods

7.3.1 Chemicals and enzymes

Carboxymethylcellulose (CMC), polygalacturonate from citrus peel (PGA) and HPLC grade dichloromethane and media components were purchased from Carl Roth (Karlsruhe, Germany). Technical grade alizarin, glycidylmethacrylate (GMA), sodium persulfate as well as cellulase (endoglucanase) and pectinase from *Aspergillus sp.* from Sigma Aldrich (St. Louis, USA) were used. In addition, purified endoglucanases (EGS and EGT) from the respective fungi *Gloeophyllum sepiarium* and *Gloeophyllum trabeum* and PG 1 (39.5 kDa) polygalacturonase from *Sclerotium rolfsii* and the pectat lyase from *Bacillus pumilus* were produced and purified as previously described [30-32].

7.3.2 Staining of polysaccharide raw materials (CMC and CMC/PGA)

For the incorporation of alizarin, 10.0 g of the polysaccharide (CMC or CMC/ PGA blends) and 0.4 g alizarin were dispersed in 50 mL dichloromethane and heated up to 50 °C under reflux conditions. After 1 h, the solvent was removed by filtration and the polysaccharide containing alizarin was dried under vacuum. To reduce the risk of contamination, the stained CMC and CMC/PGA was sterilised by UV irradiation overnight. The water was sterilised by ultrafiltration.

7.3.3 Cross-linking of polysaccharide hydrogels (CMC-GMA; CMC/PGA-GMA)

The preparation of glycidylmethacrylate (GMA) modified polysaccharide was carried out according to a modified procedure previously described [34]. Briefly, 2.5 g of the stained polysaccharide, either CMC or CMC/PGA, was dissolved in 100 mL distilled water. Thereafter, 2.5 (LD) or 5.0 mL (HD) (to obtain two hydrogels with two different linker backbone ratios 1:1 and 2:1 w/w) of GMA solution and 0.5 mL 6 M HCl to lower the pH, was added and incubated for 48 h at 40 °C. The samples were stored at 4°C.

7.3.4 Polymerisation of the modified polysaccharides

Two different polymerisation processes were used in this work; namely polymerisation by elevated temperature and by UV light irradiation.

7.3.4.1 Polymerisation by elevated temperature

The modified polysaccharide solution was poured into a heat resistant glass cup with an approximated base area of 10 × 20 cm, until a thickness of 5.0 mm of the CMC-GMA or CMC/PGA-GMA was reached. To start the radical polymerisation process, 50 mg sodium persulfate as radical source was added and the mixture was heated up to 60 °C for 30 minutes. After cooling down to room temperature, the hydrogel was cut into 1.5 x 1.5 cm pieces. The hydrogel was washed with distilled water and transferred to 20 mL glass containers for the enzymatic experiments.

7.3.4.2 Polymerisation by UV light irradiation

30 g of the modified polysaccharide solution was mixed with 30 mg sodium persulfate and 30 mg Irgacure and placed under a UV lamp until the solution showed typical hydrogel like properties (40 to 60 min). The formed hydrogel was cooled down to room temperature overnight and stored at 4 °C under sterile conditions.

7.3.5 Enzyme triggered release experiments

1 g wet mass of the hydrogels (CMC-GMA and CMC/PGA-GMA) was added to 10 mL 25 mM citrate buffer (pH 4.5). Thereafter, 100 µL of enzyme solution with different activities as indicated below were added. 25 mM citrate buffer pH 4.5 was used for dilution of the enzyme stock solutions. The samples were incubated at room temperature for 4 up to 48 hours. 250 µL of sample was taken from the supernatant and 25 µL of a 1 M glycine buffer pH 10 were added. After centrifugation for 5 min at 5000 x g, 200 µL were transferred into a transparent polystyrene 96 well plate , purchased from Greiner bio-one (Kremsmünster, Austria). The released alizarin was quantified photometrically using a Tecan Infinite 200 Platereader (Tecan, Maennedorf, Switzerland) at wavelengths between 490 and 510 nm.

7.3.6 Cultivation media for fungi and bacteria induced release experiments

A salt solution containing 2.5 g L^{-1} KH_2PO_4, 3.0 g L^{-1} K_2HPO_4, 2.0 g L^{-1} $(NH_4)SO_4$, 0.2g L^{-1} $MgSO_4$ x 7 H_2O and 5 mL L^{-1} of a micronutrient solution [35] was separately autoclaved from the C-source solution consisting 1 g L^{-1} glucose and 1 g L^{-1} polygalacturonate and 1 g carboxymethylcellulose. The pH value was set to pH value of 5.0. Incubation of microorganisms was started by adding an amount of fungi and bacteria cultures directly from a PDA media plate. The test samples were incubated for 4 days at 25°C.

7.3.7 Enzyme assays

Endoglucanase, polygalacturonase and pectat lyase activities were measured as previously described [30-32]. Essentially reducing sugars released from PGA and CMC were quantified [36-38].

7.3.8 Characterization of Polysaccharide hydrogels

Raman spectra were recorded using a Renishaw Raman System-1000 spectrometer, equipped with a 633 nm HeNe Laser linked to an Olympus Microscope with a 50x magnification objective (Gloucestershire, Great Britain). This microscope focussed the laser to spot size of about 1-2 µm. Surface analysis of the thermal polymerised hydrogels as well as of the hydrogels using UV irradiation for polymerisation, was done using a Philips 30 XL 30 SEM and the UV Zeiss Evo 60 (Carl Zeiss, Oberkochen, Germany). All micrographs were taken at electron energy of 5 keV in a high vacuum mode.

7.4 Results and Discussion

7.4.1 Modification of CMC

In this study, bioresponsive devices based on polysaccharide hydrogels for the detection of pathogenic/contaminating microorganisms were constructed. To improve the stability of the bioresponsive hydrogels, the polysaccharides were modified by covalent functionalisation using methacrylic groups. Two different ratios between linker molecules and the polysaccharide backbone were applied (LD: GMA:PS = 1:1 and HD: GMA:PS = 2:1), while polymerisation was initiated either with sodium persulfate and a temperature of 60°C, or by UV light irradiation with Irgacure as a radical initiator. The modification process was monitored by using Raman spectroscopy (Figure 30).

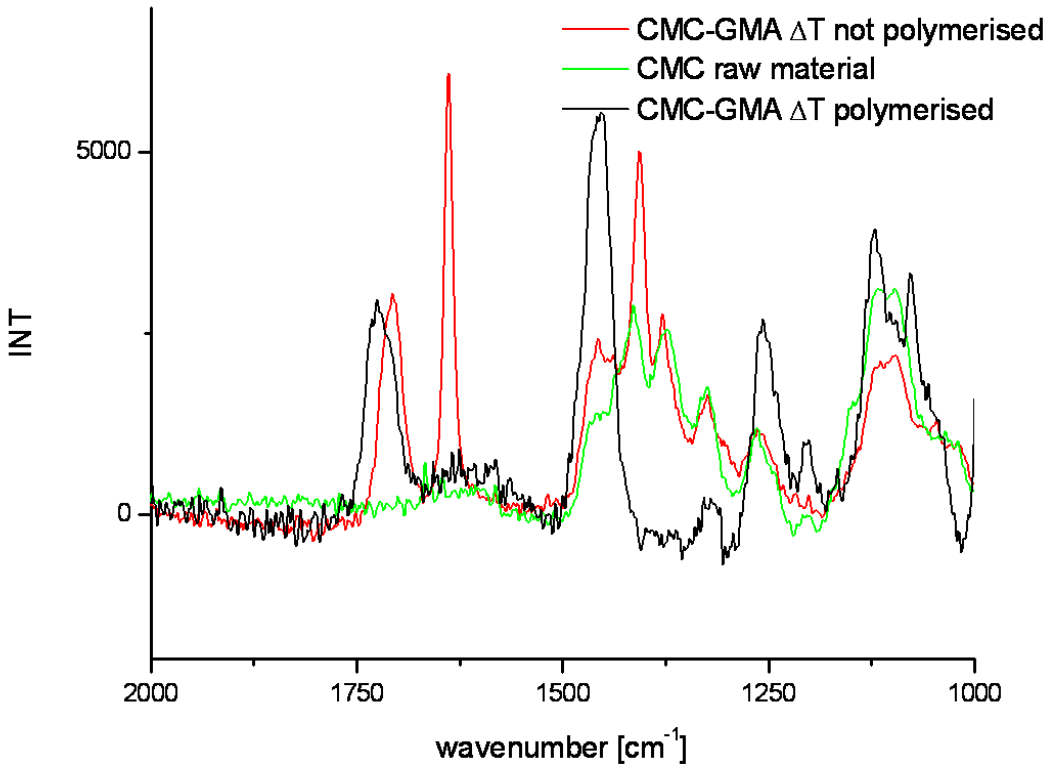

Figure 30 Raman spectrum of CMC, CMC-GMA not polymerised and CMC-GMA polymerised at 60°C (Settings: 100% laser power, 10 accumulation measurements and 50 x magnification objective). To alleviate the peak arrangement, a simple baseline correction was done.

Overlaid Raman spectra of pure CMC, CMC modified (CMC-GMA) and thermal polymerised (CMC-GMA) hydrogels are shown in figure 2. Significant intensity peaks occur at 1640 and 1450 cm^{-1}. The intensity peak located at 1640 cm^{-1} represents a not polymerised CMC sample modified with methacrylic acid. According to the literature [38], this peak corresponds to C=C stretching of the double bond in methacrylic side groups. Expectedly upon polymerisation the double bond disappeared due to the formation of new $-(CH)_2-$ links between the methacrylic monomers. These $-(CH)_2-$ links give rise to an intensity peak at 1450 cm^{-1}; this peak was only visible in the Raman spectrum of the polymerised CMC-GMA hydrogels.

The potential of cellulose based hydrogels for other controlled release systems has previously been discussed. Naturally derived carbohydrate polymers have unique physical and biochemical properties, which make them suitable for bioengineering applications [39]. Beside biocompatibility, cellulose derivates are degradable and carry chemical groups available for modification [40]. The modification of the polysaccharide was done with the linkage of methacrylate groups which was also investigated by Reeves et al [40]. Crosslinking with methacrylate improved stability of the hydrogels on one hand, on the other hand decreased gel stiffness and a better degradability after enzymatic incubation was described [40]. The stability of the methacrylic cross-linked hydrogels was determined over a period of four weeks at room temperature without remarkable auto degradation processes. As enzymes are released only in presence of microorganisms, their application as triggers allows a higher selectivity compared to well describe physical triggers like pH, temperature changes or swelling processes. However, cellulose is not degradable by human enzymes, which is a great advantage for possible applications in medicine.

7.4.2 Enzyme triggered release of alizarin from bioresponsive polymers

To follow the enzymatic degradation of modified CMC-GMA, alizarin as a model substance was incorporated into the hydrogels. The release of alizarin can be measured by a simple photometric method. To enhance the signal, the pH was elevated before spectrophotometric analysis of the supernatant leading to a colour change from orange to dark purple, with a significant decrease of the molar extinction coefficient.

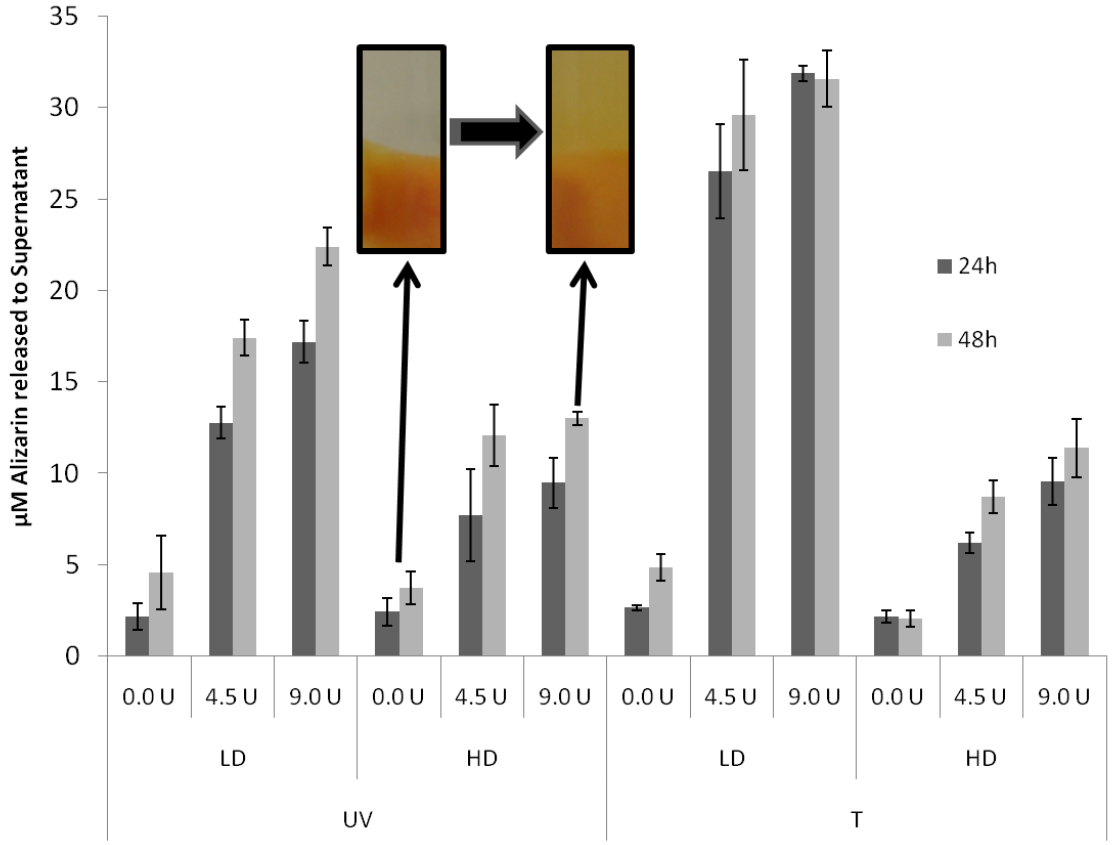

Figure 31 Release of alizarin from CMC-GMA hydrogels triggered by 4.5 and 9.0 U/g hydrogel endoglucanase from *Gloeophyllum sepiarium* compared to a blank (0.0 U). CMC-GMA was either thermally cross-linked (T) or cross-linked by UV light irradiation using Irgacure as a radical starter. The low density (LD) and high density (HD) hydrogels were prepared with CMC/GMA ratios of 1:1 and 1:2 (w/v).

The *Aspergillus* sp. cellulase was able to hydrolyse the CMC-hydrogels with different extents of cross-linking leading to release of alizarin (Figure 31). HD hydrogels exhibited a remarkable lower release of alizarin compared to LD hydrogels, both in case of temperature as well as UV polymerisation. Obviously the limiting factor is the sterical accessibility of CMC to the enzyme. The higher release seen for the thermally polymerised when compared to UV polymerised LD CMC-GMA could be due to partial hydrolysis of CMC caused by elevated temperature [41] during polymerisation, thus leading to an enhanced access of the enzyme. This could explain the higher release of alizarin in case of thermally polymerised LD CMC-GMA. However, auto-diffusion of alizarin does not seem to be affected by the polymerisation technique, since for both controls a similar release of 2.5 µM alizarin was seen. Compared to the UV polymerised LD CMC-GMA, a maximum alizarin release was seen after 24 h in case of the thermally polymerised LD CMC-GMA, while the maximum for UV

polymerised LD CMC-GMA was not reached after 48 hours of incubation. Additionally, the maximum amount of alizarin released from UV-HD CMC-GMA was only less than half of the amount measured for T-LD CMC-GMG, indicating that a major part of the UV-HD CMC-GMA hydrogel is resistant to enzymatic hydrolysis.

Extracellular enzymes, capable of hydrolysis of cellulose derivatives like carboxymethylcellulose, are released by a variety of microorganisms [18, 19]. Expectedly, successful hydrolysis of the CMC-GMA hydrogels could be observed. In nature, cellulose is enzymatically hydrolysed by the synergistic action of endo-ß-1,4-glucanases, cellobiohydrolases and ß-glucosidases. It has been suggested that endoglucanases randomly cleave cellulose into smaller fragments generating new ends which are hydrolysed end-wise by the action of cellobiohydrolases. These latter enzymes are also thought to erode crystalline regions of cellulose making them more susceptible to endoglucanase attack [20, 42]

These enzymes are secreted by a range of bacteria like *B. subtilis, B. cereus or Clostridia sp.* [29] as well as by fungi like *Aspergillus sp.* [24-26].

7.4.3 Characterisation of Polysaccharide hydrogels

Surface analysis of the polymerised hydrogels using SEM was performed to investigate enzymatic hydrolysis more in detail. Upon incubation with cellulase, the surface roughness of the different CMC-GMA hydrogels increased compared to the controls, thus indicating enzymatic hydrolysis. In the enzyme treated samples wrinkles appeared, while deep clefts are visible at a higher magnification (Figure 32 and Figure 33).

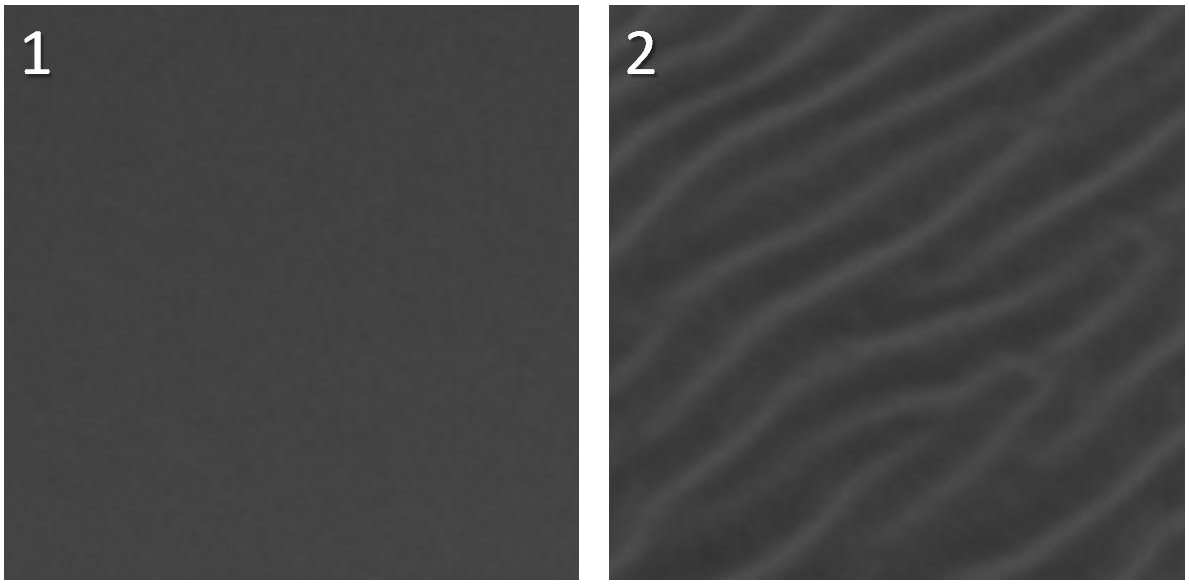

Figure 32 Surface morphology changes of CMC-GMA hydrogels after incubation with endoglucanase from *Gloeophyllum sepiarium*. SEM pictures (Philips Zeiss Evo 60) of UV polymerised CMC-GMA hydrogel without enzymatic treatment (1) and after (2) treatment with 90 U ml^{-1}

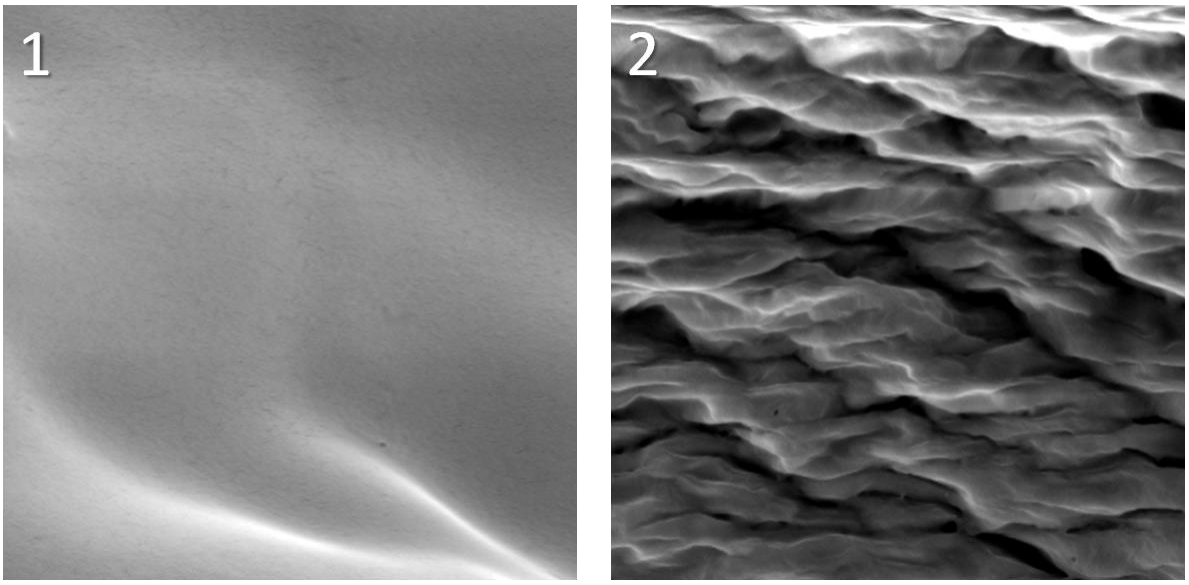

Figure 33 Surface morphology changes of CMC-GMA hydrogels after hydrolysis with endoglucanases from *Gloeophyllum sepiarium*. SEM pictures (Philips Zeiss Evo 60) of thermally polymerised CMC-GMA hydrogel without enzymatic treatment (1) and after (2) treatment with 90 U mL^{-1} endoglucanase. Samples were stabilised with glutaraldehyde, lyophilised and coated with gold (image width: 10 µm).

Compared to the UV polymerised CMC-GMA, the thermal polymerised hydrogel showed a much more pronounced change in surface morphology after enzymatic incubation. This increased roughness correlates well with the enhanced release of alizarin shown in Figure 31.

7.4.4 Blends of bioresponsive polymers

To widen the specificity of the bioresponsive system, a second polysaccharide, namely polygalacturonic acid (PGA), was integrated into the hydrogels. CMC-GMA was blended at a ratio of 1:1 with a glycidylmethacrylate modified polygalacturonic acid (PGA-GMA) as previously described [43]. The blend was cross-linked via temperature or UV polymerisation.

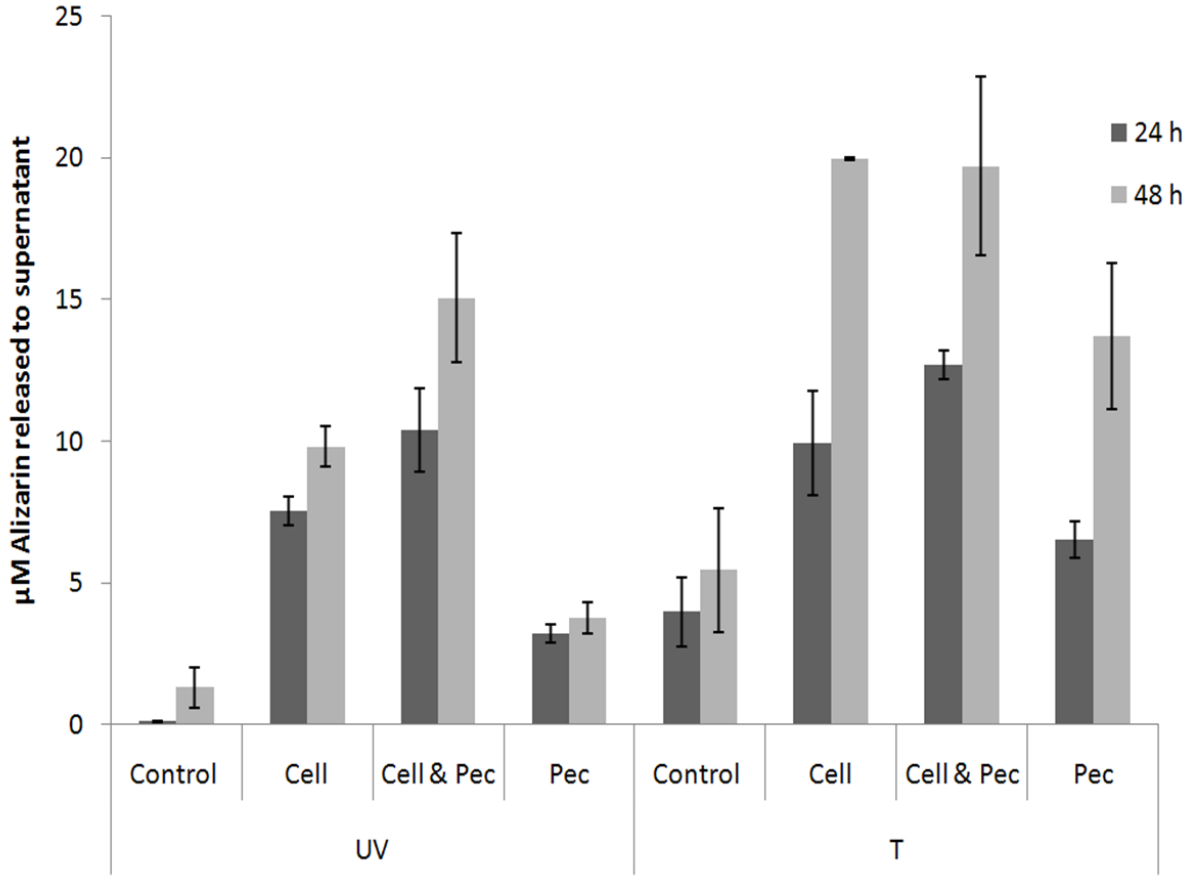

Figure 34 Release of alizarin from CMC/PGA – GMA hydrogels after incubation with endoglucanase and pectinase. The hydrogel was polymerised either by elevated temperature (T) or by UV irritation (UV). Activities of the used enzymes: "Cell": Purified endoglucanase from *Gloeophyllum sepiarium*.

Incubation of the CMC/PGA-GMA hydrogel blends with different enzymes led to an enzyme triggered release with both pectat lyase and endoglucanase. As there were used identical activities of the enzymes, the CMC fraction of CMC/PGA-GMA hydrogel moieties seems to be more susceptible to enzymatic hydrolysis than the PGA fraction (Figure 34). A combination of both enzymes led to an increased release for both hydrogels tested which was, however, not additive. Interestingly, the UV-polymerised hydrogels showed a considerable higher stability with less auto-diffusion of alizarin (control). This difference between thermal and UV polymerisation was not observed for CMC-GMA hydrogels (Figure 34).

The addition of a second enzyme substrate into a bioresponsive system thus indeed allowed a response to different enzymes (*i.e.* cellulases and pectinases). This will widen the range of microorganisms which can be detected with such bioresponsive systems. Polygalacturonic acid can be degraded by pectinases, as well as by polygalacturonases or pectat lyases [14, 32, 43]. All enzymes, which leads to a breakdown of pectin containing substrates are produced and secreted by numerous microorganisms, fungi [27, 28, 31, 32] and various bacterial species [44, 45].

Microorganism plays a major role in infection and contamination while both bacteria and fungi are able to damage food products by secreting enzymes [46]. Consequently, beside release experiments with commercial enzymes the bioresponsive polymer composites were finally tested with bacterial and fungal species. As representatives for contaminating/pathogenic fungi *Aspergillus niger* and *Trichoderma epidermidis* and as bacterial example *Bacillus subtilis* were used. Figure 35 demonstrates that these model organisms were able to trigger the release of alizarin due to production of extracellular enzymes. Although it is thus clearly possible to qualitatively detect the presence of microbes the signal (caused by enzymes) cannot be directly related to cell numbers since enzyme activities vary greatly between individual strains and environmental conditions [47]. A combination of polygalacturonic acid and carboxymethylcellulose for the preparation for bioresponsive devices is a good possibility to increase the sensitivity of the system to detect a broad range of potentially pathogenic or contaminating microorganism.

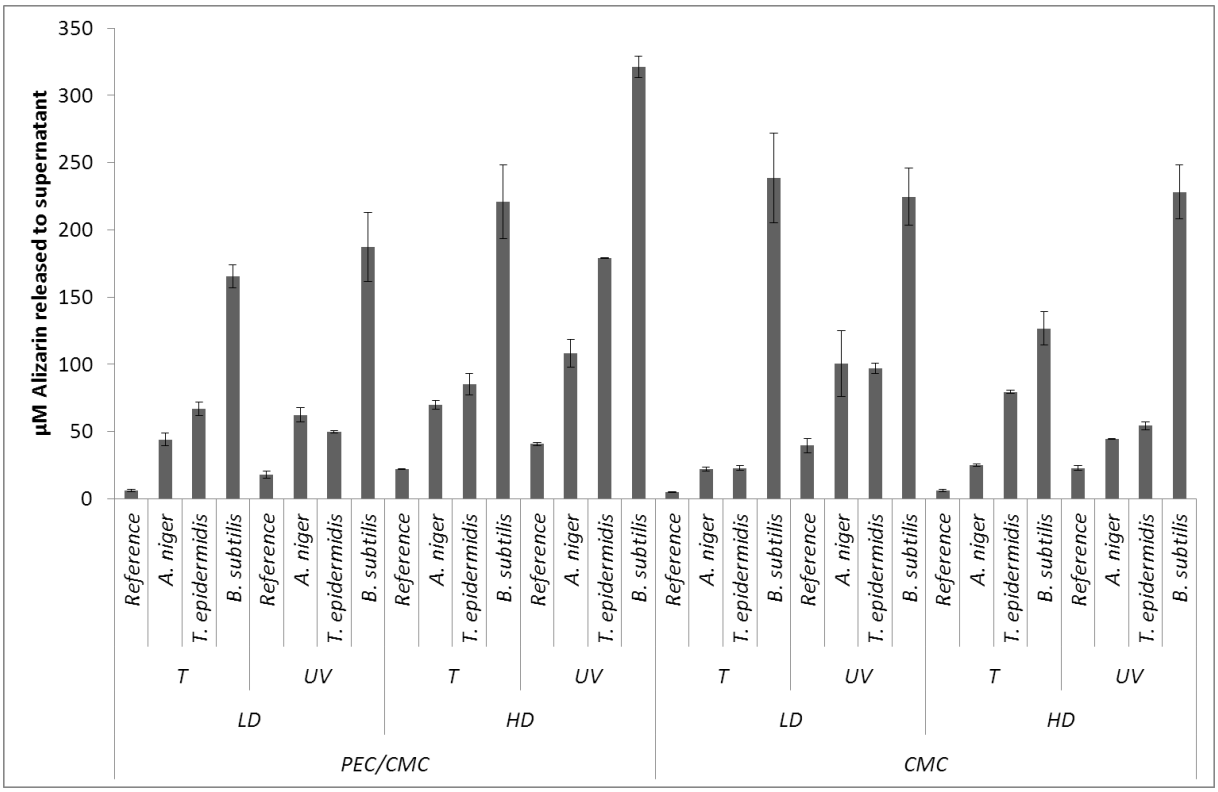

Figure 35 Triggered alizarin release from bioresponsive devices by potentially pathogenic / contaminating microorganisms. CMC-GMA was either thermally cross-linked (T) or cross-linked by UV light irradiation using Irgacure as a radical starter. The low density (LD) and high density (HD) hydrogels were prepared with CMC/GMA (labelled as PEC/CMC) ratios of 1:1 and 1:2 (w/v).

7.5 Conclusion

The construction of bioresponsive polymers described in this study and its application can be adapted depending on the enzyme pattern secreted by the target pathogenic/contaminating organisms. These systems would be suitable especially to detect the presence of *Aspergillus* spe. as they secrete (varying) proportions of cellulases and pectinases. Invasive aspergillosis is the most common mold infection worldwide due to an increasing number of immunosuppressed patients [48] resulting in high mortality rate of about 80% in high risk patients. Additionally, this system can also be used for the detection of contaminating microorganisms in medical applications and food technology [49, 50]. The potential of microbial detection was successfully demonstrated with bacteria (*Bacillus subtilis*) and fungi (*Aspergillus niger*, *Trichoderma epidermidis*). In future, alizarin could be displaced by an active agent like an antifungal or an antibacterial compound which would likewise be released in a controlled way by enzymes secreted by pathogenic microorganisms.

7.6 Acknowledgement

This study was performed within the COMET K-Project MacroFun. Financial support from the FFG, the SFG, the COST Action 868 and the Province of Styria is gratefully acknowledged. Important measurements were done by Cornelia Rainer and Ulrike Gewessler.

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8 SIGNAL ENHANCEMENT IN POLYSACCHARIDE BASED SENSORS FOR INFECTIONS BY INCORPORATION OF CHEMICALLY MODIFIED LACCASE

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Keywords: Controlled release, enzyme trigger, Bioresponsive polymer, lysozyme, laccase

8.1 Abstract

Bioresponsive polymers (BRPs) allow the detection of potentially pathogenic microorganisms. Here, peptidoglycan and cellulose based hydrogels were constructed with potential for diagnosis of wound infection or *e.g.* Aspergillosis, respectively. These systems respond to extracellular enzymes from microbes or enzymes secreted from the human immune system in case of infection. Laccases as "Enhanzymes" were incorporated into these devices for signal and stability enhancement when compared to simple dye release based systems. To retain the Enhanzymes within the BRPs, they were either PEGylated (Laccase_PEG) to increase size or methacrylated (Laccase_MA) to allow covalent attachment to the polysaccharide matrices. PEGylation of *Trametes hirsuta* laccase led to a fivefold increase in size to 270 kDa according to size exclusion chromatography (SEC). Likewise, successful methacrylation of the laccase was demonstrated by using RP-chromatography while SEC analysis proved covalent attachment of the enzyme to the methacrylated polysaccharide matrix. Upon incubation of peptidoglycan based BRPs with fluid from infected wounds, the difference to controls was four times higher for Laccase_PEG based signalling when compared to simple dye release. Similarly, the control signals (*i.e.* leaching) were considerably reduced in case of Laccase_MA incorporated in cross-linked PG and CMC hydrogels for signalling. In addition, Laccase_MA catalysed colour formation enhanced the signal dramatically with factors between 100 and 600 fold. Laccase_MA was demonstrated to oxidise silica gel immobilised ferulic acid incorporated into the BRP with clearly visible colour changes of 4.5 ΔE units according the CIELab concept upon incubation by trigger enzymes as well as infected wound fluids.

8.2 Introduction

Various microorganisms, with pathogenic potential, release enzymes into the environment to acquire nutrients or to sustain their position against rivals. Terms like microorganisms and contamination/pathogenesis are therefore interconnected especially in the field of food products *e.g.* meat and meat products or medical applications like blood products and in wound care [1-3].

Established methods for detection of potentially pathogenic microorganisms like PCR or standard microbial techniques [4-6] are rather expensive and time consuming. An alternative strategy is therefore the development of detection systems comprising of biopolymers and using extracellular bacterial enzymes as triggers. Usually, simple environmental triggers like pH or temperature changes are used [7, 8]. The usage of extracellular enzymes as trigger signals, however, can improve the specificity against the presence of bacteria or fungi, thus leading to the controlled release of dyes or *e.g.* of antimicrobial drugs. The implementation of such bioresponsive polymers (BRPs) into packaging systems in food industry or bandage materials could generally avoid and reduce the risk of contaminations and infections. Recently, the prognostic value of different human enzymes concerning detection of beginning wound infection was shown [9-11]. Similarly, the potential of extracellular enzymes from food contaminating enzymes as triggers for controlled release from BRPs were demonstrated [12]. However, in both cases high sensitivity and stability is crucial. In immunology, the well-established ELISA assay makes use of an enzyme catalysed reaction to produce and enhance a colour signal. Here, we demonstrate that the sensitivity of BRPs can be enhanced by integration of enzymes catalysing a secondary colour forming reaction which concomitantly reduces background signals due to leaching of dyes. Laccases were selected to catalyse this secondary signal enhancing reaction and consequently termed “Enhanzymes”. As examples we used BRPs detecting lysozyme in infected wound fluids and cellulases secreted by potentially pathogenic microorganisms (e.g. Aspergillosis), respectively.

Elevated levels of lysozyme in wound fluids can be used for detection of wound infection [9]. Lysozyme is a well-known cell wall degrading enzyme discovered by Jan Fleming at the beginning of the last century [13]. The interaction of lysozyme and peptidoglycan as well as its degradation process are well described in literature [14]. Stained or unstained peptidoglycan from cell wall (*Micrococcus lysodeiktikus*) can be used as substrate to determine lysozyme activity. Here the fragile peptidoglycan was combined with a matrix polymer *e.g.* agarose or methacrylated carboxymethylcellulose forming a stable, enzyme responsive network.

As methacrylated carboxymethylcellulose (CMC_MA) itself can serve as enzyme substrate, the usage of cellulase as a trigger enzyme was tested for further application. Cellulases are able to hydrolyse the β-1,4-D-glucan linkage of the cellulose molecule to produce mainly glucose, cellobiose or cello-oligosaccharides [15, 16].

Aspergillus sp. are ubiquitous dimorphic fungi which are present worldwide [17]. Some of them cause human diseases like Aspergillosis [18]. *Aspergillus niger, Aspergillus flavus* and *Aspergillus terreus* are frequently responsible for human infection, mainly of the lung [19, 20]. *Aspergillus sp*. are well known for the secretion of cellulases [15, 21-23]. Among bacteria, *Streptomyces spp.* cause a broad range of human diseases *e.g.* lung abscess or pneumonitis [24, 25], while infections of the central venous catheter are affected by *Cellulosimicrobium cellulans* [26]. Likewise, these bacteria are known to secrete cellulases [27]. Apart from pathogenic microorganisms, a wide range of (food) contaminating microorganisms produce cellulases (together with other carbohydrolases) to access their nutrients. Beside natural cellulose substrates, cellulases are able to hydrolyse modified polysaccharides like carboxymethylcellulose (CMC) or polygalacturonic acid (PGA) [16, 28, 29]. Here, CMC hydrogels were used to respond to cellulases as trigger enzymes. To enhance their stability, methacrylic groups were inserted to serve as covalent cross-linkers building up a hydrogel network by simple radical polymerisation [12, 30].

Both PG and CMC hydrogels were loaded with laccases as Enhanzymes. Laccases (E 1.10.3.2) are multicopper containing oxygen oxidoreductases. They are able to oxidise various substances like phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, hydroxyindols, benzenethiols as well as inorganic/organic metal compounds by reduction of molecular oxygen to water [31, 32].

Biochemical and technical applications are well described in literature [33-39]. Recently, a technique for immobilisation of laccase substrates, *e.g.* ferulic acid, on different surfaces, was presented [40, 41]. Therefore and due to the fact they do not require other co-substrates than oxygen laccases were selected to catalyse a colour forming reaction within bioresponsive hydrogels.

8.3 Material and Methods

8.3.1 Chemicals

Carboxymethylcellulose (CMC, MW 70000 g mol^{-1}), Tetramethylethylendiamin (TEMED), buffers (succinic acid, glycine and potassium phosphate), ethanol absolute, ferulic acid and acetonitrile were purchased from Carl Roth (Karlsruhe, Germany). Monomethoxypolyethyleneglycol (MW 5000 g mol^{-1}) was supplied by Fluka (Buchs, Switzerland).

Glycidylmethacrylate solution (GMA), sodium persulfate, peptidoglycan from *Micrococcus lysodeiktikus* ATCC No. 498, 2,2-azinobis-(3-ethylbenzothiazoline-6-disulfonic acid)(3-aminopropyl)triethoxysilane (APTS), N-hydroxybenzotriazole (HOBT) , N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDAC), cyanuric chloride and remazol brilliant blue (RBB) were supplied by Sigma Aldrich (St. Louis, USA).

Ultrafiltration was performed with Vivaspin 20 centrifugal concentrator MWCO 30000 Da (Sartorius Goettingen, Germany).

8.3.2 Enzymes

Commercial cellulase from *Aspergillus niger* was obtained by Sigma Aldrich (St. Louis, USA) as well as lysozyme from chicken egg white. The laccase from *Trametes hirsuta* was produced and purified as previously described [42-44].

8.3.2.1 Modification of laccase

For PEG modification of the laccase (Laccase_PEG), 0.8 mmol of methoxypolyethylene glycol were dissolved in 100 mL of dried toluene and activated with 2.4 mmol cyanuric chloride in the presence of 60 mmol sodium carbonate at 40°C for 40 hours. Thereafter, the activated polymer was precipitated in 300 mL of petroleum ether. 2 mL of the diluted *T. hirsuta* laccase solution (1 mg mL^{-1} protein) were mixed in 100 mL sodium borate buffer (0.1 M, pH 9.3) containing 3 g of 2-O-methoxypolyethyleneglycol-4,6-dichloro-s-triazine. The reaction mixture was shaken for 2 hours at room temperature according to previous work of Marc Schroeder [45, 46].

For the modification of *Trametes hirsuta* laccase with functional methacrylic groups (Laccase_MA), 4.5 mL of *T. hirsuta* laccase solution (4.9 mg mL^{-1} protein content, 18.3 U mL^{-1}) was diluted with

25 mL 0.1 M glycine buffer pH 4.5. After adding 1 mL glycidylmethacrylate solution (GMA) the reaction was shaken overnight at 25 °C.

Both modified laccases (Laccase_PEG and Laccase_MA] were purified and concentrated by using ultrafiltration with a Vivaspin 20 centrifugal concentrator MWCO 30000 Da and washed several times with 50 mM succinic buffer pH 4.5. Thereafter, the modified laccases were purified and characterised by hydrophobic interaction chromatography (HIC), used for Laccase_PEG and reversed phase (RP) for Laccase_MA. Measurements were carried out using an ÄKTA purifier 900 system (Amersham, Uppsala, Sweden). The UV/VIS signals were detected at 280 nm and 610 nm. The system was equipped with an Octyl Sepharose XK50/20 column (Laccase_PEG) and a resource RCP 3 mL column (Laccase_MA), respectively. For binding of Laccase_PEG a phosphate buffer (50 mM, pH 7.0) containing $(NH_4)_2SO_4$ at different concentrations (0.6 M, 1.0 M, 2.0 M and 3.0 M) was used. The gradient volume with a linear flow rate of 1 mL min^{-1} was 15 column volumes to elute with the final buffer, a phosphate buffer (50 mM, pH 7.0). The mobile phase for the elution of the Laccase_MA from the RCP column was succinate buffer (50 mM, pH 4.5) containing 5 % acetonitrile at a linear flow rate of 0.4 mL min^{-1}. After purification the modified laccases were concentrated by ultrafiltration (Vivaspin MWCO: 30000) up to a protein concentration as indicated below.

The size increase of the laccases upon chemical modification was determined by size exclusion chromatography (Laccase_PEG) and reversed phase chromatography (Laccase_MA). For gel filtration on the above-named purifier, a Superdex 75 HR 10/30 column (Amersham Pharmacia) was used. The sample volume was 200 µL and detection was done at 280 nm. Elution was performed using a phosphate buffer (0.1 M, pH 7.0) containing sodium chloride (0.1 M) at a flow rate of 0.4 mL min^{-1}. Determination of the molecular weight was done with a protein calibration kit obtained from Sigma Aldrich. The size increase of Laccase_MA was also demonstrated with size exclusion chromatography, performed with an HPLC system (DIONEX P-580 PUMP, Dionex Cooperation, Sunnyvale, USA), equipped with an ASI-100 automated sample injector and a UV light detector (Dionex UV/VIS 340U), adjusted to 205 nm. As stationary phase an YMC-PACK DIOL 6 500*8mm and an YMC-PACK DIOL 12 300*8mm were used in series. As mobile phase deionized water containing 0.1 mM NaN_3 was used. The flow rate was set to 0.6 mL min^{-1}, the sample volume to 40 µL and the column temperature to 25° C. As references protein standards obtained from Sigma Aldrich were used.

8.3.3 Enzyme assays

To determine laccase activity, ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-disulfonic acid)) was used as substrate according to Niku-Paavola [47]. 200 µL of aqueous ABTS solution (10 mM) were mixed with 650 µL sample. The increase in absorbance was measured for 5 minutes at 420 nm to get a better slope at low concentrations using a Hitachi 910 spectrometer. The activities were calculated after Liu [48]. The amount of enzyme which converts 1 µmol ABTS per minute is defined as one unit (ε_{ABTS} = 36000 $M^{-1} cm^{-1}$). Cellulase activities were determined according to Baily [49] using CMC as substrate for cellulase. Protein concentration was determined by a Roti®-Nanoquant kit obtained from Carl Roth (Karlsruhe, Germany).

8.3.4 Immobilisation of ferulic acid as laccase substrate

Immobilisation of ferulic acid on silica gel was done according to previous work [40, 41]. Briefly, 20 g of silica gel were immersed in 60 mL 20 % APTS diluted with ethanol absolute. After adding of 500 µL 6 M HCl, the reaction mixture was stirred at 40 °C for 4h. After cooling down, the modified silica gel was filtrated and washed with 70 % ethanol 5 times and dried overnight under high vacuum conditions. To couple the ferulic acid to the amino group of the modified silica gel, 200 mg ferulic acid were activated by stirring in 80 mL ethanol absolute containing 200 mg EDAC and 14 mg HOBT at room temperature for 1 hour. Subsequently, 20 g modified silica gel were added to the reaction mixture and were stirred for another hour at room temperature. The ferulic acid coupled silica gel was filtrated and washed 5 times with ethanol absolute. After drying under vacuum conditions the coupled silica gel was stored at room temperature in an evacuated desiccator.

8.3.5 Dyed peptidoglycan

To visualise the degradation of peptidoglycan by lysozyme activity, the peptidoglycan itself was dyed according to previous work [9]. 100 mg cell wall from *Micrococcus lysodeiktikus*, 500 µg remazol brilliant blue (RBB), 250 µg sodium sulphate and 100 µg sodiumcarbonate were dissolved in 1mL double distilled water and were shake for 10 min at 25°C and for another 5 min at 65 °C. To separate the dyed peptidoglycan, the reaction mixture was centrifuged at 12000 x g for 5 min. To remove the unbound dye, the pellet was washed several times with double distilled water until no colour has detectable in the washing solution.

8.3.6 Preparation of Bioresponsive Polymers

8.3.6.1 Carboxymethylcellulose (CMC) and peptidoglycan (PG) hydrogels

Hydrogels containing peptidoglycan were prepared by using agarose as scaffold matrix. An agarose solution (1 % (w/v)) was prepared in 0.1 M phosphate buffer, pH 7.0 and melted in a microwave oven. 3.12 mg peptidoglycan from *Micrococcus lysodeiktikus* was mixed with 1 mL hot agarose on a magnetic stirrer to obtain a homogenous blend.

CMC based hydrogels contains 5 % (w/v) CMC in aqueous solution and were dried after incorporation of peptidoglycan and modified laccase. To immobilise laccases, 50 µL of Laccase_PEG were mixed thoroughly with 100 µL of the hydrogel solution. Alternatively, the hydrogels were loaded with alizarin by mixing 0.1 g alizarin with 100 mL hydrogel solution. The hydrogels (thickness 3 mm) were allowed to polymerise for one hour at 25 °C, cut into pieces of 15 mm x 15 mm and washed several times with 50 mM phosphate buffer pH 7.0.

8.3.6.2 Cross-linked carboxymethylcellulose (CMC_MA) and peptidoglycan (PG_MA) hydrogels

Methacrylation of polysaccharides was carried out based on modified approaches previously described [12; 30]. Briefly, 2.5 g of polysaccharide were dissolved in 100 mL distilled water by stirring at 40 °C. Afterwards, 5 mL of glycidylmethacrylate (GMA) solution and 0.5 mL 6 M HCl were added. The reaction was stirred for 48 hours at 40 °C. The samples were stored at 4 °C. To polymerise the hydrogel, 1 mL of modified laccase (Laccase_MA), unmodified laccase (2.38 mg mL^{-1} protein content) or 15 mg alizarin were mixed with 15 g CMC_GMA, 20 mg of sodium persulfate and 10 µL TEMED. The mixture was poured into a glass container (5 cm x 7 cm). After polymerisation for 30 minutes, the hydrogels (thickness 3 mm) were cut into pieces of 15 mm x 15 mm and washed several times with distilled water to get rid of unpolymerised CMC_MA, PG_MA, laccase or dye until no laccase activity or colour was detectable in the washing solution. In another experiment, immobilised laccase substrates were incorporated into sandwich devices. Therefore, 1 g wet mass of bioresponsive hydrogels was assembled with 0.5 g ferulic acid modified silica gel in a 20 mL glass container.

8.3.6.3 Device system

To protect the fragile hydrogel operating matrix it was covered with an enzymatic activated PET membrane modified after previous work of Anita Eberl [50].

A 5 cm x 7 cm sized piece of hydrogel was combined with a 6 cm x 8 cm sized modified PET membrane on both sides. To avoid a passage beyond the membrane it was glued baggy together. After assembling the device was incubated according to the other controlled release experiments.

8.3.7 Controlled Release Experiments

As trigger enzymes, lysozyme (7000 U mL^{-1}) and cellulase from *A. niger*, (0.88 and 1.75 U mL^{-1}), were used. The hydrogel samples were transformed into 20 mL glass containers. After various time intervals as indicated below, samples were taken, centrifuged at 5000 rpm for 5 minutes to remove polymeric particles and tested for laccase activity or dye release. Alternatively, colour change on the surface of sandwich devices containing silica gels with immobilised ferulic acid as laccase substrate were followed with a Colorlite sph 850 (Colorlite: Innovative colour measurements; GERMANY) instrument.

8.4 Results and discussion

Bioresponsive Polymers (BRPs) could indicate the presence of pathogenic microorganisms based on reaction with their extracellular enzymes or enzymes secreted by the immune system. Here, signal enhancing enzymes (Enhanzymes) were incorporated into peptidoglycan and CMC based BRPs both to enhance sensitivity and to reduce background noise due to leaching of dyes. To avoid leaking out of the BRPs, the Enhanzymes were chemically modified with two approaches. First, the size of the enzyme was increased by covalent attachment of PEG. Secondly, laccases were modified to carry methacrylic functionalities to allow covalent attachment to the polysaccharide matrices (Figure 36).

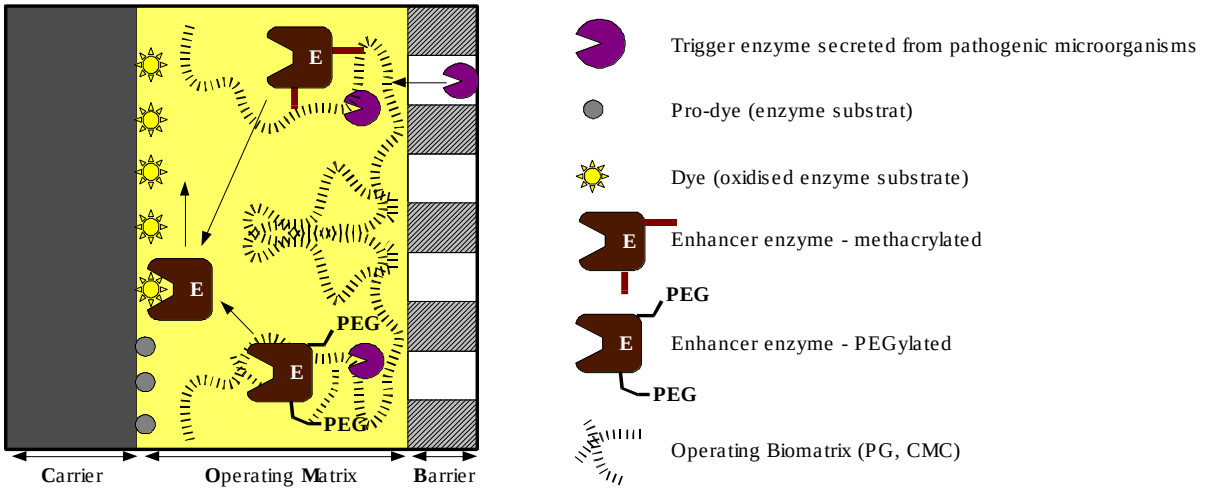

Figure 36 COMB concept for bioresponsive polymers (BRPs) with incorporated Enhanzymes for signalling. Laccase substrates (in this case ferulic acid) are immobilised on silica gel as carrier layer.

Laccases as Enhanzymes were entrapped in a (cross-linked) polysaccharide matrix (peptidoglycan PG or carboxymethylcellulose). To avoid leaching, the laccases were either covalently linked to the polysaccharide matrix via methacrylation or they were PEGylated to increase molecular weight. A barrier layer (PET membrane) allows trigger enzymes to enter the device but prevents Enhanzymes from leaching. Upon hydrolysis of the polysaccharide matrix by trigger enzymes secreted by pathogenic microorganisms (*i.e.* lysozyme, cellulose), the Enhanzymes are mobilised and induce a colour reaction on the immobilised substrate.

Covalent attachment of PEG to the *Trametes hirsuta* laccase led to a considerable fivefold increase in size to 270 kDa (peak at 10.1 mL) when compared to the native laccase (peak at 14.8 mL; Figure 37). However, due to different hydrodynamic volumes of PEG and proteins (*i.e.* the standards) the PEG modified laccase may show an apparent slightly higher molecular weight [45]. The modified laccase almost retained its specific activity with 0.21 U mg^{-1} compared to 0.25 U mg^{-1} of the native enzyme. Thus, coupling of 2,4,6 trichloro-s-triazine activated PEG might have taken place to ε-amino group of

lysine residues far away from the active site area [51]. This PEG coupling strategy was previously used for the enlargement of proteases for protein fibre functionalisation [52].

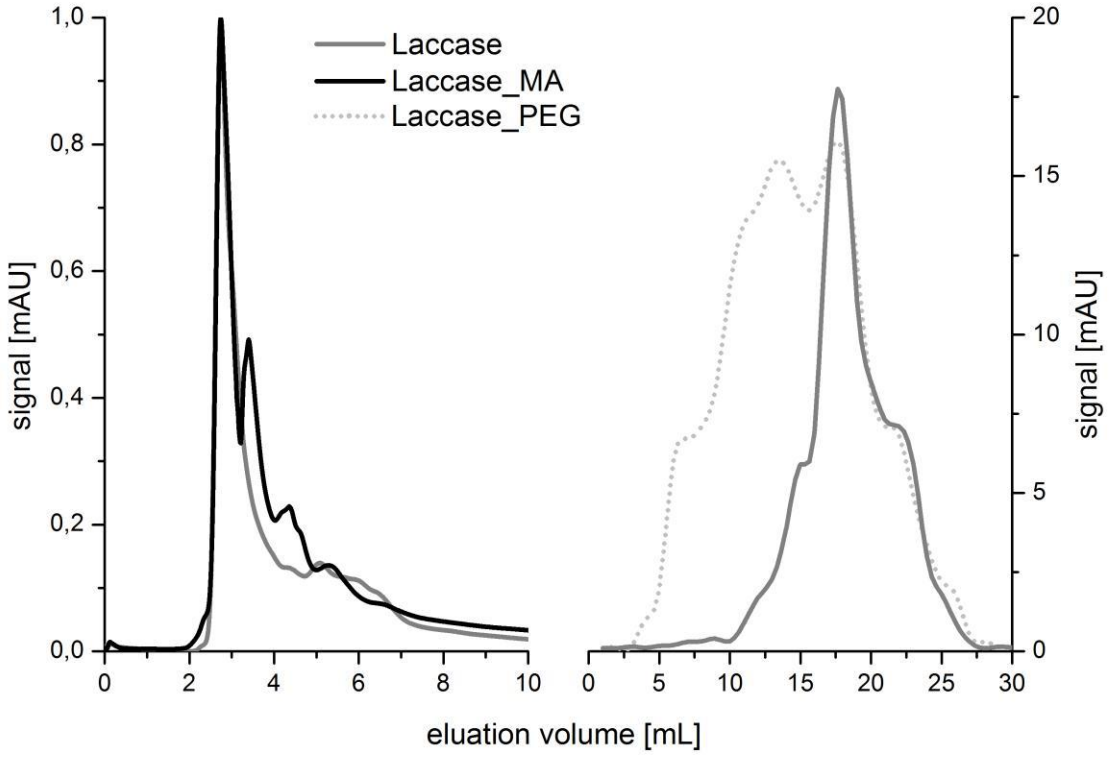

Figure 37 Chromatographic analysis of laccase from *Trametes hirsuta* (Laccase) and modified Laccases (Laccase_MA: methacrylated Laccase; Laccase_PEG: PEGylated Laccases) left: reversed phase chromatogram of Laccase_MA; right: size exclusion chromatogram of Laccase_PEG.

After separation of methacrylic acid modified laccases with reversed phase chromatography, three laccase active areas were seen (Figure 37). When compared to the control (2.74 mL), the peaks eluting at 3.40 mL and 5.28 mL can be attributed to methacrylated laccase. Compared to the not modified laccase (0.25 U mg^{-1}) the specific activity of the modified laccase was reduced to about 50% (0.13 and 0.11 U mg^{-1} in peaks at 3.40 mL and 5.28 mL). Obviously methacrylation of the enzyme at or close to the active site will result in loss of activity. The fact that distinct laccase active fractions were found may indicate different extends of methacrylation. The fractions of peak 2 were concentrated by ultrafiltration (Vivaspin MWCO 30000) up to a protein concentration of 37.6 mg mL^{-1} and a final laccase activity of 4.6 U mL^{-1}.

For the detection of infection in wounds peptidoglycan based BRPs were recently presented [9]. The increase in transparency of *Micrococcus lysodeiktikus* peptidoglycan hydrogels due to hydrolytic activity of lysozyme was not sensitive enough to detect lysozyme in infected wound samples.

Incorporation of dyes (*i.e.* RBB) enhanced sensitivity [53, 54] and indeed allowed detection of enhanced lysozyme levels in infected wounds [9]. However, increased sensitivity of such systems would be highly desirable to bring down incubation times. In this study, we found that lysozyme can liberate laccase in PG hydrogels as indicated by colour forming oxidation of ABTS (Figure 38). After 30 minutes of incubation, 26 nmole of ABTS were oxidised compared to only 14 nmole of RBB dye released within the same time interval. Thus, compared to simple release of dyes, the incorporation of laccase as Enhanzymes can magnify the signal by a factor of 2 after only 30 minutes. In absence of lysozyme, 0.5 nmole of ABTS were oxidised in the control experiment when Laccase_PEG was incorporated. Especially after longer incubation times leaching of RBB in the absence of trigger enzyme was seen. This can especially be a problem when low levels of trigger enzymes are to be detected. When incubating the device with fluid from infected wounds, the absolute response obtained with RBB was higher, however, when compared to the control (no wound fluid) the signal increased 26 times in case of Laccase_PEG when compared to a factor of 6.2 seen for RBB release.

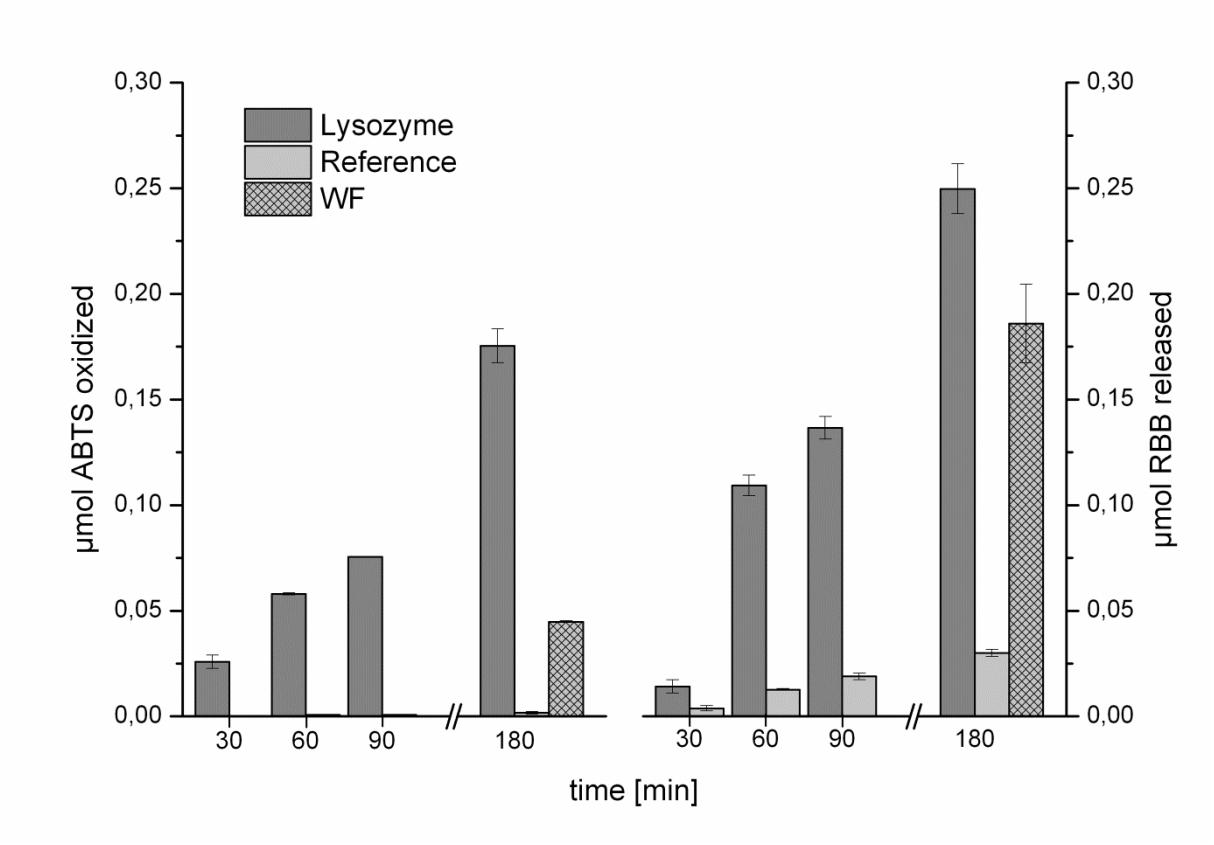

Figure 38 Response of peptidoglycan based BRPs upon incubation with lysozyme (5000 U mL^{-1}) and wound fluid (WF) as triggers. The systems contained either Laccase_PEG (left side) or remazol brilliant blue (RBB) (right side). The response was quantified via oxidation of ABTS by Laccase_PEG (left) or via release of RBB (right) and was compared to controls containing buffer only (*i.e.* no lysozyme).

As an alternative to PEGylation, covalent attachment of laccase into PG via methacrylation was investigated. In addition, PG hydrogels were cross-linked with this strategy. We and others have previously demonstrated that methacrylation of polysaccharides followed by chemical cross-linking is a powerful strategy to increase stability and reduce leaking of controlled release devices [12, 30]. Using this technique hydrogels loaded with dyes or loaded with laccases as Enhanzymes were constructed. In general, hydrogels are well known in medical applications [55]. Commonly, covalently cross-linked hydrogels show higher stability when compared to other well established ways to create hydrogels by using *e.g.* alginate in calcium chloride solution [56, 57].

Like with the PG / Laccase-PEG system, incorporated Laccase_MA was found suitable for signal enhancement (Figure 39). Compared to the release of alizarin, liberation of Laccase_MA with concomitant oxidation of ABTS lead to a 350 fold and 1800 signal enhancement after 24 and 48 hours, respectively. The signal enhancement for native laccase was even higher; however, the control values in the absence of trigger enzymes were also unacceptably high. This demonstrates the importance of covalent attachment of the laccase within the polysaccharide matrix. Considering this important prevention of leakage a lower overall signal obtained with the immobilised laccase seems to be well acceptable.

The controlled release of Laccase_MA was also successfully realised triggered by infected wound fluids according to the results of the controlled release experiment from an agarose peptidoglycan based devices.

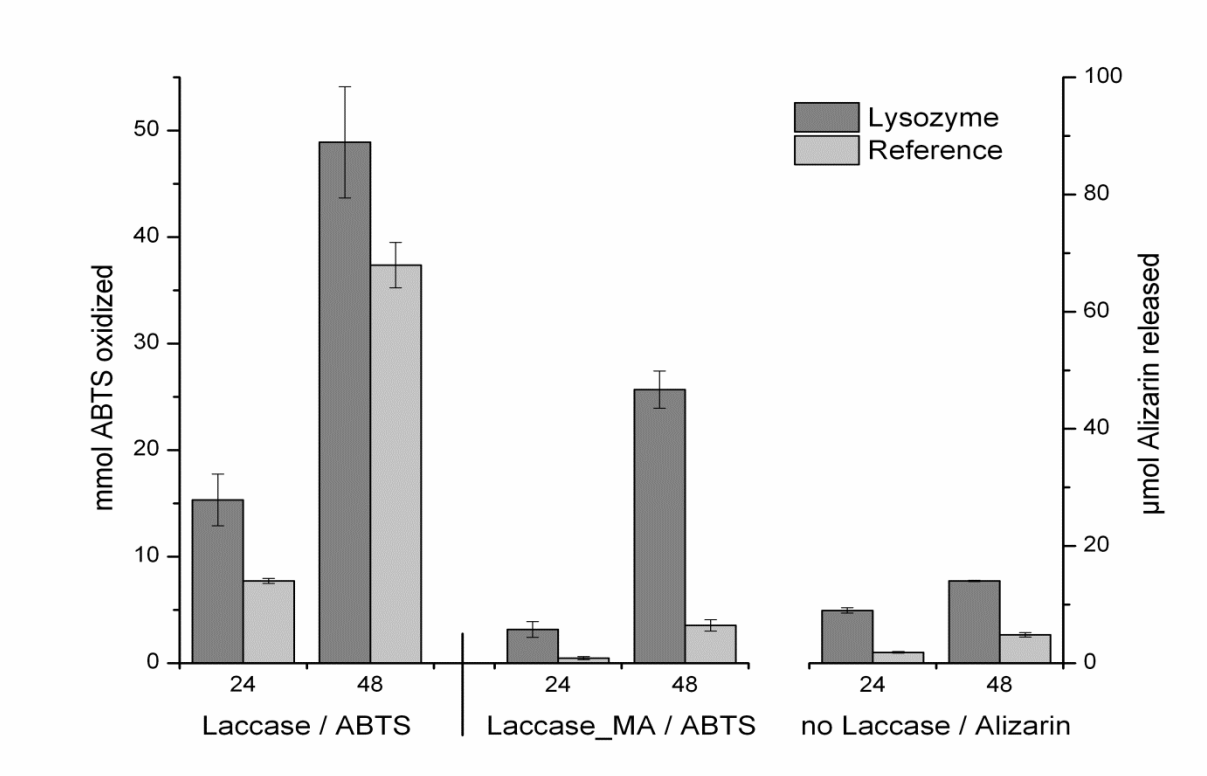

Figure 39 Response of cross-linked PG based BRPs upon incubation with lysozyme as trigger. The systems contained either Laccase_MA or alizarin. The response was quantified via oxidation of ABTS by Laccase_MA (left) or via release of alizarin (right) and was compared to controls containing buffer only (i.e. no lysozyme).

It has to be noted, that infected wound fluid may contain other enzymes than lysozyme potentially hydrolysing PG (and thus producing a signal). On the one hand, combinations of MMP-9 and lysozyme or elastase and lysozyme have previously shown to enhanced hydrolysis of peptidoglycan [9]. This indicates the ability of proteases to hydrolyse peptide bonds of PG [58, 59]. Moreover, these specific devices contained agarose as a gel forming constituent. Consequently, bacteria present in chronic wounds which produce agarases (*e.g. Pseudomonas spp.*) may potentially contribute to hydrolysis of the device [60-62].

In a next step, cross-linked CMC hydrogels were prepared as bioresponsive polymers to detect cellulases secreted by contaminating and / or pathogenic organisms causing for example Aspergillosis. Upon incubation with cellulase, Laccase_MA was successfully liberated from CMC-hydrogels leading to oxidation of ABTS (Figure 40).

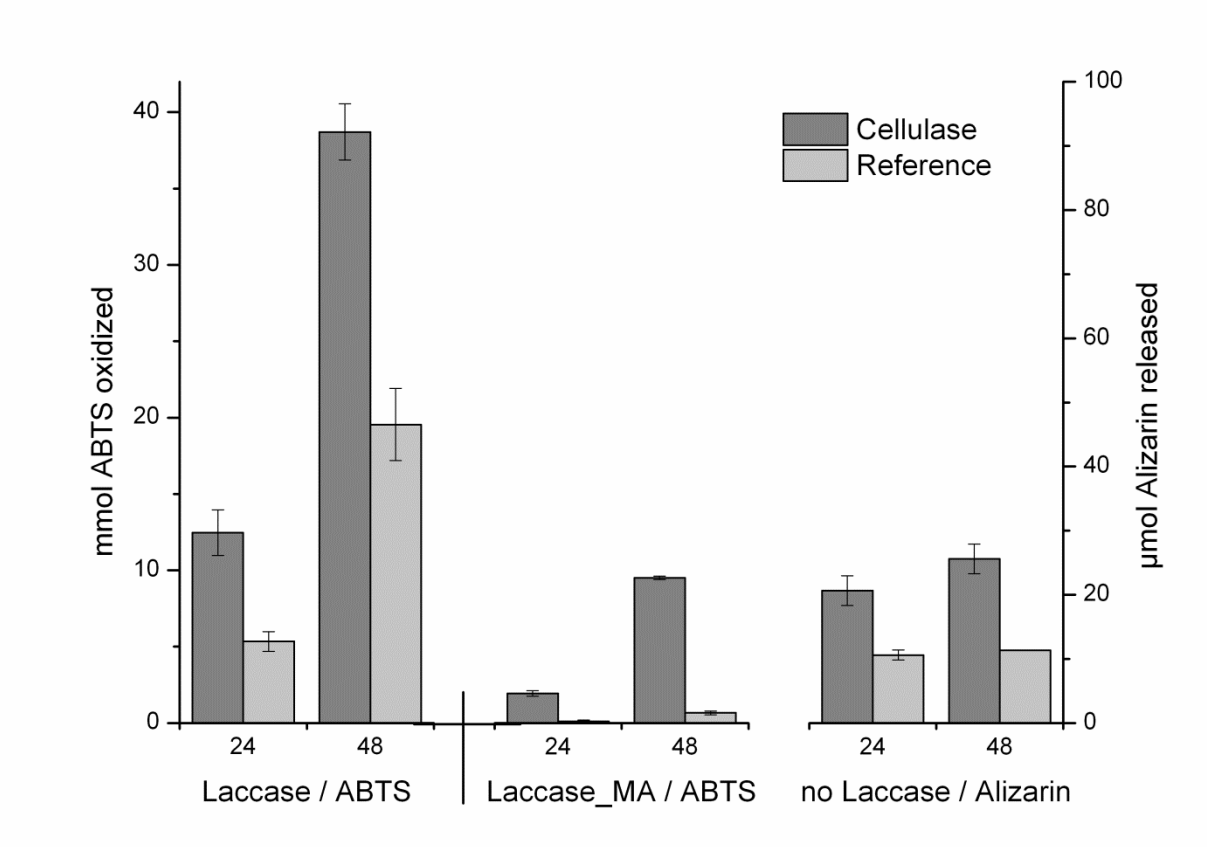

Figure 40 Response of cross-linked CMC based BRPs upon incubation with cellulase as trigger. The systems contained either Laccase_MA or alizarin. The response was quantified via oxidation of ABTS by Laccase_MA (left) or via release of alizarin (right)

Compared to simple release of a dye (alizarin), the enhancing effect by laccase was 600 fold after 24 hours and 1500 fold after 48 hours of incubation with cellulase as trigger enzyme (Figure 40). However, despite the high signal enhancement, in absence of the trigger enzyme still a high signal was measured due to leaching of the laccase from the matrix. To avoid leaching, the laccase was covalently linked to the polysaccharide matrix. Indeed, covalent entrapment of Laccase_MA in the CMC_MA hydrogel dramatically reduced the signal of the control (*i.e.* without cellulase). However, the overall signal was lower (Figure 40). Still, the amplification of the signal (oxidised ABTS compared to release alizarin) was around 100 and 370 fold after 24 and after 48 hours of incubation with cellulase as trigger. Thus, covalent entrapment of laccase as enhancer enzyme in CMC based BRPs can both reduce leaching and dramatically boost the signal when compared to dye release.

Obviously, after liberation of Laccase_MA oligosaccharides still covalently bound to the enzyme will limit laccase activity. Indeed, an increased size of the Laccase_MA from 62 kDa [44] to a molecular weight of approximately 67 kDa of the laccase liberated from CMC was seen (Figure 41). This increase would roughly correspond to an average cello oligomer degree of polymerisation of

approximately 10 to 15 remaining attached to the enzyme. Expectedly no increase of molecular weight was found for the liberated native laccase.

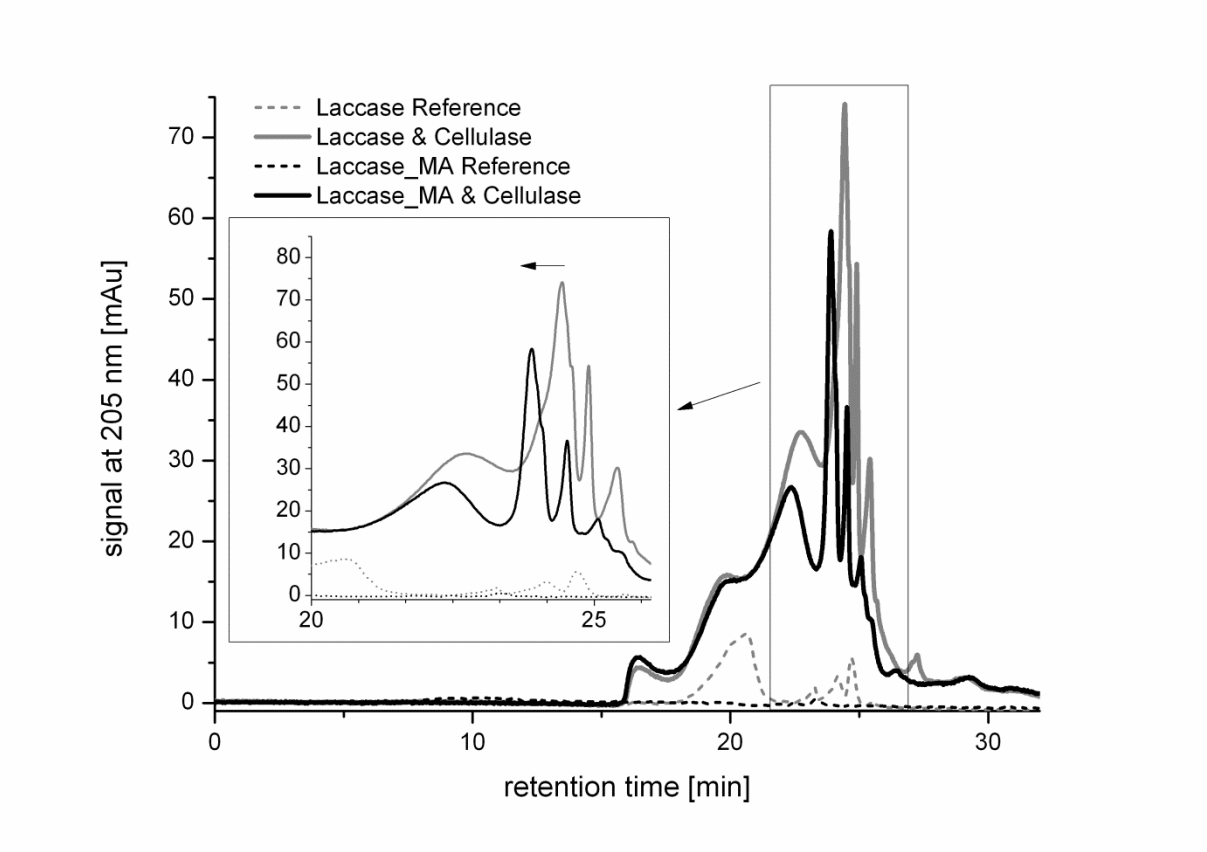

Figure 41 HPLC - size exclusion analysis of native and methacrylated laccase after liberation from cross-linked CMC cellulase.

Based on the promising results obtained with BRPs containing Enhanzymes the potential of immobilised colour forming substrates for the latter was assessed. This would ultimately allow the construction of a simple multi-layer test strips according to the COMB concept (Figure 36). For this purpose, laccase substrates were covalently attached to silica gel as carrier [40]. The operating matrix as next layer consisted of PG_MA or CMC_MA with covalently attached Laccase_MA. Finally, the system was covered with a PET membrane as barrier layer. The colour change of the immobilised ferulic acid was determinated by a solid phase colour measurements system. Upon incubation of these devices with either lysozyme or cellulose as triggers, indeed considerable colour changes were seen (Figure 42). These changes were quantified as ΔE values according to the CIELab concept. This clearly demonstrate that Laccase_MA liberated by either lysozyme or cellulose from PG_MA and CMC_MA hydrogels can oxidise silica gel immobilised ferulic acid resulting in a colour change from light grey to brownish yellow.

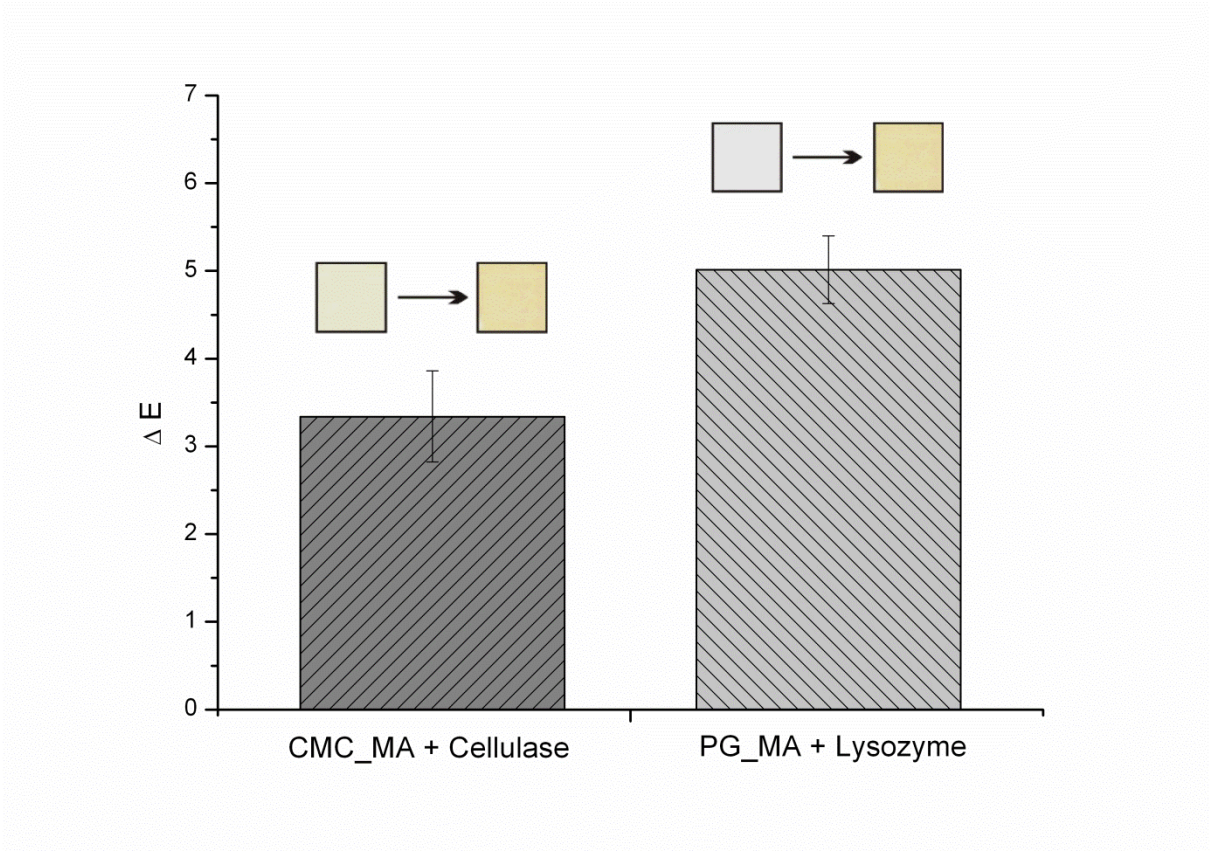

Figure 42 Response of cross-linked CMC_MA and PG_MA based BRPs upon incubation with cellulase and lysozyme as triggers, respectively. Oxidation of silica gel immobilised ferulic acid with quantified as ΔE values according to the CIELab concept. Inlet: pictures of the CMC_MA device before and after incubation with cellulose.

8.5 Acknowledgements

This study was performed within the COMET K-Project MacroFun. Financial support from the FFG, the SFG and the Province of Styria is gratefully acknowledged. The technical assistance by Cornelia Rainer is acknowledged.

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9 GENERAL SUMMARY AND CONCLUSION

The main goal of this thesis was the development of a sensoring system triggered by enzymes, released by potentially pathogenic microorganisms which can be incorporated in packaging systems of food and medical industry. Here, a bioresponsive polymer system (BRP) sensitive to these extracellular enzymes was developed. Based on literature studies for enzymes produced by potential pathogenic and contaminating microorganisms, pectinases, cellulases and lysozyme were chosen as trigger enzymes to detect bacteria or fungi [1-25].

As first biopolymer, polygalacturonic acid (PGA, pectin) was chosen and functionalised into a bioresponsive polymer (BRP) forming hydrogels. A common method to do this is the simple dropping of a pectin solution into a calcium chloride solution. The pectin will build up egg-box like structures around the Ca^{2+} ions and forms a hydrogel [26, 27]. Alizarin, as model substance, was loaded into the hydrogel and released by pectinase activities. The calcium chloride based hydrogel was not stable enough for usage in a BRP especially in aqueous environments since the autodegradation level was too high.

A good possibility to stabilise a hydrogel matrix is organic crosslinking. This was done by covalent esterification with glycidylmethacrylate [28]. The modified hydrogel was polymerised radically by three different methods. First, sodium persulfate was used as radical source in combination with elevated temperature (75 °C for 20 to 30 minutes). Second, a chemical radical starter, namely Tetramethylethylendiamin (TEMED) was used in combination with sodium persulfate as radical source. Finally UV light irradiation in combination with a photoinitiator (Irgacure from BASF, Ludwigshafen, Germany) was used to start the polymerisation process. As additional benefit of UV light treatment the hydrogel is sterilised this is important when dealing with microorganisms.

The chemically modified hydrogel showed improved long-time stability and nearly the same enzymatic degradability compared to the simple production with a Ca^{2+} ion solution. Therefore, further experiments were done with the methacrylated hydrogels.

The polygalacturonic acid based hydrogel was successfully tested with pectinases from *Aspergillus species* as well as with living pathogenic cell cultures (*Yersinia entercolitica* and *Bacillus subtilis*) regarding controlled release.

Both bacterial species were able to degrade the BRPs and release the model dye alizarin in an enzymatically controlled way. To protect the fragile bio based hydrogel, an enzymatically modified PET membrane was used as a barrier layer on the hydrogel. The membranes were enzymatically

modified to increase the numbers of hydroxyl groups on the surface [29-32]. This allowed an improved adhesion of the modified biopolymer to the PET membrane. This device system, consisting of a loaded degradable matrix and protection membrane was also tested with pectinases and showed, that the amount of released dye decreased but the long-time stability of the whole system was enhanced significantly.

To sum up, the first part of the thesis deals with the controlled release of alizarin as a model dye from a polygalacturonic acid based hydrogel. Two different ways to produce the hydrogels were tested. The common dropping of a dissolved polygalacturonic acid into a calcium chloride solution showed drawbacks. Due to that fact a chemical modified and covalent cross-linked hydrogel was established. This was achieved by methacrylation processes. The radical polymerised hydrogels were successfully tested with pure enzymes as well as growing bacterial species. To protect the system from physical damages it was covered with an enzymatic modified PET membrane. This device system was also tested successfully with pectinases.

The second part of the thesis deals with the development of another BRP as alternative to the pectin related polygalacturonic based hydrogel.

After good results with methacrylated polygalacturonic acid based hydrogels the next goal was the development of cellulose based methacrylated BRPs. Similar to the modification process of PGA, carboxymethyl cellulose (CMC) was modified with glycidylmethacrylate. The modification itself was monitored and determined by RAMAN spectroscopy.

Again, the polymerisation process was started with elevated temperature in combination with sodium persulfate, TEMED and UV light irradiation with Irgacure as radical starter.

The same functional group for polymerisation (methacrylate) allows the blending of the two methacrylated polysaccharides (PGA and CMC) in various ratios. This allows the development of a biobased hydrogel which can be degraded by different enzymes. It was successfully demonstrated that a CMC / PGA mixed polymer can be hydrolysed by cellulases as well as pectinases. This increases the number of bacteria or fungi species which can be detected by this BRP system. Consequently, these results demonstrate that the BRP specifity to trigger enzymes (i.e. to the microorganisms secreting with) can be nicely tuned by combination of different functionalised biopolymers. This boosts the usage of the BRP system to monitor bacterial contamination. The enzymatic hydrolysis of the hydrogel was investigated in detail on one hand, similar to the PGA experiments with alizarin as test substance and on the other hand with scanning electron spectroscopy.

The third part of the thesis was focused on the replacement of alizarin by much more complex substances for an enzymatic induced release. The most significant disadvantage of a simple dye is that it is not able to catalyse further chemical reactions after the release. Moreover, the release of molecules from the device may prevent its application for on-patient testing. Consequently immobilised substrate in combination of (trapped) enhancer enzymes was investigated.

Laccase from *Trametes hirsuta* was used for these modification experiments. Laccases are oxidoreductases (EC 1.10.3.2) which are able to oxidise various substrates like phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, hydroxyindols or benzenethiols [33-35].

To optimise the controlled release of laccase within a BRP by trigger enzymes, the enzyme itself was modified by two different strategies of chemical modification. First, a size change was done by covalent linkage of polyethylene glycol (PEG) molecules to the laccase surface, resulting in an increased molecular weight of 3000 g mol^{-1}[36-40]. The increased size of the laccase minimised its diffusion through the BRP in absence of a trigger enzyme remarkably.

As an alternative strategy, laccases were modified by a covalent linkage of methacrylic groups to the surface of the enzyme. In contrast to the PEG modification, methacrylic groups allow the covalent crosslinking to the polysaccharide based matrix by radical polymerisation. This immobilises the laccase and reduces the risk of diffusion without presence of a trigger enzyme. The combination of methacrylated laccase and methacrylated biopolymers (either CMC or PGA) was successfully tested with pectinases as well as cellulases [28, 41].

As third biopolymer, peptidoglycan from bacteria cells walls (*Micrococcus lysodeiktikus*) was used as a substrate for lysozyme [42-44]. Lysozyme is well known in literature as extracellular enzyme which can be detected in infected wounds, *e.g.* decubitus ulcer [1, 45] and thus indicate wound infection.

The fragile peptidoglycan was stabilised by embedding into a BRP matrix. Two matrices were tested, namely agarose and methacrylated CMC. The combination agarose/peptidoglycan was loaded with PEG modified laccases [36, 38, 39] which was successfully released upon incubation with lysozyme as trigger enzyme. Likewise, the system peptidoglycan/methacrylated CMC was charged with methacrylated laccase which was again released upon incubation with lysozyme.

To enhance the signal of the BRP system, ferulic acid as substrate for laccase , was immobilised on the carrier layer of the device [46, 47]. The released laccase was then able to catalyse the oxidation of ferulic acid and results in a colour change of the substrate. Compared to the release of a simple dye molecule the usage of released enzyme allows enhancing the signal up to a 800 times due to the catalysed colour reaction.

To sum up, BRPs developed in this thesis are suitable systems for the detection of bacterial or fungal contamination. Compared to established methods, *e.g.* PCR based methods [48-51], BRPs can be a simple and cheap alternative to avoid contaminations of a huge range of applications. Three biopolymers (CMC, PGA and peptidoglycan) were used as source to establish a BRP system. The quality of the BRP was improved by chemical modification and crosslinking processes. Finally, a simple dye was replaced by a much more complex enzyme system realised by chemical modifications of laccase by size increasing (PEG) and covalent crosslinking into a matrix (methacrylated laccase).

These combinations allow the design of a stable and applicable bioresponsive system as detection device for bacterial and fungal contaminations in an online way.

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10 PUBLICATIONS

10.1 Poster

2007

Hasmann A, Wehrschuetz-Sigl E, **Schneider K**:
Bioresponsive Polymers for Wound Treatment: COST 868 Action: Biotechnical Functionalization of renewable Polymeric Materials. Graz, Austria (2007)

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Rollett, A., **Schneider K**, Fischer R, Marold A, Flock T, Guebitz GM, Schroeder M:
Immobilization of biomolecules on soluble and insoluble polymers: 237th American Chemical Society National Meeting & Exposition, Salt Lake City, USA (2009)

2011

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Schneider K, Hasmann A, Wehrschuetz-Sigl E, Flock T, Gewessler U, Schiffer D, Guebitz GM: Carboxymethyl cellulose based bioresponsive Polymers used for enzymatic controlled release: 19th Annual BEPS Meeting, Vienna, Austria (2011)

10.2 Oral presentations

2007

Francesko A, Hasmann A, **Schneider K,** Wehrschuetz-Sigl E, Guebitz GM, Tzanov T:
Enzymatic acivity in wound flluids: COST 868 Working Group Meeting, Graz, Austria, (2007)

2009

Schneider K, Hasmann A, Rollett A, Marold A, Flock T, Guebitz GM:
Polysaccharides based bioresponsive polymer composites: RCPE Symposium, Graz, Austria, (2009)

Schneider K, Rollett A, Schroeder M, Kaufmann F, Guebitz GM:
Trigger enzymes for pectin based bioresponsive polymers: 237th American Chemical Society National Meeting & Exposition, Salt Lake City, USA (2009)

10.3 Papers

2010

Rollett A, Schroeder M, **Schneider KP**, Fischer R, Kaufmann F, Schoeftner R, Guebitz GM:
Covalent immobilisation of protease and laccase substrates onto siloxanes, Chemosphere 80 (2010): 922 – 928

2011

Schneider KP, Rollett A, Wehrschuetz-Sigl E, Hasmann A, Zankel A, Muehlebach A, Kaufmann F, Guebitz GM:
Bioresponsive systems based on polygalacturonate containing hydrogels, Enzyme and Microbial Technology 48, 4-5 (2011): 312-318.

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Novel Peptidoglycan Based Diagnostic Devices for Detection of Wound Infection, Diagnostic and Microbial Infection Disease, in press

Hasmann A, Gewessler U, Hulla E, **Schneider KP**, Francesco A, Wehrschuetz-Sigl E, Schintler M, Van Palen J, Guebitz GM, Wehrschuetz-Sigl E:
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2012

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10.4 Patents

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11 ACKNOWLEDGEMENTS

“Time to say Thank you”, es ist an der Zeit einmal danke zu sagen. Vier Jahre habe ich mit meiner Dissertation verbracht und natürlich wäre es nicht ohne die unermüdliche Unterstützung vieler Helferleins möglich gewesen.

Als erstes möchte ich Georg Gübitz dafür danken dass er mir die Möglichkeit gegeben hat meine Doktorarbeit in einem für mich fast schon perfekten Projekt zu verfassen. Bei Fragen, speziell beim Publizieren, hat Prof. Gübitz mich immer mit Rat und Tat unterstützt. Neben den wissenschaftlichen Erfahrungen die ich in den letzten vier Jahren sammeln konnte ermöglichte mir Georg auch den Einblick ins Projektmanagement, deswegen: „Danke Georg“!

Als Nächstes möchte ich meinem ersten Projektleiter Marc Schröder danken, mit dem ich die Richtung der Dissertation schon relativ früh festlegen konnte, wovon ich bis zum Ende profitieren konnte.

Nach dem Wechseln von Marc Schröder nach Luxemburg übernahm Eva Sigl die Projektleitung. Von meiner Seite kann ich echt nur sagen die Zusammenarbeit mit Eva hätte nicht besser sein können, sowohl fachlich, wie auch privat, deswegen ein riesiges „DANKE EVA“

Andrea Hasmann, bzw. jetzt Andrea Heinzle, begleitet mich schon seit dem ersten Studientag. Nachdem Andrea ihre Aufgabe als meine Betreuerin meiner Diplomarbeit beendet hatte und ich ebenfalls mit der Doktorarbeit begann war sie immer für Diskussionen zu haben. Neben beruflicher Verbundenheit möchte ich mich auch an dieser Stelle für eine echte persönliche Freundschaft bedanken, die viele Dinge in den letzten Jahren sehr erleichtert hat.

Weiters möchte ich Markus List an dieser Stelle erwähnen der neben seiner Eigenschaft als guter persönlicher Freund auch immer ein guter Diskussionspartner für Syntheseprobleme war.

In den letzten vier Jahren hatte ich auch die Ehre Projektstudenten und Diplomanten zu betreuen und abgesehen von super Ergebnissen sind wahre Freundschaften entstanden. Als Projektstudenten durfte ich mit Alexandra Rollett, Alexander Schenk, Veronika Perz, Dustin Kramer sowie Christof Stangl zusammenarbeiten. Mit Alexandra Rollet durfte ich anschließend noch im Rahmen ihrer Diplomarbeit zusammen arbeiten. Es war eine echt schöne Zeit, deswegen „Danke Alex“.

Abgesehen von Alexandra durfte ich noch Annemarie Marold, Teresa Flock und Claudio Crivaro durch ihre Diplomarbeiten begleite. Danke für die schöne Zeit, spezielle Institutsausflüge oder auch für den gemeinsamen USA Aufenthalt mit Anne, Alex, Teresa und Stefan.

Ich will nicht versuchen alle aufzuzählen mit denen ich in den letzten Jahren eine schöne Zeit am Institut für Umweltbiotechnologie in der Petersgasse verbringen durfte, deswegen mal ein riesig großes pauschales DANKE.

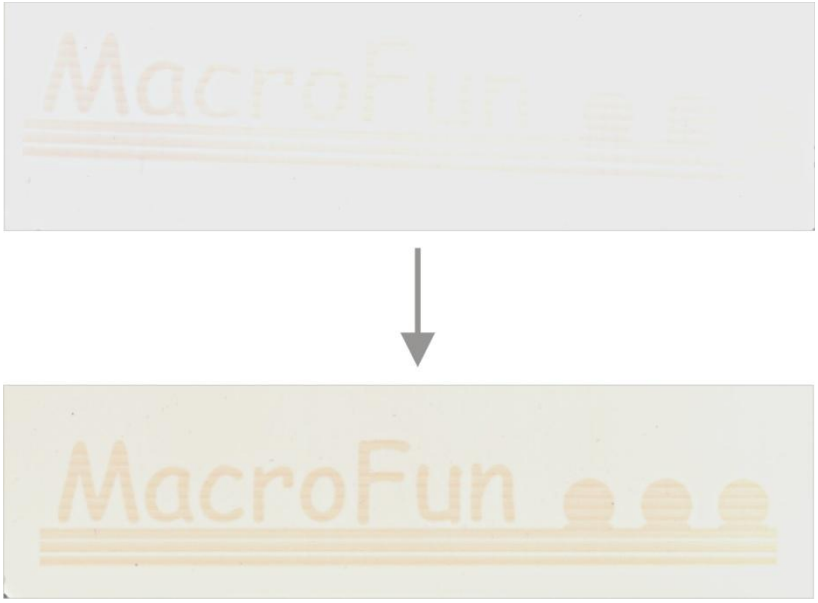
Natürlich wäre eine solche Arbeit ohne die bedingungslose Unterstützung der Familie nicht denkbar. Deswegen danke an meine Mutter Gertrude, meine Schwester Stefanie, meinen Großeltern und allen andern die in den letzten Jahren Gejammer ertragen und aushalten mussten. Ein riesen Danke!!!

Als letztes möchte ich jener Frau danken, die mittlerweile auch meine Frau ist, Ulrike Schneider, geb. Richter. Sie war die treibende Kraft hinter mir. Unermüdlich hat sie mich bestärkt und wieder aufgerichtet wenn dunkle Stunden mich nahe ans Aufgeben gebracht haben. Sie allein weiß wie viel Kraft und Ausdauer notwendig waren um so weit zu kommen. Ein riesiges, riesiges Danke mit einer großen Schleife drauf.

Sollte ich jemanden vergessen haben, bitte verzeiht mir, es war keine Absicht. Ich möchte nun mit einem großen D A N K E E U C H A L L E N endgültig schließen.

PS:

und es geht doch:



12 EIDESSTATTLICHE ERKLÄRUNG - STATIONARY DECLARATIONS

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Graz, am …… ……..

(Unterschrift)

STATUTORY DECLARATION

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

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date (signature)

13 CURRICULUM VITAE

DI Konstantin Philipp Schneider

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Schulische und Universitäre Ausbildung

Aug.-Sept. 2010	*Forschungsaufenthalt an der School of Materials, University of Manchester; Arbeitsgruppe Dr. S. J. Eichhorn*
Seit 2007	***Doktoratstudium der technischen Wissenschaften an der TU Graz***
	Studienzweig: Biotechnologie, Biochemie und Lebensmittelchemie
	***Dissertation** am Institut für Umweltbiotechnologie*
	Titel: „Bioresponsive polymer systems for medical and technical applications“
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Okt. 2000-Sep. 2007	***Studium der Technischen Chemie an der Technischen Universität Graz***
	Studienzweig: Biochemie, Biotechnologie und Lebensmittelchemie
	***Diplomarbeit** am Institut für Umweltbiotechnologie*
	Titel: „Biodegrading enzymes present in wound fluids“
	Betreuer: Univ. Prof. DI Dr. Georg Gübitz
	Abschluss: Dipl. Ing. am 19.09.2007

1991-1999	***BG & BRG Fürstenfeld***
	Fachbereichsarbeit aus Chemie: „UV-VIS Photometrie in Theorie und Praxis anhand ausgewählter Beispiele“
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Zusatzqualifikationen und Sprachen

Sprachkenntnisse	*Englisch (verhandlungssicher), Französisch (Grundkenntnisse)*
EDV-Kenntnisse	*MS Office, Photoshop, Chemoffice, Origin, Scifinder, u. a.*
Projekttätigkeiten	*Mitarbeit beim Einreichen eines Patents*
	Fachartikel in internationalen Fachzeitschriften
	Finanzmanagement von geförderten F&E Projekten
	Koordination zwischen Firmenpartner und universitären Partnern
Analytik und Anwendungen	*HPLC-MS, GC-MS, FTIR und Raman Spektroskopie, Proteinreinigung (Präparative LC bzw. SDS-PAGE), NMR, Scale-up und Reaktorfermentation bis 10L*
Geräteverantwortlicher	*GC-MS, FTIR und Raman Spektroskopie*
Weiterbildung	*Grundlagen der Qualitätssicherung in Pharma-, Lebensmittel- und Biotechnologie, Ausbildung zum Ersthelfer*

Beruflicher Werdegang

Seit 2007 ***Dissertation:*** *am Institut für Umweltbiotechnologie*

Andere Tätigkeiten:

- *Betreuung von Lehrveranstaltungen als Studienassistent (Grundlagen Chemie und Biowissenschaften; Bioremediation)*
- *Betreuung und Anleitung von Projektmitarbeitern, 4 Diplomanten sowie 5 Projektstudenten.*
- *Koordination der Zusammenarbeit mit internationalen Firmen (CIBA Special Chemicals, Schweiz; BASF, Deutschland; Profactor, Österreich und Macopharma, Frankreich)*
- *Projektmanagementerfahrung (Investitionen, Bestellungen, Projektanträge; Patentanträge)*
- *Erfahrungen bei Vorträgen und Postern, eingereicht bei internationalen Konferenzen (XVI International Conference on Bioencapsulation. Dublin; 2011 MRS Fall Meeting & Exhibit, Boston; 237th American Chemical Society National Meeting & Exposition. Salt Lake City und andere)*
- *Forschungsaufenthalt Jul. 2010 – Sept. 2010 an der School of Materials, University of Manchester in der Arbeitsgruppe von Dr. S.J. Eichhorn.*

Okt.2006-Sept.2007 *Diplomarbeit: Diplomarbeit am Institut für Umweltbiotechnologie:*

Im Rahmen der Diplomarbeit wurden Wundflüssigkeiten aus menschlichen Decubitus ulcer Wunden auf Enzymakivitäten als Auslöser für Controlled Release Systeme untersucht. Dazu wurden Enzymassays wie SDS-PAGE modifiziert und optimiert.

Andere Tätigkeiten: *Geräteverantwortlicher für Kleingeräte wie pH-Meter, UV-VIS Spektrometer und Zentrifugen.*

Jul.-Sept. 2003 *Fresenius Kabi Austria, Research and Development:*

Analysen und Methodenentwicklung in der pharmazeutischen Produktentwicklung

Jul.-Sept.2002 *Fresenius Kabi Austria, Research and Development:*

Routineanalytik im Rahmen der Qualitätssicherung in der pharmazeutischen Produktion