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List of Abbreviations

CMC	carboxymethylcellulose
UV light	ultra violet light
TEMED	Tetramethylethylenediamine
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
B	Blank
C	(enzymatic hydrolysis with) cellulase
P	(enzymatic hydrolysis with) pectinase
C+P	(enzymatic hydrolysis with) cellulase and pectinase
ddH_2O	Double distilled water
GMA	glycidylmethacrylate
LD	low degree of cross-linking
MD	medium degree of cross-linking
HD	high degree of cross-linking
PVA	polyvinylalcohol
PEG	polyethylene glycol
CA	carminic acid
LiCl	lithium chloride
NaCl	sodium chloride
HCl	hydrochloric acid
$Na_2S_2O_8$	sodium persulfate

List of used Chemicals

Name	Company	Town, Country
α-amylase from Bacillus amyloliquefaciens	Sigma-Aldrich	St. Louis, USA
activated polyvinylalcohol	Ciba Specialty Chemicals Inc	Marly, Switzerland
Alizarin (tech 85%)	Sigma-Aldrich	St. Louis, USA
Bismarck Brown R	Sigma-Aldrich	St. Louis, USA
Carboxymethylcellulose sodium salt	Carl Roth	Karlsruhe, Germany
carminic acid	Sigma-Aldrich	St. Louis, USA
Cellulase from *Aspergillus species*	Sigma-Aldrich	St. Louis, USA
Cyanuric chloride	Sigma-Aldrich	St. Louis, USA
Dextran T 100	Sigma-Aldrich	St. Louis, USA
Dextran T 40	Carl Roth	Karlsruhe, Germany
Glycidylmethacrylat	Sigma	St. Louis, USA
HCl	Sigma-Aldrich	St. Louis, USA
Irgacure	BASF	Ludwigshafen, Germany
laccase from Myceliophthora thermophila	Novozyme	Bagsvaerd, Denmark
Lithium chloride	Roth	Karlsruhe, Germany
Methacrylic anhydride	Sigma-Aldrich	St. Louis, USA
Methylmethacrylate	Sigma-Aldrich	St. Louis, USA
Mono-methoxy polyethylene glycol (MW 5000 g/mol)	Fluka	Buchs, Switzerland
N-Methyl-2-pyrrolidone	Sigma-Aldrich	St. Louis, USA
n-Pentan	Carl Roth	Karlsruhe, Germany
N,N-Dimethylacetamide	Sigma-Aldrich	St. Louis, USA
N,N-Dimethylformamide	Sigma-Aldrich	St. Louis, USA
Na_2HPO_4	Sigma-Aldrich	St. Louis, USA
$Na_2S_2O_8$	Sigma-Aldrich	St. Louis, USA
NaCl	Sigma-Aldrich	St. Louis, USA
NaH_2PO_4	Sigma-Aldrich	St. Louis, USA
Pektin C	Carl Roth	Karlsruhe, Germany
Pectinase from *Aspergillus aculeatus*	Sigma-Aldrich	St. Louis, USA
Pectinase from *Aspergillus niger*	Sigma-Aldrich	St. Louis, USA
Triethylamine	Sigma-Aldrich	St. Louis, USA

Zusammenfassung

Ziel dieser Arbeit war es, ein bioresponsives System zu entwickeln mit dem kontaminierende Mikroorganismen z.B. in Lebensmittelverpackungen detektiert werden können.

Einen Lösungsansatz für diese Problemstellung bietet die Herstellung von Hydrogelen auf Basis von Polysacchariden, welche mit den Enzymen der kontaminierenden Organsimen reagieren, wodurch in weiterer Folge eine visuell oder photometrisch leicht detektierbare Farbreaktion ausgelöst wird.

Für diesen Zweck wurden drei bioabbaubare Materialien (Dextran, Carboxymethylcellulose und Pektin) mit Methacrylgruppen modifiziert. Hydrogele daraus konnten erfolgreich mittels Einsatz dreier verschiedener Polymerisationsmethoden vernetzt werden, was mittels FTIR Messungen nachgewiesen wurde. Da Dextranhydrogele sehr fragil sind, wurden diese mit Polyvinylalkohol verstärkt.

Die enzymatische Hydrolyse der Hydrogele durch Trigger-Enzyme von kontaminierenden oder pathogenen Mikroorganismen wurde optimiert. Die Hydrolysereaktion wurde mit SEM Messungen verifiziert und mit HPLC Messungen überwacht.

Um den Hydrolyseprozess zu visualisieren, wurde mit der Freisetzung von Substanzen experimentiert und photometrische Messungen wurden durchgeführt.

Unterschiedliche Farbstoffe und signalverstärkende Enzyme wurden in die Hydrogele eingeschlossen. Um die Sensitivität zu verbessern, wurden die eingeschlossenen signalverstärkenden "Enhanzer" -Enzyme modifiziert, um ihr Molekulargewicht zu vergrößern oder um die Möglichkeit zu kreieren, sie mit dem Hydrogel kovalent zu vernetzen. Durch Inkubation dieser bioresponsiven Systeme mit kontaminierenden Mikroorganismen bzw. deren extrazellulären Enzyme wurde das Enhanzer-Enzym freigesetzt und katalysierte eine Farbreaktion. Mit diesem neuen Ansatz wurde ein Verstärkungseffekt um den Faktor 800 erzielt.

Abstract

The aim of this work was to create a bioresponsive system that is able to detect the presence of contaminating organisms e.g. in food packaging. An approach to solving this task was the production of hydrogels, which could react with enzymes of these contaminating organisms whilst e.g. creating a color reaction that is easily detectable visually or photometrically.

For this purpose three biodegradable materials (dextran, carboxymethylcellulose and pectin) were modified with methacrylate groups and their hydrogels were successfully produced by three different cross-linking methods, which was demonstrated with FTIR analysis. As dextran hydrogels were quite fragile, stability improvement with polyvinyl alcohol was tested.

Enzymatic hydrolysis of the hydrogels by trigger enzymes from contaminating or pathogenic microorganisms was optimized. The hydrolysis reaction was verified with SEM measurements and monitored with HPLC measurements.

To visualize the hydrolysis process, the release of substances was experimented with and photometric measurements were made. Various dyes were enclosed in the hydrogels as well as enhancer-enzymes together with pro-dyes, which upon oxidation produce color and thus enhance the detectable signal. To improve the signal, the enclosed enhancer-enzymes were also modified to increase their size or create the opportunity of cross-linking them with the hydrogel network. With this new method an enhancement of a factor 800 could be reached.

Introduction

General Introduction

This thesis was a small part of a project dedicated to the production of a bioresponsive detection system for fungi and bacteria, e.g. involved in the contamination of alimentary products. This system contains a carrier layer, an operating matrix and a barrier. The carrier layer “carries” the operating matrix. The operating matrix is the bioresponsive heart of the whole system and the barrier layer e.g. a membrane, separates the product from the operating matrix and only allows certain molecules, e.g. enzymes, of contaminating bacteria/fungi to pass. A system like this could be integrated in food packaging material and indicate a contamination. This could be achieved with a color change by dye released from the bioresponsive detection system upon its hydrolysis by enzymes of the contaminating organisms. “Best before dates” are an estimation but products can on the one hand spoil earlier, e.g. if they were not stored properly at all times, or on the other hand at times the store needs to toss them out because the “Best before date” has expired although they are still fine to use.

Replacing the old empirical system with a new in-situ bioresponsive system would thus bring more safety for consumers and safe stores a lot of money, as they do not need to discard of products that are actually still salable.

The goal of this thesis was to produce hydrogels that could be used as bioresponsive unit for the detection system. Enzymes of contaminating organisms should be able to interact with the hydrogel (bioresponsive system), e.g. partial hydrolysis in order to produce a color reaction to indicate their presence. The bioresponsive system should be able to respond to a wide range of contaminating organisms i.e. their extracellular enzymes. Naturally the response should be high enough to clearly see when the products are contaminated. The system should have a high stability and auto diffusion of the e.g. dye should be kept at a low level.

Hydrogels in biomedicine have been around for a few decades. The first biomedical hydrogel was synthesized in 1955 by professors Lim and Wichterle. Hydrogels are natural or synthetic homo- or co-polymer networks that can adsorb water or saline up to 1000 times their weight. Hydrogels can be applied in many different areas as their properties can easily be influenced by synthesis related factors (e.g. cross-linker, method and degree of cross-linking) or by the compounds used to make the gels. (Janik 2010)

For our application dextran, carboxymethylcellulose and pectin will be chosen. Natural hydrogels often do not provide enough stability e.g. pectin could be used to form gels, however,

they are not of a very stable nature. (Akbuga 1996) (Robyt 1997) To produce stable gels with these substances methacrylate groups could be introduced into their structure by different methods, which could be radically cross-linked and form covalent bonds.

Different dyes could be used to visualize the hydrolysis process and as model for substances that could be released. Another point of interest is the incorporation of enzymes, such as laccases from *Trametes hirsuta*, in the hydrogels.

One released enzyme has the possibility to react with several pro-dye molecules incorporated in the system, which leads to an enhanced color reaction. This could lead to a higher sensitivity of the system.

To minimize auto diffusion of the laccase it could be modified with different methods, such as the increase of the molecular weight of the laccase with polyethyleneglycol or introducing methacrylate groups to the laccase to crosslink it within the methacrylated biomatrices.

To improve the stability of fragile dextran gels, blends with polyvinyl alcohol were tested in this thesis. PVA hydrogels are known for their mechanical stability and their ability to retain much water. This quality makes them suitable for medical applications like contact lenses or wound dressings. Wound healing could be improved with these gels as the proper amount of moisture kept them from becoming irritated and tissue reconstruction is improved.

Biomatrices and their modification and degrading enzymes

Three different biomatrices were investigated in this thesis for the production of hydrogels for the bioresponsive unit. Dextran, carboxymethylcellulose and pectin are well described in the literature and all of them are known well in the food industry. To all of the biomatrices methacrylate groups were introduced. This opens up the possibility to easily create blends of all of the biomatrices that could improve the functionality of the bioresponsive system, as it would be sensitive to a wider range of enzymes. The modified biomatrices were then polymerized radically to ensure sturdy polymers because covalent bonds were formed. (Schneider 2012)

Dextran

Dextran is a homoglycan and consists of glucose units, which are mostly connected by 1,6-glycosidic bonds within the backbone and 1,3-glycosidic bonds in the branches. (Robyt 1997)

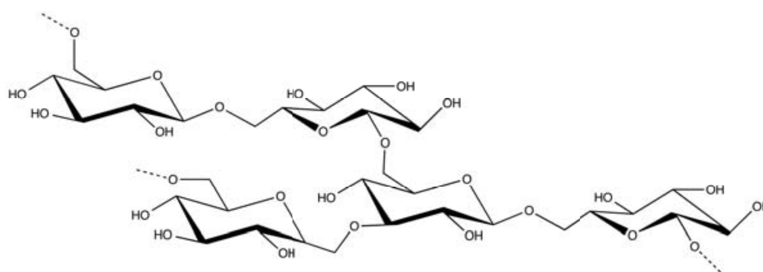

Figure 1 Structure of the homoglycan dextran

It is very common in bacteria. It is a water binding polysaccharide preventing the cells from drying out. (J. Koolman 2005) Bacteria also secrete dextrans to be able to adhere better on smooth surfaces e.g. tooth decay. (Ray 2004) In medicine, dextrans are used to expand the volume of blood transfusions (R. Terg 1996) or as carrier for medicinal substances. (Mehvar 2000) In the sugar industry, dextran is a problem because the sugar solution gains viscosity and the sucrose yield is decreased by its presence. (Kahalikova 2005)

α-Amylase is able to degrade the 1,6-glycosidic bonds of dextran as a side reaction. (Y. Sakano 1985) α-amylase from Bacillus amyloliquefaciens was used in this thesis because hydrogels are very fragile and a slow release is wanted.

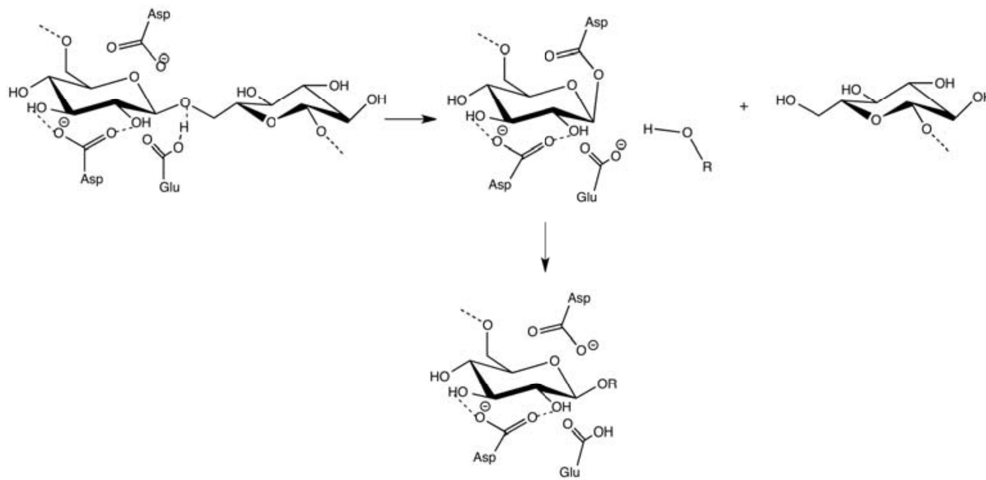

Figure 2 Mechanism of the enzymatic hydrolyzation of dextran with α-amylase (Maarel)

Three different methods were used in this thesis to modify dextran: modification with glycidylmethacrylate (van Dijk-Wolthuis 1995), methylmethacrylate (A. Yu. Men'shikova 2001) (Yasuhiko Onishi 2005) (Yasuhiko Onishi∗ 1978) and methacrylic anhydride (Kim 2000).

A blend with methacrylated PVA was made to improve the stability of dextran.

In the literature blends with various biodegradable materials and polyvinyl alcohol (PVA) have been described. Hydrogels with PVA and dextran or chitosan could be used for drug delivery (Maltinti 1999). Hydrogels with starch and PVA could be used as dialysis membrans. (MG Cascone 2001) or potentially as artifical skin. (Kunal Pal 2006)

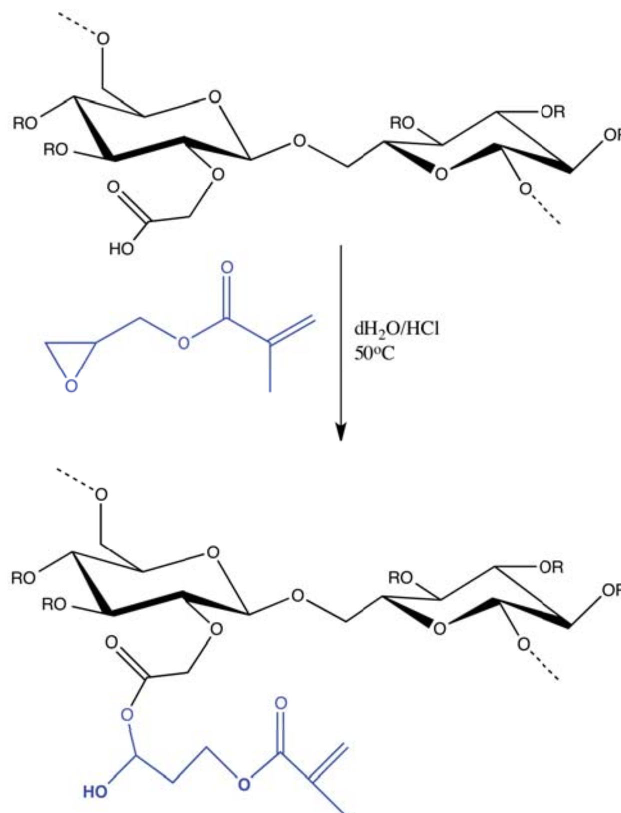

Figure 3 Modification of dextran with glycidylmethacrylate based on(J.F.A.S. Maior 2008)

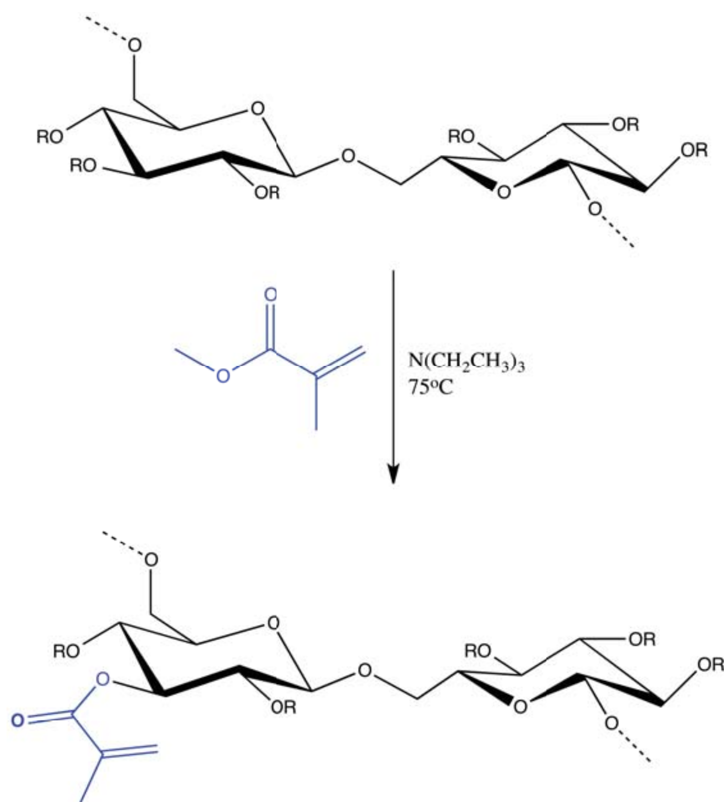

Figure 4 Modification of dextran with methylmethacrylate based on(J.F.A.S. Maior 2008)

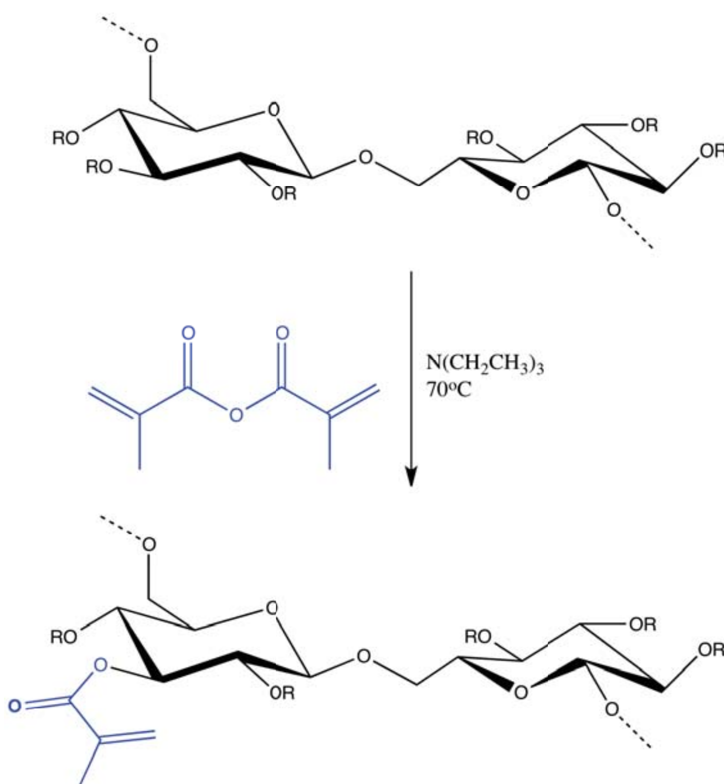

Figure 5 Modification of dextran with methacrylate anhydride based on(J.F.A.S. Maior 2008)

As dextran hydrogels are quite fragile, blends with PVA can be produced. Hydrogels based on the dextran/PVA blends are two-phase systems, however interactions can occur between the two components. (MG Cascone 2001). According to the literature there is no evidence of bond formation in these gels. (R. Jayasekara 2004)
However PVA can be methacrylated as previously described (Mühlebach 1997) and then crosslinked with methacrylated dextran.

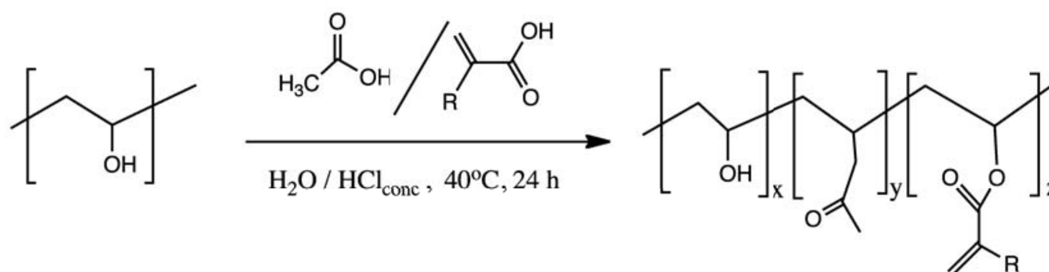

Figure 6 Modification of polyvinyl alcohol with methacrylic acid (Mühlebach 1997)

PVA was modified by A. Mühlebach with methacrylic acid at 40°C. Because of the methacrylate groups it is possible to crosslink PVA radically.

Pectin

Pectins are heteroglycans that are very rich in galacturonic acid.

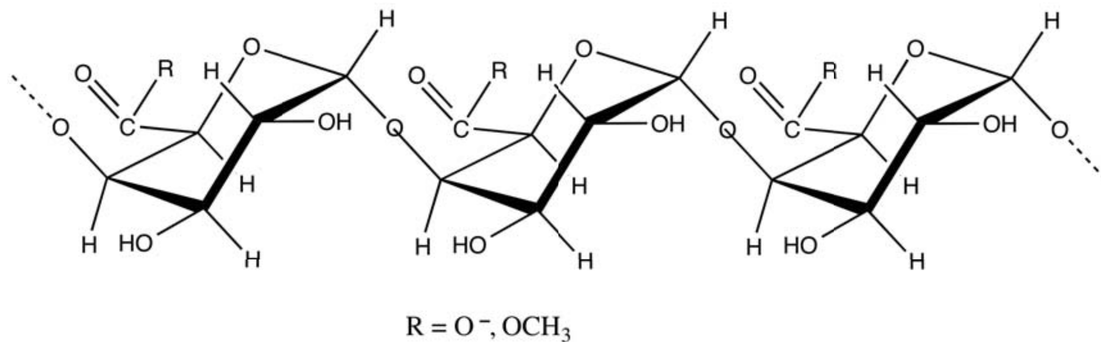

Figure 7 Structure of the heteroglycan pectin modified from (BL. Ridley 2001)

In nature pectin is a very important component in plants e.g. in peels from apples, citrus fruits, etc.

Because of its gelling properties pectin is often used in the food industry e.g. for jam production. (Willats 2006) Pectinases hydrolyze pectin. There are several kinds of: Protopectinases, polygalacturonases, lyases and pectin esterases (Jayani 2005) In this work a pectinase from *Aspergillus niger* and *Aspergillus aculeatus* were used. It hydrolyzes pectin randomly in the middle of the chain.

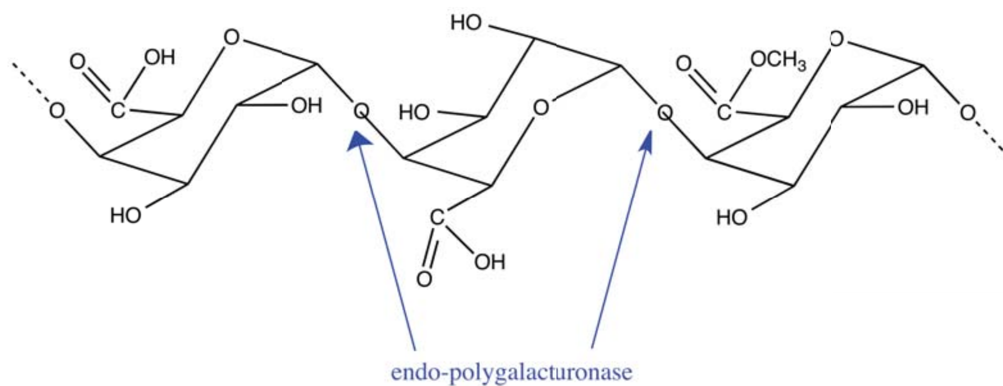

Figure 8 Hydrolyzation of pectin by an endo-polygalacturonase based on(Parkin 2008) and(Y van Santen 1999)

Pectin can be modified with glycidylmethacrylate at 40 °C. (J.F.A.S. Maior 2008) (Konstantin P. Schneider and Armin Zankel 2011)

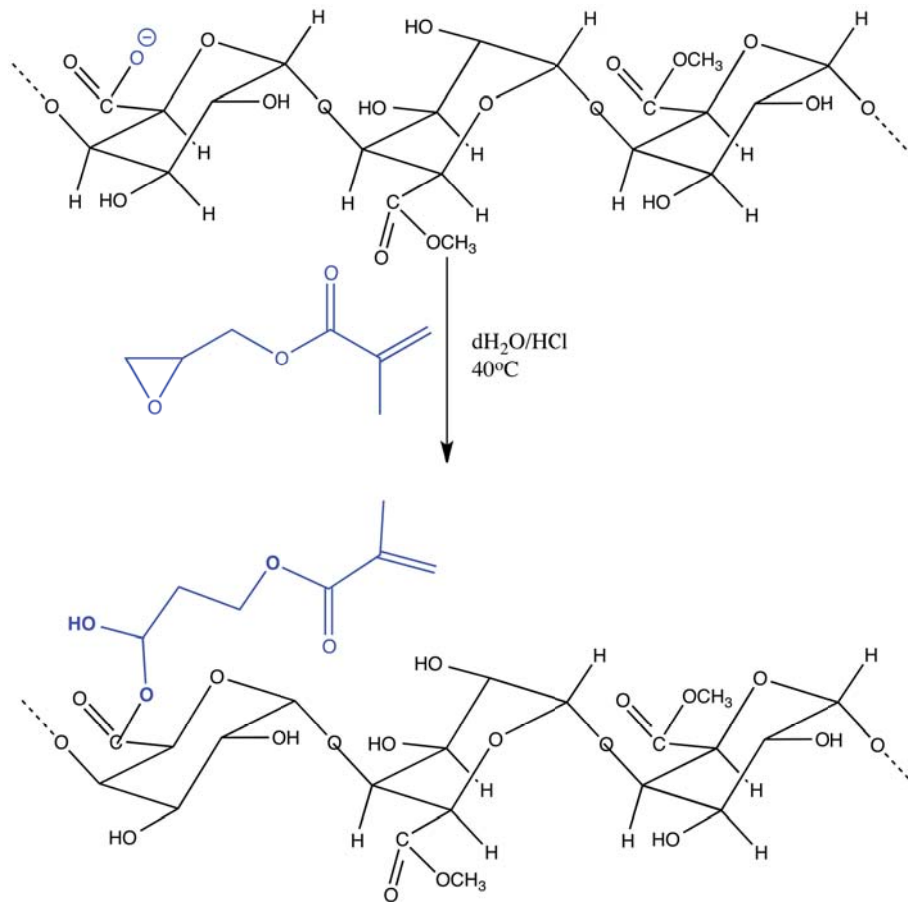

Figure 9 Modification of pectin with glycidylmethacrylate based on (J.F.A.S. Maior 2008)

Carboxymethylcellulose

When speaking of carboxymethylcellulose (CMC) in this thesis, actually the sodium salt is meant. CMC can be derived from cellulose by introducing carboxymethyl groups to some of the OH-groups.

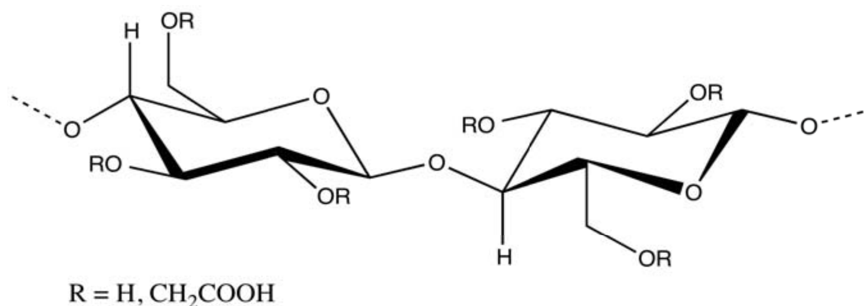

Figure 10 Structure of carboxymethylcellulose based on (M Kumar 2008)

In the food industry, CMC is used amongst others as gelling agent and stabilizer. (World Health Organisation)

CMC can be hydrolyzed by cellulases. In the figure below specifities of different cellulase types can be seen on a cellulase backbone.

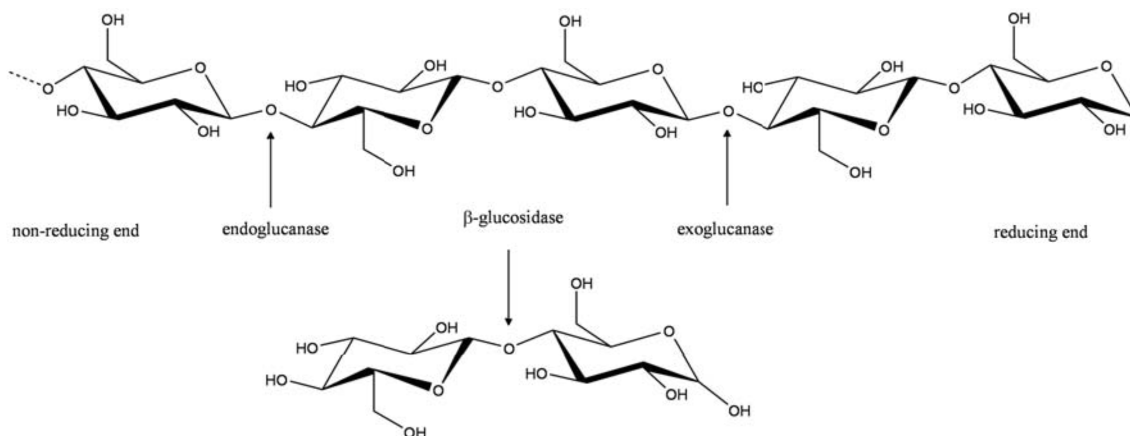

Figure 11 Enzymatic hydrolysis of cellulase (M Kumar 2008)

CMC can be modified with gylcidylmethacrylate at 40°C as previously described. (J.F.A.S. Maior 2008) (Konstantin P. Schneider and Armin Zankel 2011)

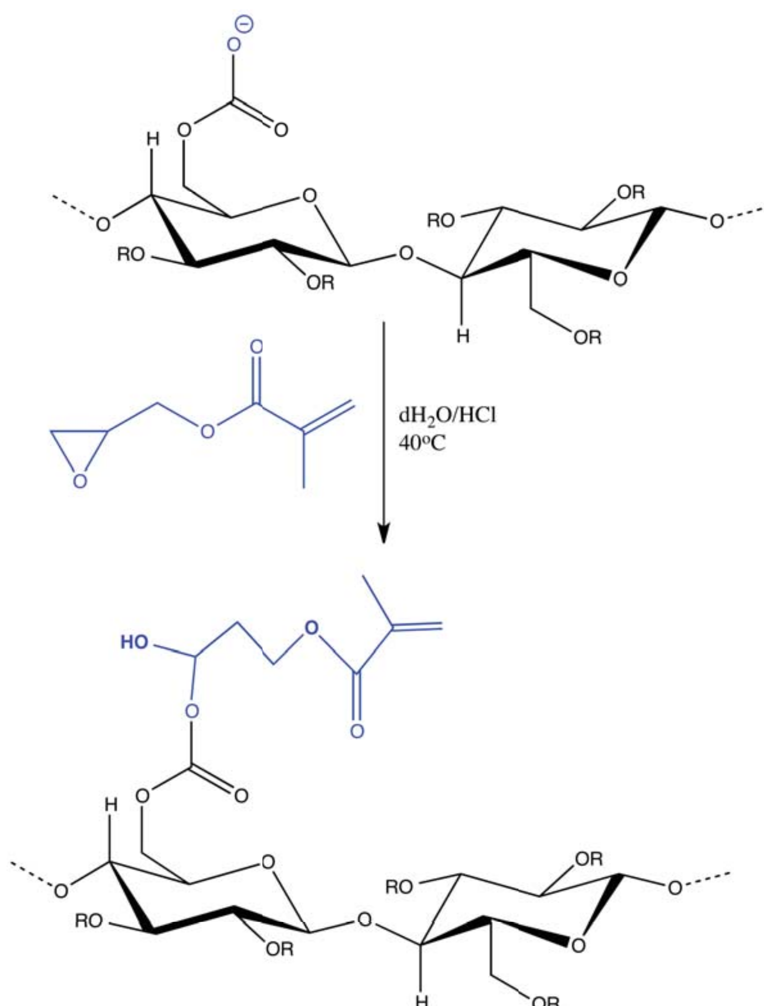

Figure 12 Modification of carboxymethylcellulose with glycidylmethacrylate based on (J.F.A.S. Maior 2008)

Loading of bioresponsive polymers

To visualize the hydrolysis process of the hydrogel different dyes were incorporated in the hydrogels. For dextran four options were investigated: Bismarck Brown R, methyl red, carminic acid and Reactive Black. Carboxymethylcellulose and pectin were dyed with alizarin.

Bismarck Brown R

Bismarck Brown R is an alkaline dye. Because of its cationic groups it can easily interact with anionic fibres. As dextran has many hydroxylgroups this dye was chosen.
The molecular weight of Bismarck Brown R is 461.39 g/mol.

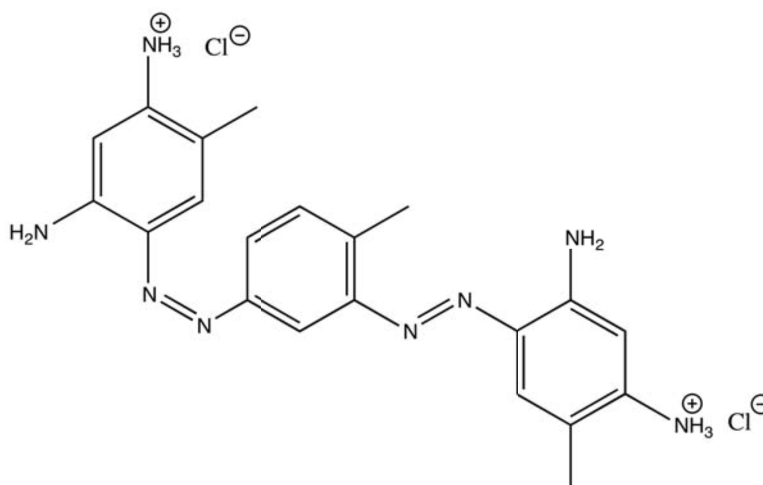

Figure 13 Structure of Bismarck Brown R

Methyl red

Methyl red is a pH sensitive azo-dye. Its color ranges from red to yellow.

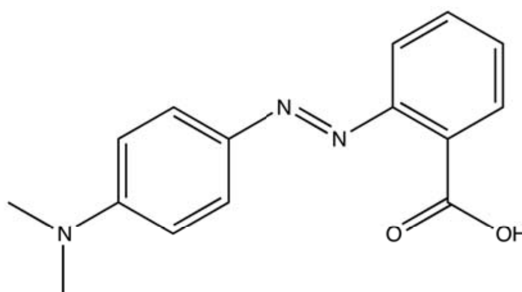

Figure 14 Structure of methyl red

As in literature described it had already been successfully encapsulated in cellulose matrices. (Van der Schueren 2012) The molecular weight of methyl red is 269.31 g/mol.

Methyl red was methacrylated with glycidylmethacrylate and an attempt was made to cross-link it into the hydrogel.

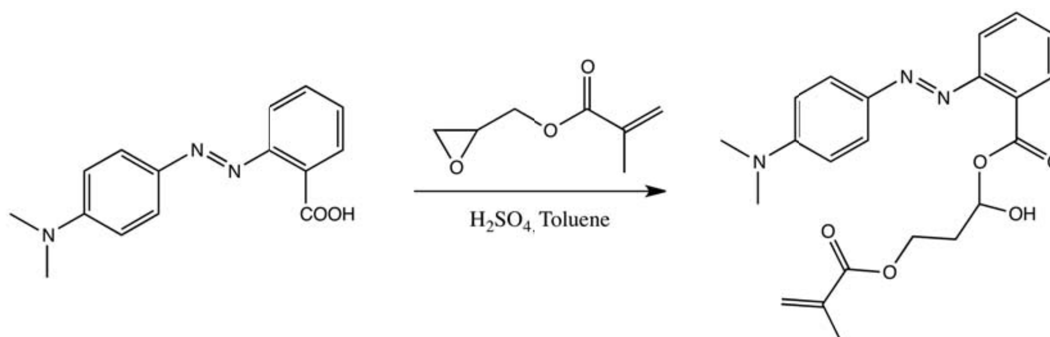

Figure 15 Modification of methyl red with gylcidylmethacrylate

Carminic Acid

Carminic acid is produced by cochineals as protection from predators. It is extracted from the dried cochineals and their eggs with hot water. The pigment carmine is an aluminium salt of carminic acid.

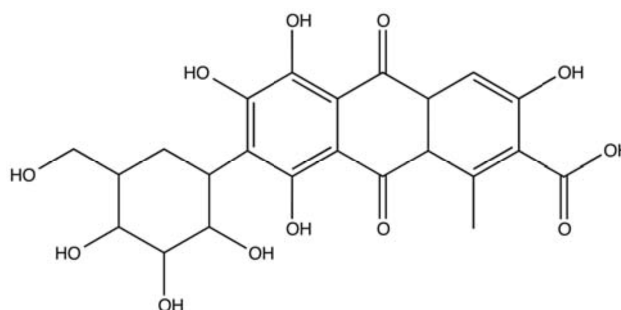

Figure 16 Structure of carminic acid

It has the food additive number E120 and because of its bright red color it is used in e.g. the beverage campari, lipstick. (E. Lück 1998) The molecular weight of carminic acid is 492.38 g/mol.

Reactive Black

In the textile industry Reactive dyes are often used for dyeing cellulosic fibres but also leather, wool, nylon and silk. (C. Yang 2005)

They produce unfading dyed fabrics because the Reactive dyes link covalently to the hydroxyl groups of the fabric. The unbound color can easily be washed away. The molecular weight of the sodium salt of Reactive Black 5 is 991.82 g/mol. (R. Stute 1974) (F. Esteves 2007)

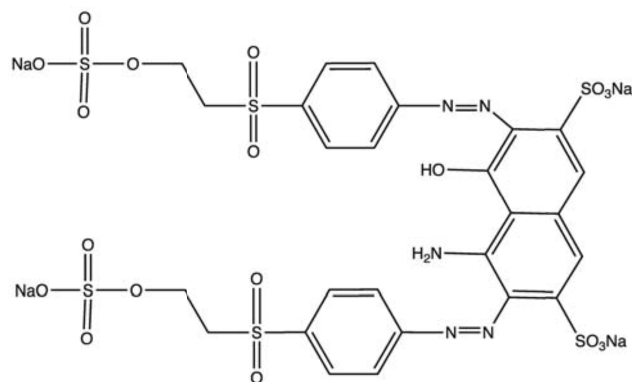

Figure 17 Structure of the sodium salt of Reactive Black 5 (2-(p-aminophenysulfonyl)enthanol sulfate ester)

Reactive Black 5 was chosen to dye dextran to investigate the influence on the color release of dyes covalently bound to the biomatrix.

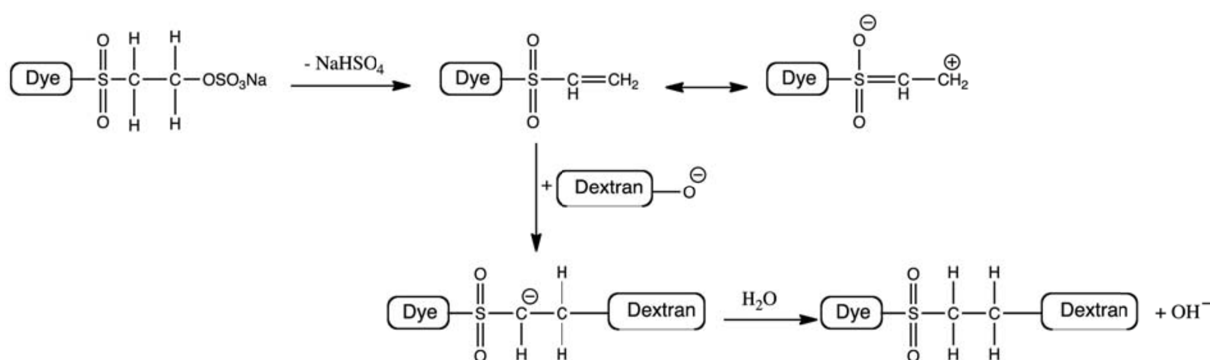

Figure 18 Dyeing reaction of sodium salt of Reactive Black 5 with dextran

Alizarin

Alizarin (1,2-Dihydroxyanthraquinone) is a pH sensitive dye used in the textile industry. Under alkaline conditions it is purple and in acidic conditions it is red-orange. The color change to a darker color improves its photometric detectability. Its molecular weight is 240.21 g/mol.

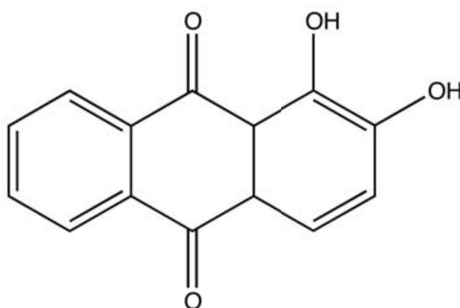

Figure 19 Structure of alizarin

Laccases are polyphenoloxidases that contain copper in their reactive center. (Thurston 1994)

They are typically produced by fungi e.g. *Trametes hirsuta*, a wood degrading fungus. (Abadulla 2000) (Phillips 2006)

Laccases are used as signal enhancers if a pro-dye is added to the system. Laccase is able to oxidize ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) as described in the chapter “Assays”

Laccase from *Trametes hirsuta* was chosen as enhancing enzyme in this thesis because laccases do not require any cofactors to work, only oxygen.

In previous experiments laccase from *Trametes hirsuta* was able do hydrolyze indigo dye which could be used in the stonewash process but also to degrade the dyes in the wastewater. (Campos 2001) On the other hand, many laccase-catalyzed biotransformations of phenolic compounds produce strongly colored reaction products.

To decrease the effects of auto diffusion of the enzyme out of the hydrogel, the laccase was modified with two different methods: one to increase its size and one to be able to crosslink it covalently with the methacrylated biomatrices.

Laccase modification was previously described. (Schroeder 2005) Polyethylene glycol is activated with cyanuric chloride to create 2-O-methoxypolyethylene glycol-4.6-dichlorotriazine. It was added to laccase from *Trametes hirsuta* to link it to the enzymes amino groups (Figure 20)

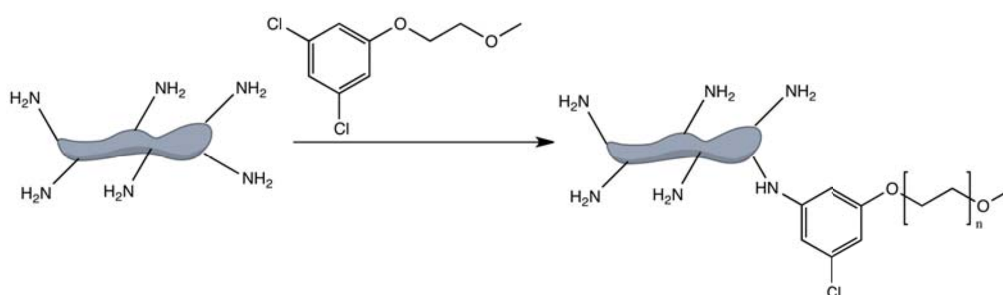

Figure 20 Modification of laccase from *Trametes hirsuta* with polyethyleneglycol (Schroeder 2005) (al 1995)

The second modification strategy involved the introduction of methacrylate groups to laccase from *Trametes hirsuta* to be able to crosslink it to the biomatrix. The reaction was previously described. (Schneider 2012)

The hydroxyl groups of the enzyme were modified with glycidylmethacrylate at pH 4.5.

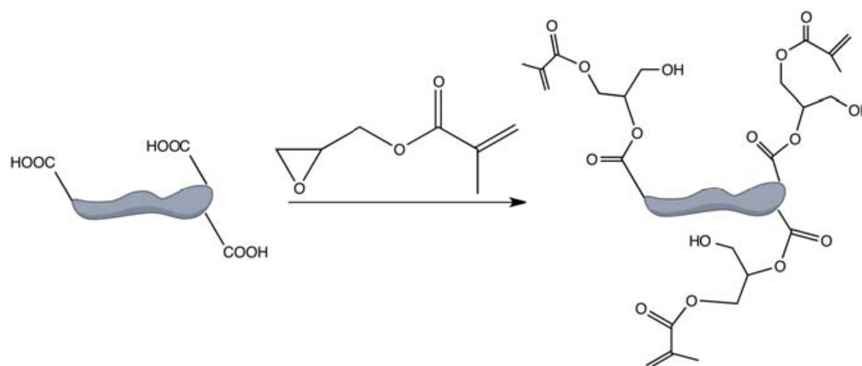

Figure 21 Modification of laccase from *Trametes hirsuta* with glycidylmethacrylate based on (J.F.A.S. Maior 2008)

Assays

Activities of the enzymes used for hydrolysis of the biomatrices in this thesis were determined with assays. The cellulase, pectinase and α-amylase assay used 3,5-Dinitrosalicylic acid (DNS).

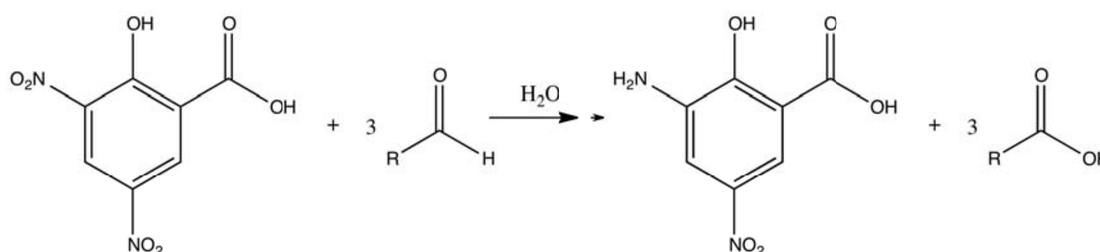

Figure 22 Reaction mechanism of the DNS assay with reducing sugars

DNS reacts with reducing molecules e.g. reducing sugars to 3-amino-5-nitrosalicylic acid, which can be detected at 540 nm. (Bernfeld 1955) (Bailey 1988)

The activity of laccases was determined with an assay that uses ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) as substrate.

The cation of ABTS is of a dark green color and is easily detectable. During this reaction oxygen is reduced to water. (Niku-Paavola 1988)

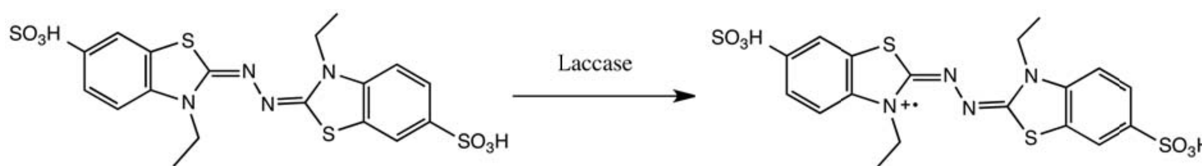

Figure 23 Reaction of laccase with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid))

Materials and Methods

Dyed Dextran based hydrogels

Dextran consists of glucose units connected by 1,6-glycosidic bonds within the chain and 1,3-glycosidic bonds in the branches. (Robyt 1997) Amongst other enzymes, α-amylase is able to digest dextran. (Y. Sakano 1985) It was possible to produce dextran hydrogels and degrade them with α-amylase. This process was visualized with the help of dyes that can be physically enclosed within the grid of the hydrogel (e.g. carminic acid) or covalently linked to the dextran backbone (e.g. reactive dyes). When the backbone was hydrolyzed, e.g. by α-amylase, the dye was released.

Dextran hydrogels are of a fragile nature. This fragility can be compensated by including polyvinyl alcohol (PVA) into the gel.

Dyeing of dextran

The role of enzymatic hydrolysis was determined by the release of dyed fragments of the dextran backbone to the supernatant. Four methods of dyeing were used. Carminic acid and Bismarck Brown are physically enclosed in the polymer whereas modified methyl red and Reactive Black and covalently bind to the sugar.

Dyeing of dextran with carminic acid

10.0 g dextran T40 and carminic acid (50 mg and 100 mg) were suspended in 100 ml n-pentane and stirred under reflux for 2 hours.

The dyed dextran was then modified with methacrylic anhydride based on work by (Kim 2000), however the dextran dyed with 100 mg carminic acid was precipitated in acetone as well as in a mixture of ethanol:isopropanol to determine which precipitation conditions deliver a better product. The precipitate was collected by vacuum filtration, dried under vacuum and stored at 4°C until further use.

Dyeing of dextran with Bismarck Brown R

10 g of dextran T40 und 0.2 g of Bismarck Brown were suspended in 100 ml n-pentane and stirred for 2 hours under reflux. Another 0.2g of Bismarck Brown were added to the suspension and it was stirred for 2 more hours. The solvent was removed under reduced pressure and the

precipitate was dried under vacuum. The dyeing process and methacrylation process with glycidylmethacrylate was done simultaneously by adding 0.5 mL HCl (6 M) to the aqueous solution of dextran and Bismarck Brown. The modification was conducted as described in in the chapter "Modification and cross-linking of dextran. This reaction was previously described by (A. Yu. Men'shikova 2001) (Yasuhiko Onishi 2005) (Yasuhiko Onishi∗ 1978)

Dyeing of dextran with methyl red

In this case, the dye and dextran were metharcylated separately. Dextran was modified with methacrylic anhydride as described in the chapter"Modification and cross-linking of dextran 1.35 g methyl red were dissolved in 50 mL THF in reflux mode. 3.3 mL glycidyl methacrylate and 50 µL concentrated H_2SO_4 were added. The reaction was stirred for 12 hours.

Dyeing of dextran with Reactive Black 5 (Cibacron Marine DP-B; Intracron Black VCKN, remazol black B)

5.30 g dextran, 0.54 g Na_2CO_3, 4.98 g Na_2SO_4, 0.28 g NaOH, 160 mg Reactive Black 5 and 100 mL distilled water were weighed into a suitable vessel and stirred in a water bath at 60°C for 2 hours. The solution was then cooled down to room temperature and part of the dyed sugar was precipitated in cold ethanol and part in acetone to determine the influence of the precipitation solvent on the color release. The precipitate was collected by vacuum filtration, dried under vacuum and stored at room temperature until further use.

Modification and cross-linking of dextran

Several methods of crosslinking dextran were applied.

Modification of Dextran with glycidylmethacrylate

5 g (dyed) dextran T40 were dissolved in 100 mL distilled water under elevated temperature (50°C). 500 µL 1 M HCl were added and the solution was stirred for 10 minutes. 5 mL glycidylmethacrylate were added. The reaction was finished after 12 hours by cooling down to 4°C. The solution was stored at 4°C until further use.
The reaction was based on previously published work by (van Dijk-Wolthuis 1995)

Modification of Dextran with methylmethacrylate

12 g (dyed) dextran T40 or T100, 200 mg LiCl and 125 mL ethanol (99.8%) were transferred into a suitable three-neck round-bottom flask. (At this point 0.2 g Reactive Blue or 0.2 g Reactive Black were added if in situ dying was wanted). 10 mL methylmethacrylate and 5 mL triethylamine were added. The reaction was stirred for 72 h at 75°C under reflux. A drying tube was used to keep out air humidity.

The reaction mixture was then cooled to room temperature and filtered with vacuum suction. The filter cake was washed with ethanol and dried under reduced pressure and stored under vacuum condition at room temperature. This reaction was previously described by (A. Yu. Men'shikova 2001) (Yasuhiko Onishi 2005) (Yasuhiko Onishi∗ 1978)

Modification of Dextran with methacrylic anhydride

10 g dextran T40 and 10 g LiCl were suspended in 100 ml solvent (N,N-Dimethylacetamide, methylpyrrolidione, N,N-Dimethylformamide or ethanol) and stirred under reflux at 90°C for 2 hours. Then the solution was cooled to 70°C and 1 ml triethylamine was added to the stirring reaction mix. After 15 minutes, 2 mL methacrylic anhydride were slowly added.

After approximately one hour another milliliter methacrylic anhydride (5 x 200 µL) and 0.5 mL triethylamine were added to the reaction which was then left to stir at 70°C for at least 5 days.

The reaction was finished when the reaction broth became clear and the magnetic stirrer was clearly visible in the solution.

The reaction was cooled down to room temperature and filtered under reduced pressure to remove solid remainders of the substrates. The filtrate was precipitated by slowly dripping it into ice-cold, stirred acetone. The precipitate was collected by filtration under reduced pressure, washed with acetone and dried under low pressure.

Since the product is hygroscopic it was stored under low pressure or at -18°C until further use.

The reaction was based on previously published work by (Kim 2000)

Production of dextran based hydrogels

The modified dextran was crosslinked by three different radical polymerization methods, which provided the advantage of a tight network as covalent bonds were formed. Having several options to conduct the polymerization reaction was a big advantage as the goal of our work was to enclose working substances into the hydrogel and some might be sensitive to one of the polymerization methods, e.g. thermosensitive substances.

We used three different polymerization methods based on the work of (Schneider 2012).

Gel formation at room temperature (TEMED)

20 mg $Na_2S_2O_8$ and 10 µL TEMED were added to 3 g of a 20 % aqueous solution of the methacrylated sugar. In case of the modification with glycidylmethacrylate, the 3 g of solution were directly taken from the reaction mix after modification. The solution was mixed and the gel formation process took place at room temperature. For the dextran solution dyed with methyl red, 5 of µL methyl red solution were added to the reaction mixture. Gel formation usually took place within 15 minutes.

Gel formation at increased temperature (ΔT)

20 mg $Na_2S_2O_8$ were added to 3 g of a 20 % aqueous solution of the modified dextran. In case of the modification with glycidylmethacrylate, 3 g solution were taken directly from the reaction mix after modification. For the dextran solution dyed with methyl red, 5 µL methyl red solution was added to the reaction mixture. The solution was mixed and the gel formation process took place at 80°C for 1-2 hours.

Gel formation under UV light (UV-light)

20 mg $Na_2S_2O_8$ and Irgacure were added to 3 g of a 20% aqueous solution of the modified dextran. In case of modification with glycidyl methacrylate, 3 g solution was directly taken from the reaction mix after modification. For the dextran solution dyed with methyl red, 5 µL methyl red solution were added to the reaction mixture. The solution was mixed and the gel formation process took place at room temperature under UV radiation for 1-2 hours.

Enzymatic hydrolysis of dextran hydrogels

After the gel formation was finished, the hydrogel was cut into pieces and the pieces were carefully rinsed with distilled water. The water was separated from the gel with a sieve and the pieces were weighed into suitable glass vessels.

For the enzymatic hydrolysis 10 mL of phosphate buffer (50 mM, pH 7) and 0.4 mL of α-amylase from *Bacillus amyloliquefaciens* (27 U/mL) were added to the polymer piece. In the case of hydrogels dyed with Reactive Black 20.2 mL of distilled water and 0.2 mL of α-amylase were used.

For the blank phosphate buffer/distilled water was used instead of enzyme.

In order to determine the degree of hydrolysis 500 µL of sample were taken at time points between 0 h to 48 h.

They were centrifuged for 5 min at 10.000 rpm. The supernatant of dyed samples was investigated with a wavelength scan from 300-700 nm.

Size exclusion experiments where used to determine the hydrolysis fragments after enzymatic hydrolyzation experiments of undyed samples. A Phenomenex Biosep-SEC-S-4000 column was used as stationary phase and double distilled degased and filtrated water with 10 % methanol (flow rate: 0.5 and 0.6 mL/min) were used as mobile phase. The analyses were performed with a HPLC (DIONEX P-580 PUMP, Dionex Cooperation, Sunnyvale, USA) in combination with a refractive index detector. For weight reference various substances were investigated concerning their retention time and a calibration curve was created.

The hydrolysis process was also investigated by performing scanning electron microscopy on untreated and treated hydrogels . The samples were hydrolyzed in with 25 µL and 50 µL of α-amylase from *Bacillus amyloliquefaciens* in distilled water. Before the measurements the samples were stabilized with 3% glutaraldehyde for 24 hours. After a 30 hour lyophilization the samples were sputtered with gold For 30 hours. The measurements were performed on a Zeiss Evo 60 (5.0 kV).

Hydrogels based on dextran with enclosed enzyme

Alternatively, enzymes can be incorporated in hydrogels instead of dyes. They can be used to enhance the signal e.g. released laccase can oxidize ABTS, which results in a dark green color of the supernatant.

Modification of the dextran with methacrylic anhydride

Dextran T100 was modified as described above in „Modification of dextran with methacrylic anhydride“ based on previously published work by (Kim 2000) However, in total 3 mL of methacrylic anhydride were added.
The experiment was performed with a PEG modified laccase from *Trametes hirsuta* (PEG-L2) by Marc Schroeder. (Schroeder 2005) Also a commercial laccase from the fungus *Myceliophthora thermophila* (MtL) was used.

Modification of laccase from *Trametes hirsuta* with polyethyleneglycol (PEG)

First methoxypolyethylene glycol (5000 g/mol) was activated and then mixed with laccase from *Trametes hirsuta* to receive a laccase enhanced with PEG groups.
Methoxypolyethylene glycol (0.8 mmol) was dissolved in 100 mL of dried toluene.
Sodium carbonate (60 mmol) and 6.0 g molecular sieves were added. Methoxypolyethylene gylcol was activated with cyanuric chloride (2.4 mmol) under agitation for 40 hours at 40°C to produce 2-O-methoxypolyethylene glycol-4,6-dichloro-s-triazine. The reaction broth was centrifuged and the filtrate precipitated in 300 mL petroleum ether.
2 mL of laccase (1 mg/mL) were united with 100 mL of sodium borate buffer (0.1M, pH 9.3). 3g of 2-O-methoxypolyethylene glycol-4,6-dichloro-s-triazine were added and the mixture was shaken at room temperature. After 2 hours the PEGylated enzyme was first purified by ultra filtration with a Vivaflow 50 (Molecular weight cut off 30000) and then with the ÄKTA.

The column was a "Hiload 26/60 superdex 200 prep grade" and a potassium phospate buffer (50 mM, pH 7) with 0.1 M sodium chloride was used as eluent.
The reaction was performed as previously described by (Schroeder 2005)

Production of hydrogels with enclosed enzyme

Blanks and samples were prepared according to Table 1 $Na_2S_2O_8$ and Irgacure as radical starters were added and then mixed carefully.

Table 1 Sample preparation of dextran hydrogels with enclosed laccases PEG-L2 (laccase from *Trametes hirsuta* modified with polyethyleneglycol) and MtL (laccase from the fungus *Myceliophthora thermophila*)

Modified dextran [g]	ddH2O [µL]	Laccase	
		PEG-L2 [µL]	MtL [µL]
0,3	2500	500	/
0,3	2950	/	50
0,3	2900	/	100

The mixtures were then irradiated for 1-2 h by UV light until the gels had hardened.
The samples were then cut into 4 pieces and weighed into suitable vessels for the enzymatic hydrolysis.

Enzymatic hydrolysis of dextran hydrogels with enclosed enzyme

10 mL of a 50 mM phosphate buffer (pH 6.9) and 300 µL of α–amylase from *Bacillus amyloiquefaciens* were added to the samples. The blanks were prepared with buffer instead of enzyme. Samples and blanks with Laccase L2 were rebuffered to pH 4.5 with a 500 mM succinate buffer (pH 4.5) before performing the activity assay with ABTS. Laccase from *Myceliophthora thermophila* worked well at pH 6.9. 1 mL sample was taken at 0 h, 1 h and 24 h.
The activity assay was performed as described in the chapter "Assays". As blank succinate buffer was used.

Also size exclusion experiments where used to determine the fragments after the hydrolysis raction. A Phenomenex Biosep-SEC-S-4000 column was used as stationary phase and double distilled degased and filtrated water with 10 % methanol (flow rate: 0.5 and 0.6 mL/min) were used as mobile phase. The analyses were performed with a HPLC (DIONEX P-580 PUMP, Dionex Cooperation, Sunnyvale, USA) in combination with a refractive index detector. For weight

reference various substances were investigated concerning their retention time and a calibration curve was created.

Mixed hydrogels based on a dextran and polyvinyl alcohol (PVA) blend

As the dextran hydrogels lacked stability, a polymer blend consisting of activated PVA (modified by A.Mühlebach, (Mühlebach 1997)) and dyed activated dextran was investigated. The PVA was used to provide improved stability and dextran ensured the biodegradability of the gel by α-amylase.

Production of hydrogels based on a blend of dyed dextran and PVA (8.5%)

Activated dextran T100 dyed with Reactive Black was prepared with methlymethacrylate as previously described in the chapter “Modification of dextran with methylmethacrylate”based on the work of by (A. Yu. Men'shikova 2001) (Yasuhiko Onishi 2005) (Yasuhiko Onishi∗ 1978) and used for a 20% solution in distilled water. Irgacure and $Na_2S_20_8$ were added to the solution accordingly. The activated PVA (8.5 %) was used as delivered.
PVA and the dextran suspensions were mixed in a plate in varying proportions (PVA:dextran) with a total sample volume of 300 µL from 8:2 to 1:9. Irgacure and $Na_2S_20_8$ were added and irradiated with UV light. The plate was kept in an oven for 10-15 min at 80°C since not all samples had fully polymerized.

Production of hydrogels based on a blend of dyed dextran and PVA (11%)

Activated dextran T40 dyed with Reactive Black was prepared with methlymethacrylate as previously described in the chapter “Modification of dextran with methylmethacrylate”based on the work of by (A. Yu. Men'shikova 2001) (Yasuhiko Onishi 2005) (Yasuhiko Onishi∗ 1978) and used for a 20% solution in distilled water. Irgacure and $Na_2S_20_8$ were added to the solution accordingly. The activated PVA (11 %) was used as delivered.
PVA and the dextran suspensions were mixed in a plate in varying proportions (PVA:dextran) with a total sample volume of 1000 µL from 5:5 to 2:8. Irgacure and $Na_2S_20_8$ were added and irradiated with UV light. The plate was kept in an oven for 10-15 min at 80°C since not all samples had fully polymerized.

Enzymatic hydrolysis of hydrogels based on a dextran and PVA blend

The samples were hydrolyzed with α-amylase from *Bacilius amyloliquefaciens*. A 1:8 and 1:15.4 dilution of the enzyme was prepared with distilled water.

To polymer blends with PVA (8.5%) 1 mL of the respective dilution was added.
The samples were kept in a shaker at room temperature and at 0, 1 and 4 h samples of the supernatant were taken,
To polymer blends with PVA (11%) 5 mL of the respective enzyme dilution were added. The samples were kept in a shaker at room temperature and at 0, 1, 2 and 24 h samples of the supernatant were taken, centrifuged for 8 min at 13.000 rpm.
A wavelength scan was performed from 300-700 nm. The peak maximum of Reactive Black was detected at λ = 580 nm.

Carboxymethylcellulose based hydrogels

Dyeing of carboxymethylcellulose with alizarin

Dyeing a biodegradable matrix with alizarin was used to measure, control and characterize an enzymatic hydrolysis process catalyzed by cellulases.

5 g carboxymethylcellulose, 0.2 g of the dye alizarin (85 % graduate) and 100 mL of n-pentane were united in a suitable vessel and heated under reflux for 2 hours. The solvent was then removed under reduced pressure and the sample was dried under vacuum. The reaction was previously described by (Schneider 2012)

Modification of carboxymethylcellulose with glycidylmethacrylate

Determination of the most suitable crosslinking degree

2.5 g of dyed carboxymethylcellulose are dissolved while stirring in 100 mL ddH2O over night at 45°C.

2.5 mL, 5 mL, and 7.5 mL of glycidylmethacrylate and 1 mL of 6 M HCl were added. The mixture was stirred over night. If the mixture turned purple, more HCl was added.

Equal amounts of carboxymethylcellulose and glycidylmethacrylate turned out to be most beneficial for our purposes. So all further experiments were carried out with this ratio.

The reaction was based on the work of (J.F.A.S. Maior 2008) (Konstantin P. Schneider and Armin Zankel 2011)

Polymerization of Carboxymethyl cellulose hydrogels (CMC_MA)

The methacrylated CMC (CMC_MA) was crosslinked by two different methods. The gels were stored at 4°C until use. The polymerization method with TEMED could not be used, as there were unfavorable interactions like e.g. the polymer turned black. The reactions were previously described by (Schneider 2012)

Gel formation at increased temperature (dT)

About 20 mL of the methacrylated CMC solution were transferred into a suitable glass form. 20 mg of $Na_2S_2O_8$ were added as radical starter. The solution was mixed and the gel formation process took place at 80°C for 1-2 hours.

Gel formation under UV light (UV-light)

About 20 mL of the methacrylated CMC solution were transferred into a suitable glass form. 20 mg of $Na_2S_2O_8$ and of Irgacure were added as radical starters. The solution was mixed and the gel formation process took place at room temperature under UV radiation for 1-2 hours.

Enzymatic hydrolysis of Carboxymethyl cellulose hydrogels

After the gel formation was finished, the hydrogel was cut into squares of about 1 cm^2 and the pieces were carefully rinsed with dH_2O. The water was separated from the gel by filtration and the pieces were weighed into a suitable glass vessel. For the enzymatic hydrolysis a succinate buffer (50 mM, pH 4.5) and a cellulase from Aspergillus *sp.* and pectinase from *Aspergillus aculeatus* were used. The reaction was previously described by (Schneider 2012)

Pectin based hydrogels

Dyeing of pectin with alizarin

5 g of pectin, 0.2g of alizarin (85 % graduate) and 100 mL of n-pentane were united in a round bottom flask and heated under reflux for 2 hours. The solvent was then removed under reduced pressure and the dyed pectin was dried at 60°C. The reaction was previously described by (Schneider 2012)

Modification of pectin with glycidylmethacrylate

5 g of (dyed) pectin were dissolved while stirring in 100 mL ddH2O at 45°C. 0.6mL 6 M HCl were added. After 5 min 5 mL glycidylmethacrylate were added. The mixture was stirred over night. In case of dyed carboxymethyl cellulose, more HCl should be added if the mixture should turn purple as this indicates an increasing pH value.
The reaction was previously described by (J.F.A.S. Maior 2008) (Konstantin P. Schneider and Armin Zankel 2011)

Production of pectin hydrogels

The modified pectin was crosslinked by two different methods; increased temperature and UV. The polymerization method with TEMED could not be used, as there were unfavorable interactions like e.g. the polymer turned black.

Gel formation at increased temperature

About 20 mL of modified pectin solution were transferred into a suitable glass form. 20 mg of $Na_2S_2O_8$ were added as radical starter. The solution was mixed and the gel formation process took place at 80°C for 1 hour.

Gel formation under UV light

About 20 mL of modified pectin solution was transferred into a suitable glass form. 20 mg of $Na_2S_2O_8$ and of Irgacure were added as radical starters. The solution was mixed and the gel formation process took place at room temperature under UV radiation for 1-2 hours.

Enzymatic hydrolysis of pectin hydrogels

After the gel formation was finished, the hydrogel was cut into squares of about 1 cm^2 and the pieces were carefully rinsed with dH_2O. The water was separated from the gel by filtration and the pieces were weighed into a suitable glass vessel. For the enzymatic hydrolysis a succinate buffer (50 mM, pH 4.5) and cellulase from Aspergillus *sp.* and pectinase from *Aspergillus aculeatus* were used. The reaction was previously described by (Schneider 2012)

Mixed hydrogels made of CMC and pectin

As we were able to successfully produce and degrade dyed hydrogels on the base of carboxymethylcellulose our next field of interest was to reproduce our results with mixtures of pectine and carboxmethylcellulose in various ratios. To measure the level of hydrolysis the concentration of released dye is measured by colorimetric detection.

The sugars were dyed and modified with gylicdylmethacrylate based on previous work of (J.F.A.S. Maior 2008)

Hydrogels with the following Pectin/CMC ratios were produced: 1:0, 2:1, 1:1, 1:2 and 0:1 and enzymatically hydrolyzed with cellulase from *Aspergillus sp.* and pectinase from *Aspergillus aculeatus*. As buffer a succinate buffer (50 mM, pH 4.5) was used. 3-fold measurements were made.

Dyeing blends of carboxymethyl cellulose and pectin with alizarin

Blends of CMC and pectine in the ratios: 2:1, 1:1, 1:2 were dyed.

30 g of a CMC/Pectine blend, 1.2g of alizarin (85 % graduate) and approximately 150 mL of n-pentane were united in a suitable vessel and heated under reflux at 50°C for 1 hour. The solvent was then removed under reduced pressure and the sample was dried under vacuum.

The reaction was previously described by (Schneider 2012)

Production of pectin and CMC hydrogels

The blends were polymerized as by 2 different methods:

with UV light and with increased temperature. The polymerization method with TEMED could not be used, as there were unfavorable interactions like e.g. the polymer turned black.

The reaction was previously described by (J.F.A.S. Maior 2008) (Konstantin P. Schneider and Armin Zankel 2011)

Enzymatic hydrolysis of mixed hydrogels

After the gel formation was finished, the hydrogel was cut into squares of about 1 cm^2 and the pieces were carefully rinsed with distilled water. The water was separated from the gel by filtration and the pieces were weighed into a suitable glass vessel. For the enzymatic hydrolysis a succinate buffer (50 mM, pH 4.5) and cellulase from *Aspergillus sp.* and pectinase from *Aspergillus aculeatus* were used. The reaction was previously described by (Schneider 2012)

Mixed and pure hydrogels on CMC and pectin base with enclosed enzyme

Unmodified as well as modified laccase was enclosed and released from hydrogels.

Two modification strategies were used and their influence on the enzymatically induced laccase release from the gel were tested. Modification with polyethyleneglycol increased the size of the laccase and modification with glycidyl methacrylate made it possible to crosslink the enzyme within the hydrogel.

Modification of laccase from *Trametes hirsuta* with glycidylmethacrylate

4.5 mL of laccase from *Trametes hirsuta* were methacrylated by adding 25 mL of buffer and 1 mL of glycidyl methacrylate. Glycine buffer (0.1 M, pH 4.5) and borate buffer (0.1 M, pH 9.3) were used to determine the best reaction conditions. The modification took place over night.

To purify the modified laccase it was transferred into vivaspins (molecular weight cut off 30000) and centrifuged at 5000 rpm for 15 minutes.

Succinate buffer (pH 4.5, 50 mM) was used to rebuffer, wash thrice and fill the enzyme up to a volume of 10mL.

To determine the amount of activity loss of the enzyme caused by the modification, purification and polymerization the laccase activity was determined via an activity assay where ABTS serves as substrate. The following samples were prepared: 1 mL of the modified laccase was polymerized at room temperature for one hour by adding 1 µL of TEMED and 5 mg $Na_2S_2O_8$, a blank, a sample before and various samples after the addition of glycidyl methacrylate, a sample after the purification process and a sample after the polymerization reaction.

The activity of the laccase was measured as described in the chapter "Assays", however, samples were diluted 1:100 before use. A mixture of 4.5 mL of dH_2O, 25 mL of glycine buffer (0.1M, pH 4.5) and 1 mL of glycidyl methacrylate served as blank.

The reactions were based on previous work of (Schroeder 2005)

Modification of laccase from *Trametes hirsuta* with polyethyleneglycol

Laccase from *Trametes hirsuta* was modified as previously described in based on the work of (Schroeder 2005)

Production of pectin and CMC hydrogels with enclosed enzyme modified with glycidylmethacrylate

The sugars were modified according to the methods described in the chapter Modification of laccase from Trametes hirsuta with polyethyleneglycol (PEG)

however without the addition of alizarin. Also double the amount of glycidylmethacrylate was used to produce a tighter network.

Following blends of CMC and pectin were modified: 1:0, 1:1, 0:1

In a suitable glass vessel 15.9 g of a modified CMC, pectin or a blend and 1 mL of unmodified or modified laccase were mixed. 20 mg of $Na_2S_2O_8$ and 10 µL of TEMED were added as radical starters. The mixture polymerized at room temperature within several minutes.

The polymer was cut in pieces and washed with buffer several times. At first the gels were enzymatically hydrolyzed after this step. However, to reduce auto diffusion to a minimum another washing step was added and the gel was then left in buffer over night under agitation.

On the next day it was washed twice for 2 hours before the incubation in order to remove any laccase that was not well within the polymer. The reaction was previously described by Schneider, Gewessler et al. 2012).

Production of pectin and CMC hydrogels with enclosed enzyme modified with polyethyleneglycol

Hydrogels with enclosed laccase from *Trametes hirsuta* were modified with polyethyleneglycol were produced by Konstantin Schneider. (Schneider, Gewessler et al. 2012)

Enzymatic hydrolysis of pectin and CMC hydrogels with enclosed enzyme

A polymer piece was transferred into a scintillation vial and 15 mL of succinate buffer (50 mM, pH 4.5) were added.

The pure hydrogels were hydrolyzed with various concentrations of cellulase from *Aspergillus sp.* and pectinase *Aspergillus niger* (485 U/mL) The blends of CMC and pectin were hydrolyzed with cellulase from *Aspergillus sp.* (175 U/mL) and pectinase *Aspergillus aculeatus* (130 U/mL), as they had more similar activities

The samples were shaken at room temperature. 1 mL of the supernatant was removed to measure the activity of the released laccase at 0, 1, 2, 4, 6, 24, 48 and 72 hours. The individual weight of the polymer pieces and volume loss by sample taking was considered in the activity calculations.

Enzyme assays

Cellulase Assay

The pectinase assay used was previously described. (Bailey 1988) The activity of pectinase was determined by the increase of reducing sugars, which caused the reduction of 3,5-dinitrosalicylic acid. This reaction went hand in hand with a color change from yellow to orange that could be detected at 540 nm.

Solutions

DNS Solution

13.98 g NaOH and 216.1 g potassium sodium tartrate were dissolved in 800 mL dH_2O. 5.16 g phenol, 7.48 g 2-hydroxy-3,5-dinitrosalicylic acid and 5.86 g sodium metabisulfite were added and filled up to 1000 mL with dH_2O. The yellow solution was stored lightproof and refrigerated.

Glucose standard solutions

As standards glucose solutions with Na-citrate buffer (50 mM, pH 4.8) were prepared in the following concentrations: 20; 10; 5; 2.5; 1.25; 0.625; 0.3125; 0.156 and 0.078 [mg/mL]

1% CMC solution as substrate

1 g CMC was dissolved in 100 mL Na-citrate buffer (50 mM, pH 4.8) on ice.

Sample preparation

The samples, blanks and standards were prepared according to the following table:

Table 2 Sample preparation for the cellulase assay

	1% CMC solution [µL]	Na-citrate buffer (50 mM, pH 4.8) [µL]	Standard solutions [µL]	Sample [µL]	DNS solution [µL]
Blank	360	40	/	/	600
Sample Blank	360	/	/	40	600
Sample	360	/	/	40	600
Standard	360	/	40	/	600

The blanks and standards were incubated for 5 min at 50°C after adding the DNS solution and the samples before. Putting the reaction mix on ice stopped the incubation. The solutions were measured at 530 nm.

Calculations

$$U/mL = \frac{(A_{530nm,\ sample} - A_{530nm,\ sample\ blank}) * 1000}{(30\min * MW_{glucose}\ [g/mol])}$$

$MW_{Glucose}$ = 180.16 g/mol

Pectinase Assay

The pectinase assay used was previously described by (BG Klug-Santner 2006). The activity of pectinase was determined by the increase of reducing sugars, which caused the reduction of 3,5-dinitrosalicylic acid. This reaction went along with a color change from yellow to orange that can be detected at 540 nm.

Preparation of the needed solutions

PGA solution

0.25 g polygalacturonic acid were dissolved in 100 mL Tris-HCl buffer (50 mM, pH 7.8).

DNS Solution

13.98 g NaOH and 216.1 g potassium sodium tartrate were dissolved in 800 mL dH_2O. 5.155g phenol, 7.48 g 2-hydroxy-3,5-dinitrosalicylic acid, and 5.86 g sodium metabisulfite were added and filled up to 1000mL with dH_2O. The yellow solution was stored lightproof and refrigerated

D-galacturonic acid standard solutions

As standards D-galacturonic acid solutions with Tris-HCl buffer (50 mM, pH 7.8) were prepared in the following concentrations [mg/mL]: 3; 1.5; 0.375; 0.1875

Sample preparation

The samples, blanks and standards are prepared according to the following table:

Table 3 Sample preparation for the pectinase assay

	PGA solution [µL]	pectinase [µL]	Standard solutions [µL]		DNS solution [µL]	Sample [µL]
Sample Blank	450	/	/	Incubation (5 min, 50°C) and put on ice	750	50
Sample	450	50	/		750	/
Standard	450	/	50		750	/

The blanks, samples and standards were heated to 95°C for 5 min and then centrifuged with 10.000 rpm for 10 minutes. 200 µL of the supernatant were measured at 540 nm.

Calculations

$$U/mL = \frac{c * f * 1000}{M * t}$$

c concentration according to standard curve

f factor dilution

M molecular Mass D-Gal acid monohydrate (212.16 g/mol)

t time

α–Amylase Assay

The amylase assay was previously described. (Bernfeld 1955) The ability of α-amylase to produce reducing groups from starch was utilized to reduce 3,5-dinitrosalicylic acid. This reaction went hand in hand with a color change from yellow to orange that was detected at 540 nm.

Preparation of the needed solutions

DNS Solution

13.98 g NaOH and 216.1 g potassium sodium tartrate were dissolved in 800 mL dH_2O. 5.155g phenol, 7.48 g 2-hydroxy-3,5-dinitrosalicylic acid, and 5.86 g sodium metabisulfite were added

and the solution filled up to 1000 mL with dH_2O. The yellow solution was stored lightproof and refrigerated

Starch Solution

1.0 g of soluble starch was dissolved in 100 mL of sodium phosphate buffer with 6.7 mM NaCl (20 mM, pH 6.9). To facilitate the solubilisation the starch solution was boiled for 15 minutes. After cooling down to room temperature while stirring the solution was filled up to its original volume of 100 ml by the addition of water. Samples for the assay were dispensed while stirring. Volume control by weight!

Maltose Standard Solution

A 0.2 % (w/v) maltose standard solution was prepared with 10 ml dH2O and maltose monohydrate. To obtain various concentrations the solution was then diluted with dH2O with the following dilution factors: 10; 5; 3,3; 2,5; 2

Sample preparation

The samples, blanks and standards were prepared according to the following table:

Table 4 Sample preparation for the α-amylase assay

	Starch solution [μL]	α–amylase [μL]	Standard solutions [μL]		DNS solution [μL]
Sample Blank	**100**	**100**	/	**Incubation (3min, 20°C) while shaking and put on ice**	**100**
Sample	**100**	**100**	/		**100**
Standard	/	/	**200**	/	**100**

Amylase was added to the sample blank after incubation time and the addition of DNS solution. All reaction mixtures were heated to 95°C for 15 minutes and then cooled down with ice. 900 μL of ddH_2O were added and 200 μL of each mixture was measured at 540nm.

Calculations

Standard Curve: A_{540nm} Standard = A_{540nm} Std - $A_{540nm\ Std\ Blank}$
Plot A versus mg maltose

Sample: Calculation of the maltose release from the standard curve

$$U/_{mL} = \frac{(A_{530nm,\ sample} - A_{530nm,\ sample\ blank})*1000}{(30\min * MW_{glucose}\ [g/mol])}$$

Assay for the determination of laccase activity

The determination of laccase activity with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was previously described by (Niku-Paavola 1988). ABTS was oxidized to a green radical cation. The absorption maximum was expected at 420 nm.

Solutions

10 mM ABTS solution: 2.74 g ABTS (548.70 g·mol^{-1}) were dissolved in 500 mL distilled water.

Sample preparation

650 µL sample were united with 200 µL10 mM ABTS solution. An absorbance time scan was performed at 420 nm for 60 s at room temperature.

Determination of the activity

$$U/_{mL} = \frac{k[abs/\min]*V_{total}[mL]*f}{V_{sample}[mL]*\varepsilon[L/(mmol*cm])*d[cm]}$$

A	activity	[U/mL]
k	slope	[abs*min^{-1}]
V_{gesamt}	total volume	[mL]
V_{probe}	sample volume	[mL]
	molar extinction coefficient	
ε	of ABTS at 420nm	[l*mmoL^{-1}*cm^{-1}]
d	Thickness (of the well)	[cm]
f	dilution factor	-

Results and Discussion

Dextran based hydrogels

Dextran based hydrogels were designed to be susceptible to hydrolytic trigger-enzymes especially α-amylases secreted by contaminating microorganisms.

To cross-link the biodegradable polysaccharide based backbones methacrylic linkers were used. To bind the linkers 3 different methods were used. The polysaccharides were modified with glycidylmethacrylate, methyl methacrylate and methacrylate anhydride.

In general all modifications were successfully carried out as described in the published works. However the modified procedures were not always successful.

Carboxymethylcellulose and pectin could be dyed and modified at the same time and it was possible to use the reaction solution from the dyeing and modification process directly for the polymerization process. In the special case of dextran it was necessary to isolate the modified polysaccharide backbone from the reaction broth of the modification. Then it was dissolved again to create an aqueous solution that was polymerized.

Based on the work of (Kim 2000) different solvents to modify the dextran in methacrylic anhydride as well as different solvents for precipitation were tested

In Table 5 the results of the solvent testing for the modification is listed. If it was possible to produce a hydrogel from dextran modified in this solvent the reaction was considered a success.

Table 5 Results of the solvent testing for modification of dextran with methacrylic anhydride in various solvents
x = production of hydrogels was successful; - = production of hydrogels was not successful

Modification method	Solvent	Result
Glycidly methacrylate	Distilled water	x
Glycidly methacrylate	Ethanol	-
Methacrylate anhydride	N,N-Dimethylacetamide (T)	x
Methacrylate anhydride	n-Methylpyrrolidon	-
Methacrylate anhydride	N,N-Dimethylforamide (T)	x

The big advantage of using N,N-Dimethylacetamide and N,N-Dimethylformamide is that the modified dextran is soluble in the solvent. However at our chosen reaction temperature of 70°C also in N,N-Dimethylformamide also the unmodified dextran was soluble and in N,N-

Dimethylacetamide not. When only the modified dextran is soluble and not the reactants purification is simplified.

In Table 6 all the solvents tested for the precipitation process of modified dextran are listed. If it was possible to isolate the modified dextran by precipitation from the reaction broth with this solvent, the precipitation with this solvent was considered a success.

Table 6 Results of the solvent testing for precipitation of dextran modified with methacrylic anhydride
-18°C = the solvent was chilled at18°C before use
x = the precipitation was successful; - = the precipitation was not successful

Solvent	Result
Petrolether	-
Diethylether	-
Acetone	X
Chloroform (-18°C)	X
Ethanol (-18°C)	X
Isopropanol	X
Ethanol: Isopropanol (1:2)	X
Ethanol: Isopropanol (1:3)	X
Ethanol: Isopropanol (3:1)	X

To sum up the optimization of the preparation of modified dextran with methacrylic anhydride N,N-Dimethylacetamide was chosen as the most suitable solvent for modification. Acetone was chosen as solvent for the precipitation of modified dextran because the precipitate dried the fastest.

Different from the modification of dextran it was possible to do the modification of pectin as well as CMC in a one-pot reaction. The benefit of this is easy handling and no purification steps were needed which means that a higher product yield is possible.

In general the production of hydrogels all the described methods used in literature worked if no dye was used. However, the polymerization was not reproducible at all times and the gels were not very stable. Maybe the dye interferes with the methacrylation reaction.

Overall the most reliable polymerization technique seemed to be elevated temperatures.

As the production of hydrogels with Bismarck Brown R was only possible with elevated temperature this dying method was discarded.

Modification of dextran with methacrylic anhydride was the most reliable method, however it was not successful at all times.

Size exclusion experiments where used to determine the hydrolysis fragments after enzymatic hydrolysis experiments.

To estimate how big the separated molecules are, standards were injected in the HPLC and over this calibration curve the size of the sample molecules were calculated. The calculated molecular weight was then divided by the molecular weight of glucose to have an idea how many glucose units this peak might be.

Not taken into account in this method were the hydrodynamic volumes of the substances and that certain molecules might interact with the column and therefore their retention might be prolonged. Also the method could still be optimized to have a better separation for molecules with low molecular weights. The calibration curve just has an R^2-value of 0.89. This method was not completely optimized and just served as a demonstration that there was a detectable reaction.

For weight reference various substances were investigated concerning their retention time and a calibration curve was created. In Figure 25 a calibration for a flow rate of 0.6 mL/min is depicted.

Table 7 List of used reference substances including their molecular weights [g/mol] and retention times [min] for size exclusion at 0.6 mL/min

Substance	MW [g/mol]	Retention time [min]
maltose	342.3	52.2
cellobiose	342.1	50.3
dextran	1000	43.4
dextran	5000	34.9
dextran	10000	32.9
dextran	50000	31.7
carboxymethylcellulose	90000	30.0
carboxymethylcellulose	250000	29.8

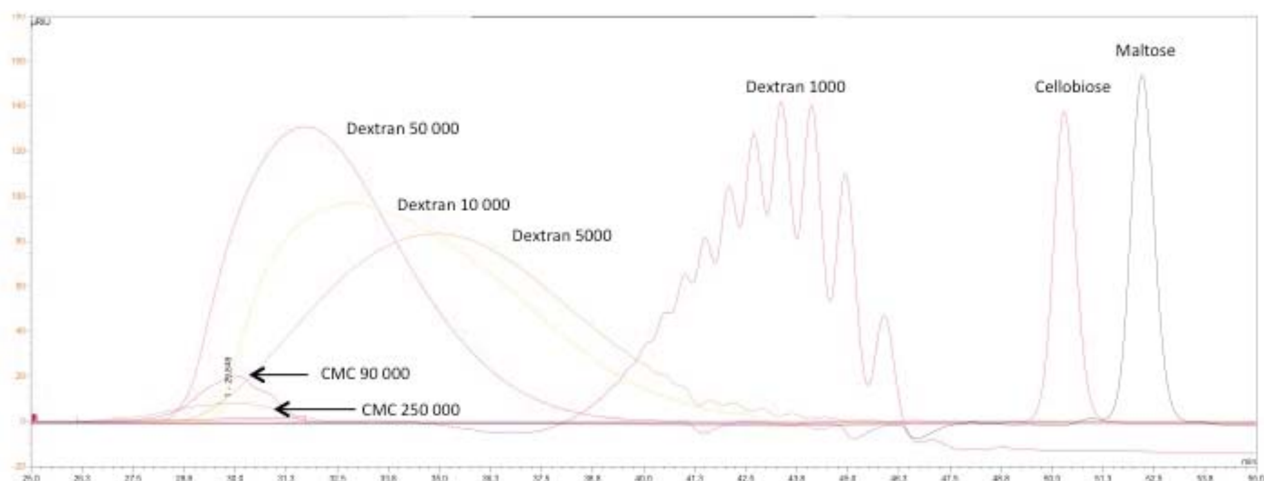

Figure 24 HPLC size exclusion measurement of reference substances. The flow was 0.6 mL/min and the refraction index was detected. Following substances were used as reference molecules: maltose (342.3 g/mol), cellobiose (342.1 g/mol) dextran (1000, 5000, 10 000, and 50 000 g/mol) and carboxymethylcellulose (90 000 and 250 000 g/mol)

It was clear that the separation capacity differed for molecular weights up to 10.000 g/mol and higher. Therefore, two curves were used to calculate the size of the separated molecules depending on their retention time.

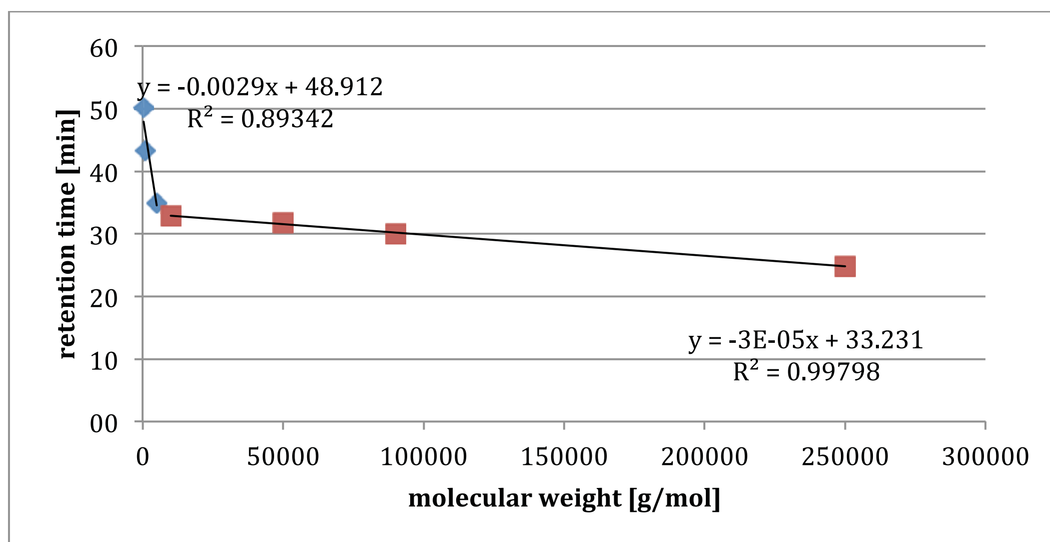

Figure 25 Exemplary calibration curve for the determination of molecular weights of separated molecules by size exclusion The blue dots represent molecules with sizes between 342.3 and 5000 g/mol and the red dots molecular weights between 10 000 and 250 000 g/mol

In this example for sample molecules with a retention time of 34 min or higher the equation y =-0.0031x + 48.912 was used to calculate their size and for sample molecules with retention times of 33 min or lower the equation y = -3E-05x+33.231 was used to approximate their size.

Reference substances were always run with the samples to take a possible peak shift into account. Calibration curves were adjusted in these cases.

Dextran was modified with methacrylic anhydride based on (Kim 2000) and hydrogels were produced using polymerization techniques involving elevated temperature and irradiation by UV-light.

The hydrogels were then hydrolyzed in sodium phosphate buffer with α-amylase from *Bacillus amyloliquefaciens* in three different concentrations: low enzyme concentration (l.e.): 50µL (0.1 U/mL), medium enzyme concentration (m.e.): 100µL (0.3 U/mL) and high enzyme concentration (h.e.): 300µL (0.8 U/mL). The supernatant centrifuged and investigated by HPLC.

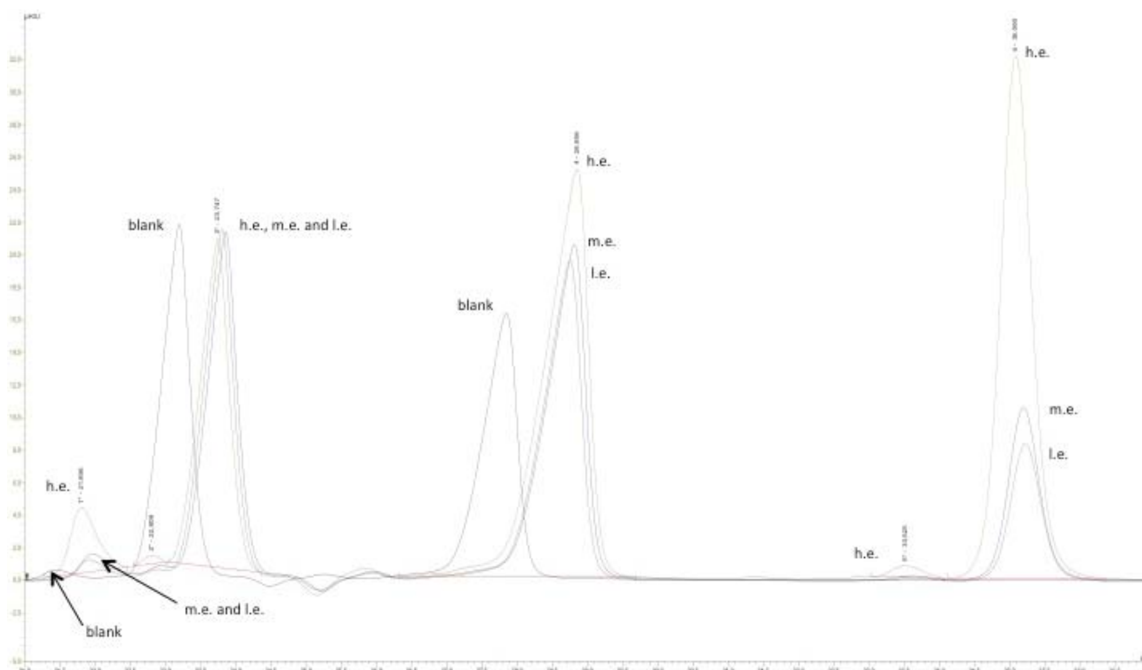

Figure 26 Hydrolysis of a dextran hydrogel modified with methacrylate anhydride and polymerized with elevated temperature after 0 hours using α-amylase from Bacillus amyloliquefaciens .The hydrogel was hydrolyzed with various amounts of α-amylase: h.e.: highest enzyme concentration (0.8 U/mL) , m.e.: medium enzyme concentration (0.3 U/mL), l.e.: lowest enzyme concentration (0.1 U/mL)

In the chromatogram at 0 hours the peaks of the blank are shifted, however because of previous experiments and the location of the rest of the peaks on the chromatogram, the retention times of the blank could easily be adapted.

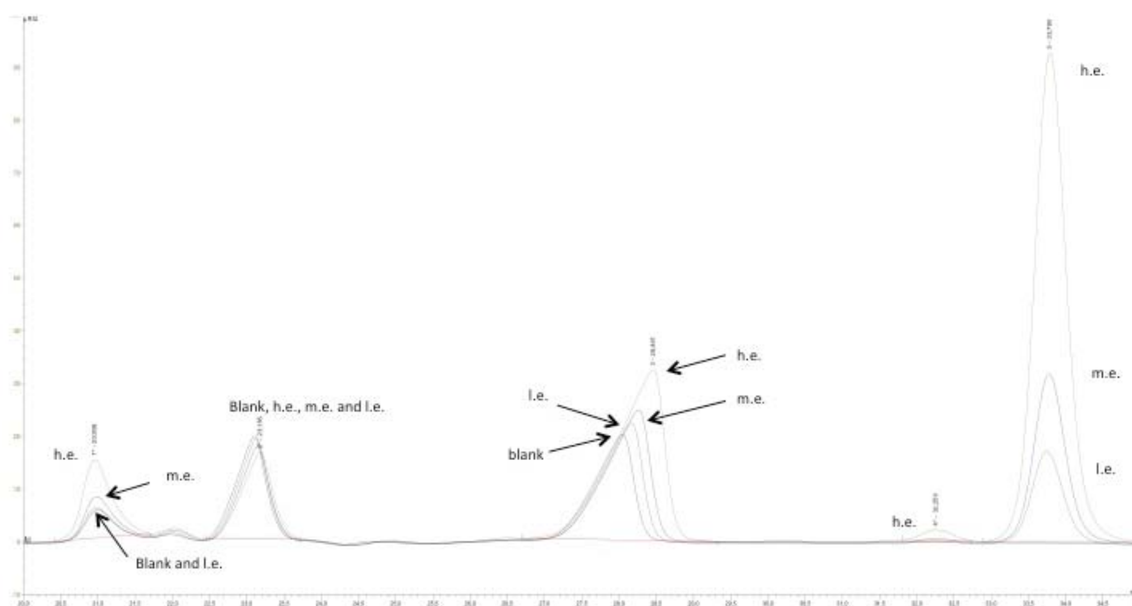

Figure 27 Hydrolysis of a dextran hydrogel modified with methacrylate anhydride and polymerized with elevated temperature after 24 hours using α-amylase from Bacillus amyloliquefaciens. The hydrogel was hydrolyed with various amounts of enzyme : h.e.: highest enzyme concentration (0.8 U/mL) , m.e.: medium enzyme concentration (0.3 U/mL), l.e.: lowest enzyme concentration (0.1 U/mL)

Table 8 List of peaks detected during HPLC size exclusion investigation of the enzymatic hydrolysis of a dextran hydrogel polymerized under elevated temperature using α-amylase from Bacillus amyloliquefaciens. Sizes of the separated molecules were estimated over a calibration curve and the possible number of glucose units was calculated

Retention time [min]	Number of calculated glucose units	Found in...
21	29	Samples and blank, increased after 24 hours
24	23	Samples and blank, no change over time
28	17	In samples and blank, increased a bit after 24 hours
33	7	Only in samples
34	7	Only in samples, increased after 24 hours

The peaks at 21 and 28 min increased especially in the sample hydrolyzed with the highest α-amylase concentration. The peak at 23 min stayed the same over time. The peaks at 33 and 34 min were only sample associated and the latter peak was clearly increased after 24 hours.
The higher the enzyme concentration was, the higher was the peak.

To investigate the bond formation in the differently polymerized hydrogels FTIR measurements were made of unmodified dextran, modified dextran polymerized with UV light, modified dextran polymerized with TEMED and modified dextran polymerized with elevated temperature. The spectra can be found in Figure 28 and the peak interpretation in Table 9. The peaks were interpreted with spectra and tables found in (Socrates 1994)

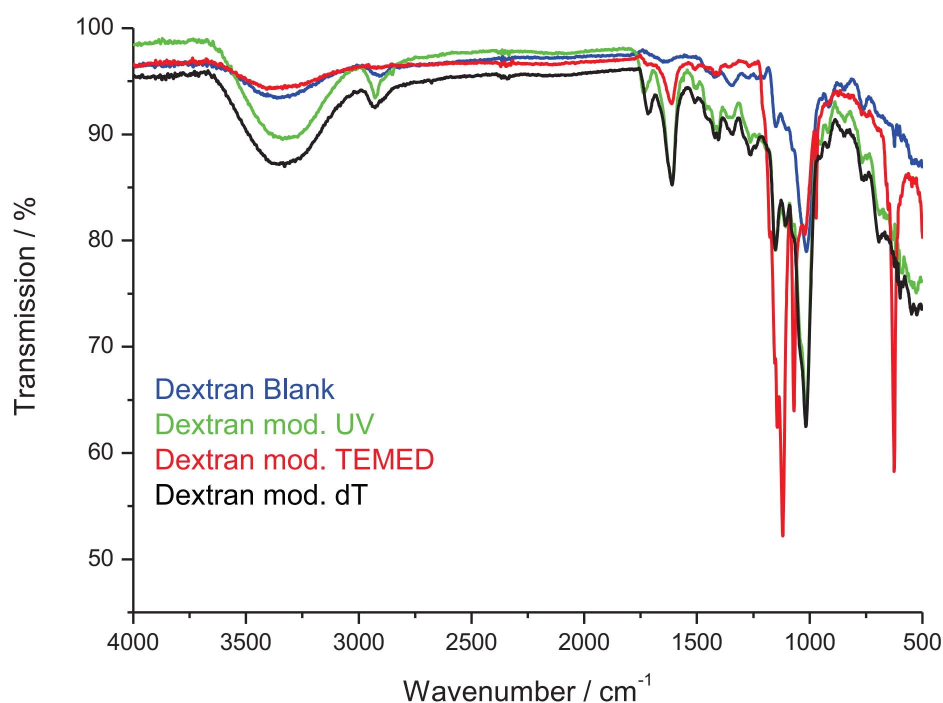

Figure 28 ATR-FTIR spectra of dextran modified with methacrylate anhydride and unmodified dextran

Table 9 Peak interpretation of the ATR-FTIR spectrum of dextran modified with methacrylate anhydride polymerized with UV irradiation, TEMED and elevated temperature and unmodified dextran

Peak	Interpretation	Associated with
3350	OH Stretching	Dextran
2928	CH Stretching	
1730	Ester	
1460-1200	CH; OH deformation	
1160-1000	CO Stretching	
1150-1085	Stretching of the sugar ring	
1340	C=C rocking	Methacrylic acid
1405	C=C deformation	
1612	C=C stretching	
1017	Skeletal vibration of MA	
950-935	C=wagging	

The hydrolysis process was investigated by performing scanning electron microscopy on untreated and treated hydrogels. The surface of the reference is still intact whereas the samples are perforated from the hydrolysis process. The higher enzyme concentration (3: 50 µL of α-amylase) clearly shows holes whereas the sample with the lower enzyme concentration (2: 25 µL of α-amylase) shows a tear Figure 29. The measurements were performed on a Zeiss Evo 60 (5.0 kV) by Konstantin Schneider during a short term scientific mission over a COST action (COST-STSM-868-6311)

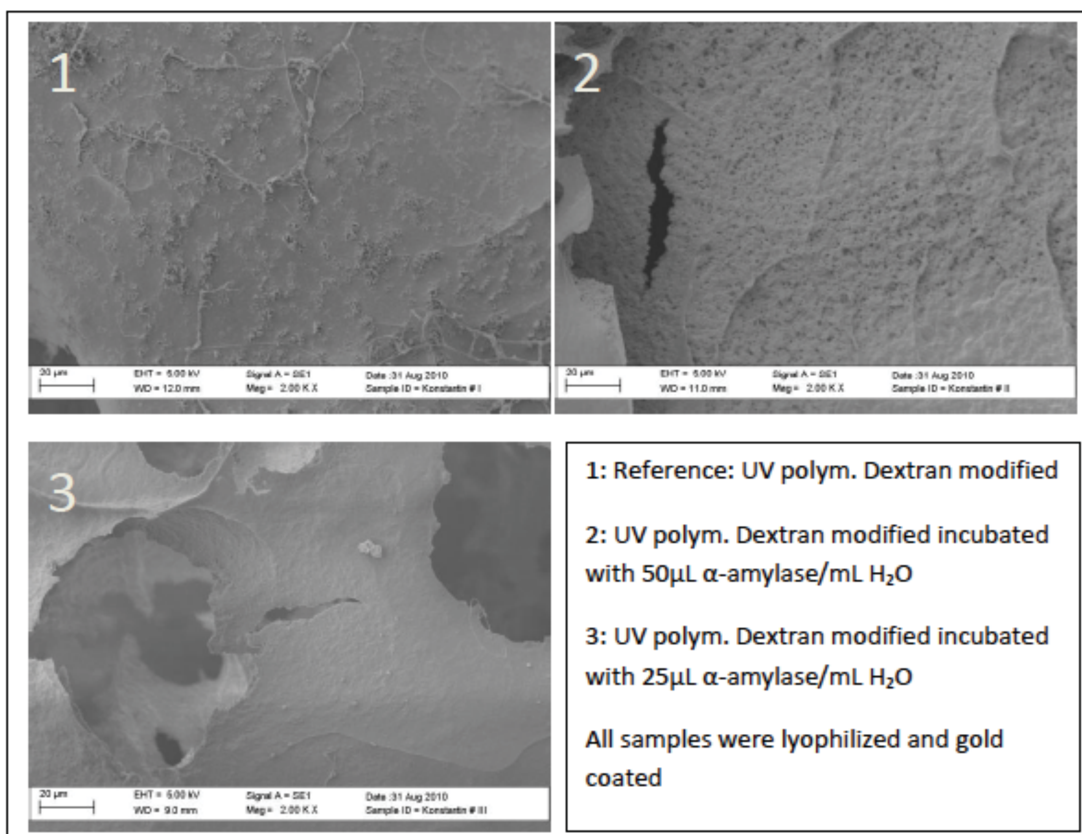

Figure 29 Scanning Electronic Microscope Measurement on a Zeiss Evo 60 (5.0 kV) of dextran hydrogels polymerized by UV irradiation and hydrolyzed with α-amylase from Bacillus amyloliquefaciens .The reference (1) is intact whereas the samples (2 and 3) are perforated due to the enzymatic hydrolysis. The polymer subjected to the higher enzyme concentration (3: 50µL of α-amylase) shows bigger holes than the polymer hydrolyzed with a lower enzyme concentration (2: 25µL of α-amylase)

Dyed dextran hydrogels

To visualize the hydrolysis process different kinds of dyes were used which were released to the supernatant upon the hydrolysis of the hydrogel. They served as direct control of the process.

Dextran polymers with Bismarck Brown R

It was planned to do the dyeing process and methacrylation process in a one-pot reaction after mixing the dye and the dextran by dispersing both substances together in n-pentane. After drying under vacuum the dying and modification process was done simultaneously by adding 0.5 mL HCl (6 M) to the aqueous solution of dextran and Bismarck Brown. After the standard polymerization procedures of elevated temperature, TEMED and Irgacure in combination with UV light it was not possible to isolate a polymerized sample in the cases of TEMED and Irgacure. Because of that reason the combination of Bismarck Brown and dextran does not seem to be the best candidate for further investigation.

Dextran polymers with carminic acid

Dextran was dyed with 50 mg and 100 mg of carminic acid (CA). To determine a dependence of the solvent the modified dextran was precipitated in, the batch dyed with 100 mg of carminic acid was split and precipitated in acetone and in a ethanol:isopropanol mixture.

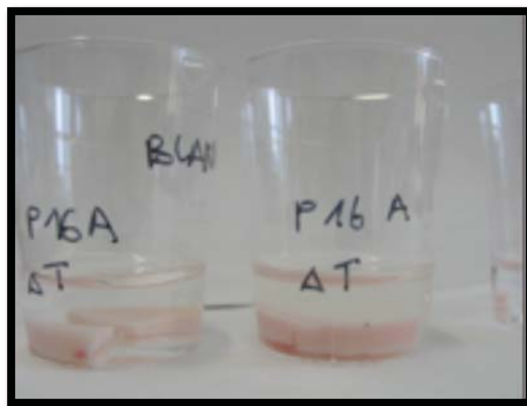

Figure 30 Dextran polymer (sample right side) and blank (left side) dyed with carminic acid and precipitated in ethanol:isopropanol and polymerized under elevated temperature

Figure 30 illustrates the fragility of the dextran gels. The blank had broken into two halves.

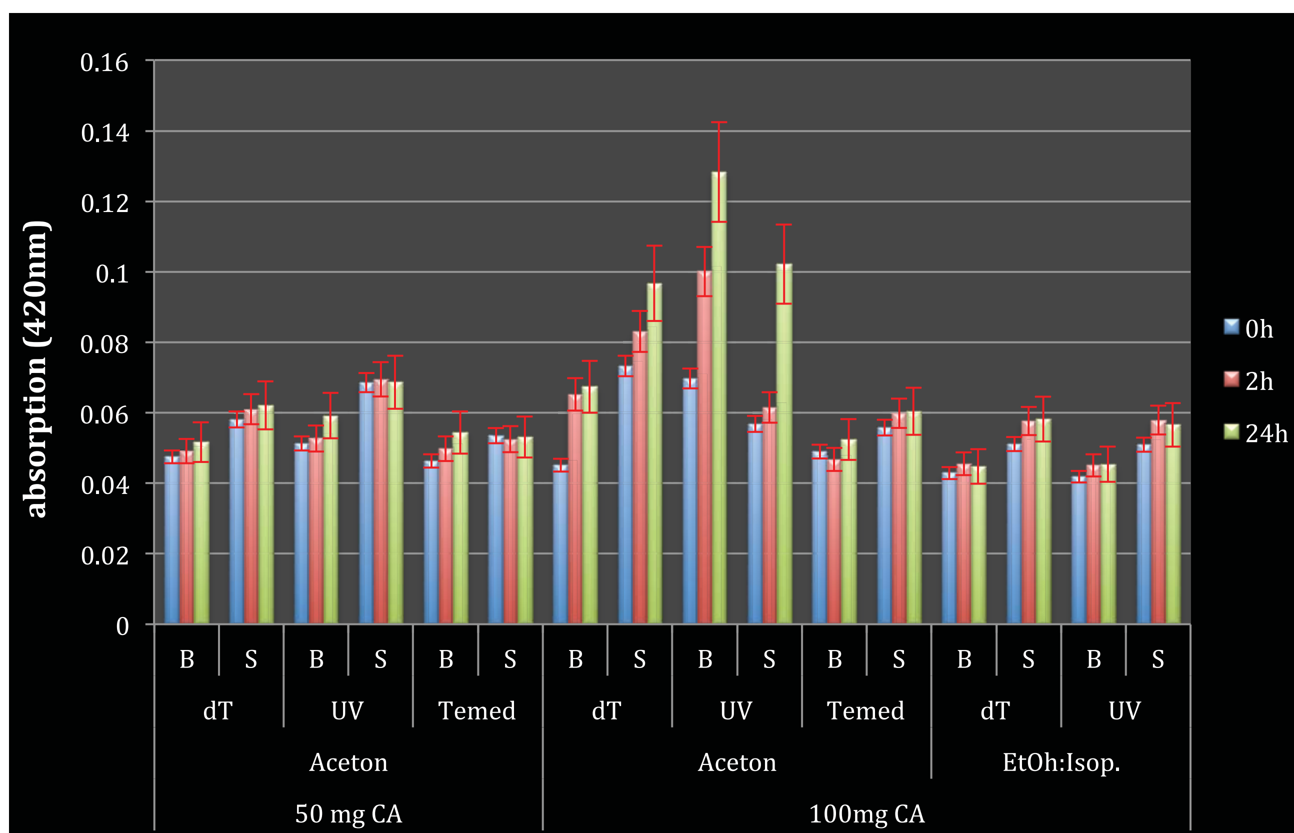

Figure 31 Hydrolysis of a dextran hydrogel dyed with 50 mg and 100 mg carminic acid (CA) using an α-amylase from *Bacillus amyloliquefaciens* (1 U/mL). Dextran dyed with 50mg of carminic acid was precipitated in acetone. Dextran dyed with 100mg of carminic acid was precipitated in acetone.and a mixture of

EtOH:Isopropanol (3:1). The gels were radically polymerized with three different methods: elevated temperature (dT), irradiated with UV-light (UV) and at room temperature with TEMED (Temed).

As expected, the color release of the hydrogel dyed with 100 mg carminic acid was higher than with 50 mg. Samples polymerized by UV irradiation delivered the best results. However, these samples often are the most fragile consequently there could have been additional contact surface for the enzyme to attack.

The solvent in which the modified dextran was precipitated seemed to have an influence on the hydrolysis of the polymer. Samples that were precipitated in an ethanol:isopropanol (3:1) mixture showed less dye release than samples precipitated in acetone.

This was one of the reasons why acetone was chosen as precipitation solvent for modified dextran in all other experiments.

Dextran polymers with methyl red

To improve the immobilisation of the dye molecule itself a modification was done by the combination of glycidyl methacrylate with methyl red. The dyeing process was successful delivering a pink powder. Two possible peak maxima were found, one at 420 nm and one at 570 nm. As for the sample peak after enzymatic hydrolysis a stronger signal was found at 420 nm, this maximum was used.

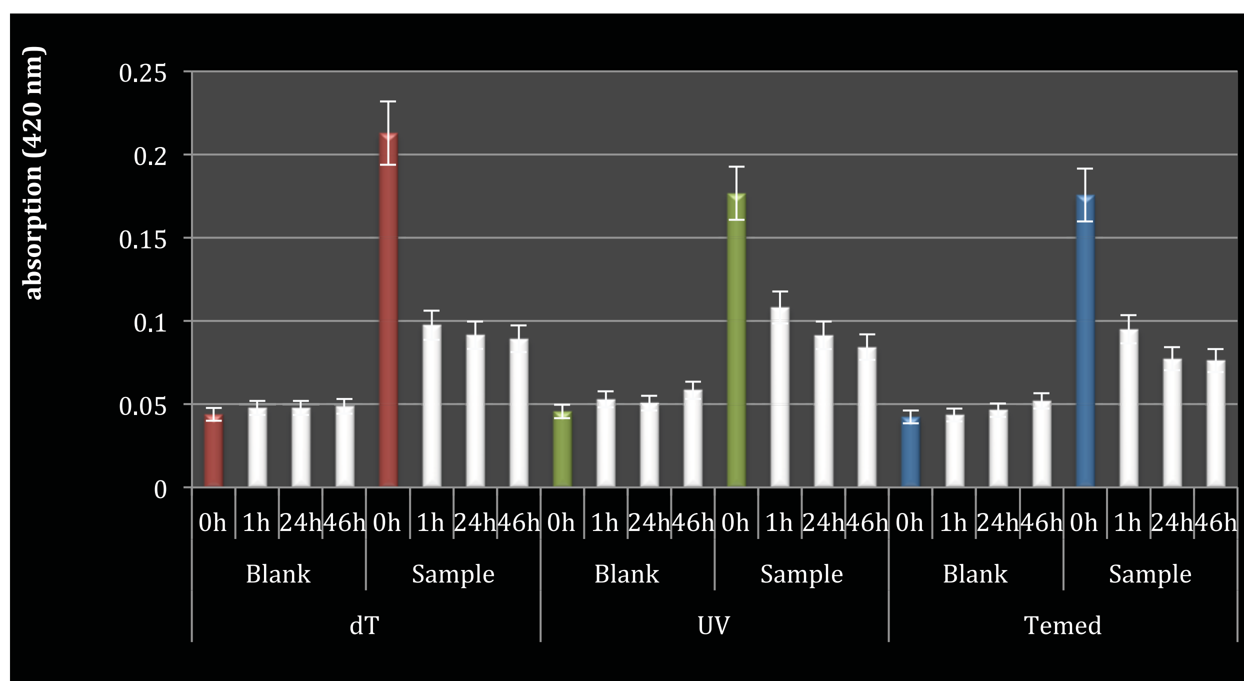

Figure 32 Hydrolysis with α-amylase from *Bacillus amyloliquefaciens* (1 U/mL) of a dextran hydrogel dyed with modified methyl red. Polymerization techniques used were elevated temperature (dT), irradiation with UV-light (UV) and with TEMED at room temperature (Temed)

Figure 32 that it was possible to release methyl red from the dextran hydrogel with α-amylase. However, the release happened within several minutes.

As for our experiments we preferred a slow release. Consequently, we decided not to continue with this substance. Auto diffusion was similar in all gels, however the hydrolysis peak of samples cross-linked with temperature was visibly higher.

Dextran polymers with Reactive Black 5 (Cibacron Marine DP-B; Intracron Black VCKN, remazol black B)

The dyeing process was successful although slight differences in the amount of dye can already have a large impact on the color. The precipitation process in alcohol was sometimes difficult as the dextran became lumpy which resulted in a long drying period and pestling process. At times during filtration the filter became clogged and elongated the washing process.

Figure 33 Dextran dyed with Reactive Black

Furthermore, it was not always possible to dissolve the dyed dextran. Dyeing the dextran during the modification seemed like a better option. However this was not successful.

Maybe there was a concurrence between the dyeing and methacrylation reaction, which resulted in less methacrylation and therefore gel formation was not possible.

Modified dextran dyed with Reactive Black 5 was precipitated in ethanol and acetone to investigate the influence of the solvent on the release of the dye. The only samples where it really seemed to have an impact were the ones modified with TEMED. When the dextran was precipitated in ethanol it did not work as well as in acetone.

In all cases there was a visible difference in color release between blank and sample, however after one hour not much more color was released.

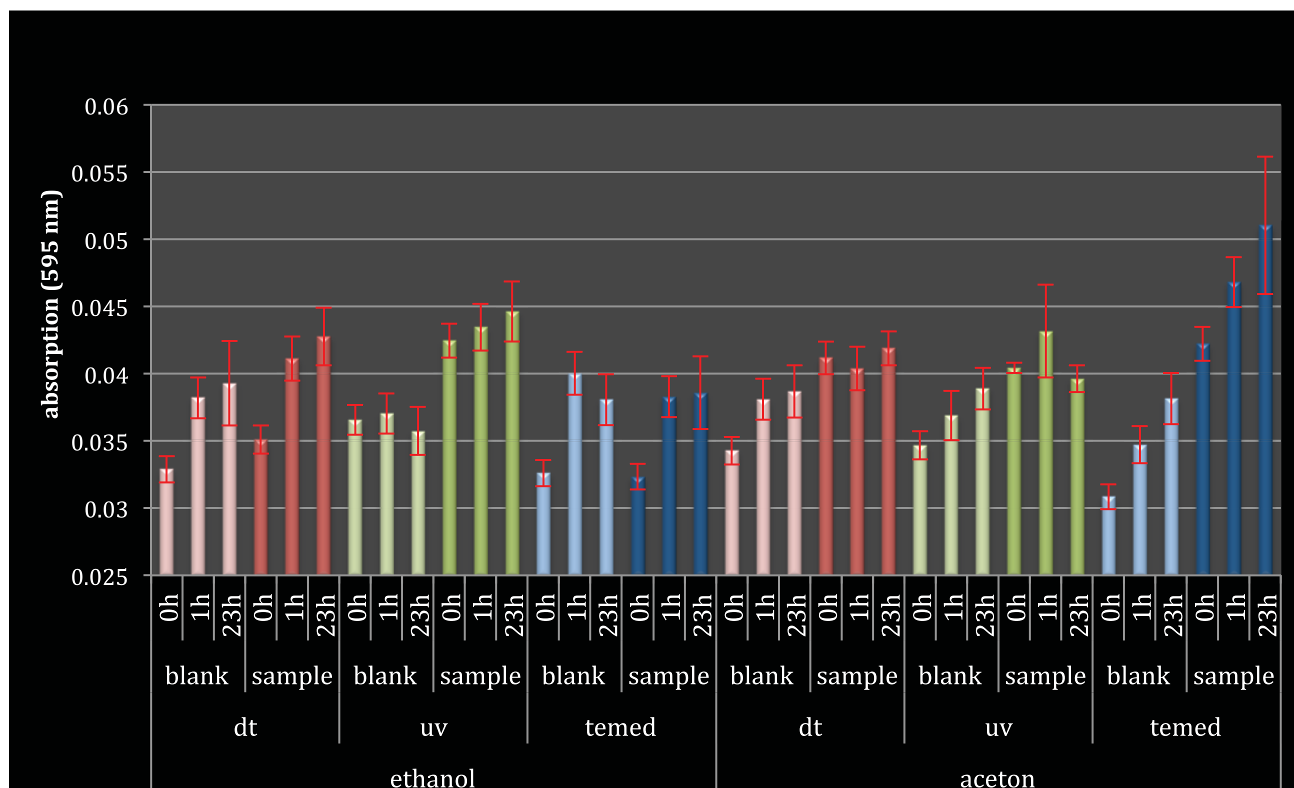

Figure 34 Hydrolysis with α-amylase from *Bacillus amyloliquefaciens* (0.26U/mL)of a dextran hydrogel dyed with Reactive Black 5. Polymerization techniques used were elevated temperature (dT), irradiation with UV-light (uv) and with TEMED at room temperature (temed). The modified dextran was precipitated in ethanol and acetone to study the influence of the used solvent on precipitation.

There was a visible difference between the blank without and the sample with α-amylase. After one hour the supernatant of the sample visibly had released more color than the blank.

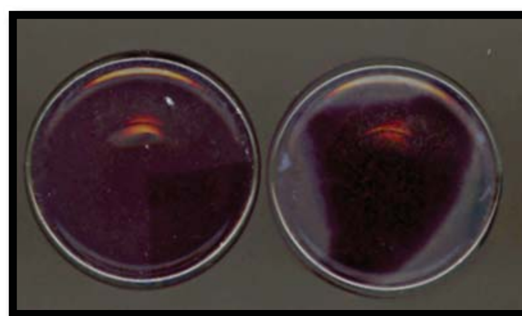

Figure 35 Enzymatic hydrolysis of a hydrogel dyed with Reactive Black 5. On the left is the sample and on the right side the blank

As the results were not satisfactory we explored other methods like enclosing enzymes into the gels instead of dye and produced dextran/PVA blends to improve the stability of the gels.

Hydrogels based on dextran with enclosed enzyme

Modification of laccase with polyethylene glycol was successful.

The purification process was finalized with the ÄKTA. The column used was “Hiload 26/60 superdex 200 prep grade”. Eluent A was a 50 mM K-phosphate buffer (pH 7) with 0.1 M NaCl. Figure 36 shows unmodified laccase from Trametes *hirsuta* at an eluent volume of 290.44 mL (fractions 96-110). The modified laccase was purified. The peak of the modified laccase was expected at a smaller volume as the separation method was based on the molecule size. The bigger the molecules are the quicker they are washed out of the column. The peak at 295.98 mL was associated with unmodified laccase and the peak at 177.40 mL was associated with the modified laccase. (Figure 37)Fractions 32-42 were collected and stored at 4°C.

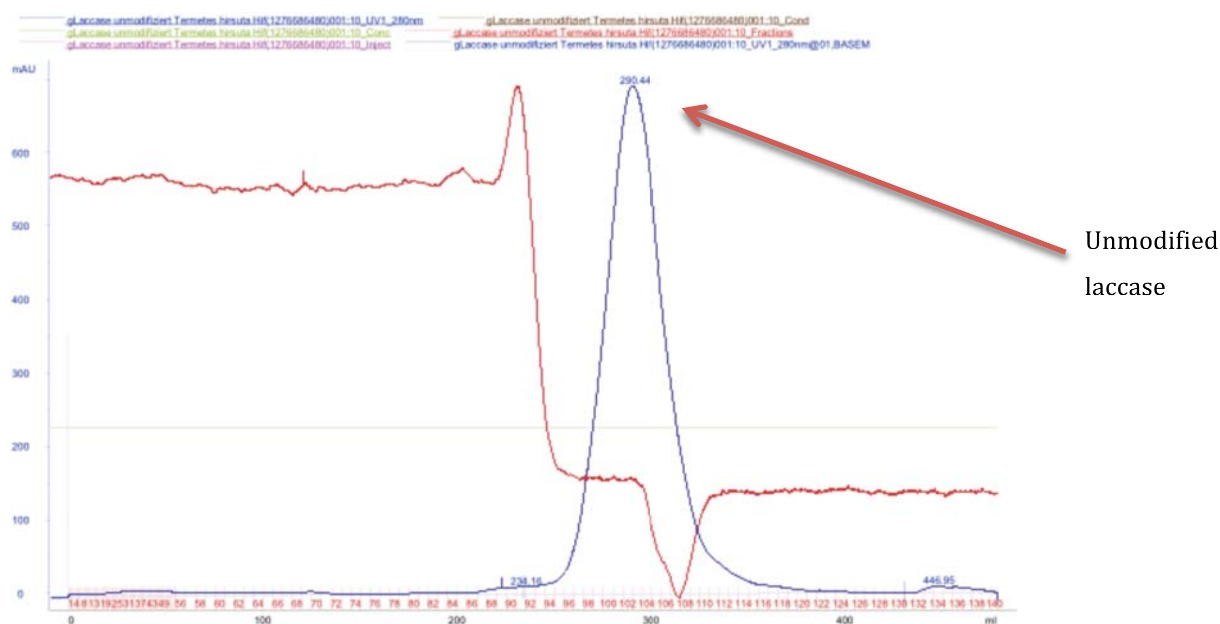

Figure 36 Chromatogram of unmodified laccase from *Trametes hirsuta.* The peak of the unmodified laccase is at 290.44 mL

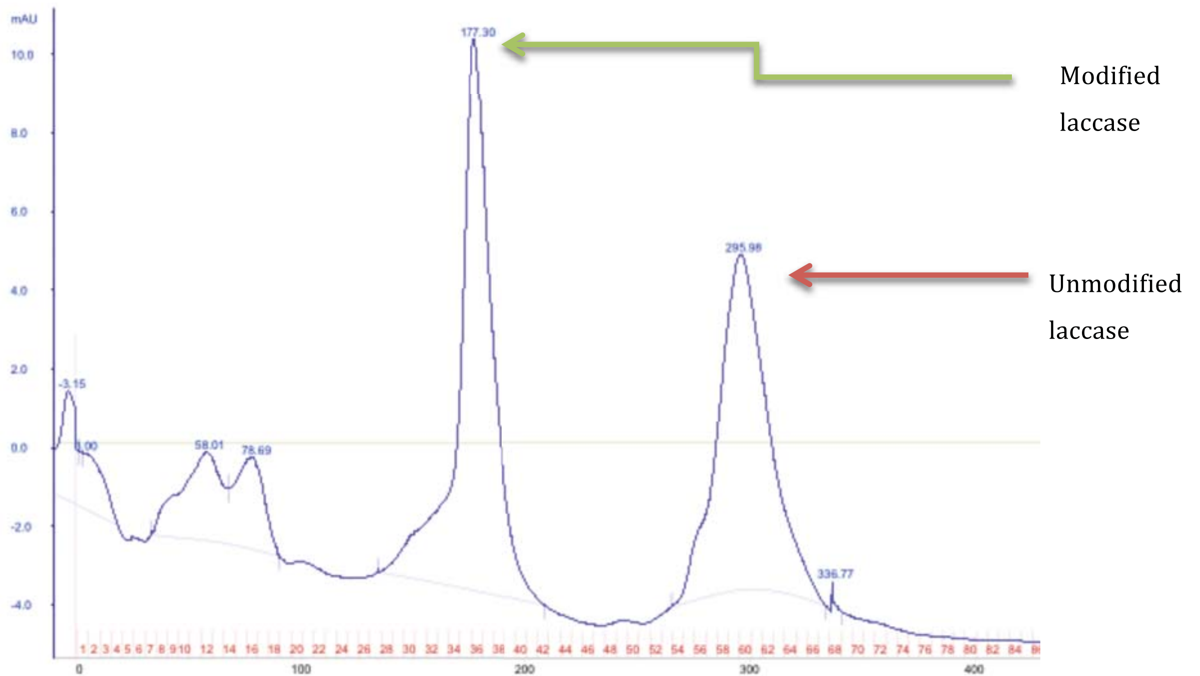

Figure 37 Chromatogram of laccase from *Trametes hirsuta* modified with polyethylene glycol .shows Unmodified laccase has its peak at 295.98 mL. The peak at 177.30 mL is associated to the modified laccase.

Production of dextran hydrogels with enclosed enzyme was successful. The dextran hydrogel with the enclosed enzymes was hydrolyzed using α-amylase from Bacillus amyloliquefaciens. In the blanks the volume of enzyme was replaced with buffer.

The enzymatic hydrolysis process was monitored over the released laccases into the supernatant via an activity assay using ABTS as a substrate. The size of formed fragments in the supernatant due to the hydrolysis process was investigated by size exclusion measurements with HPLC. For weight reference various substances were investigated concerning their retention time and a calibration curve was created. (Figure 25)

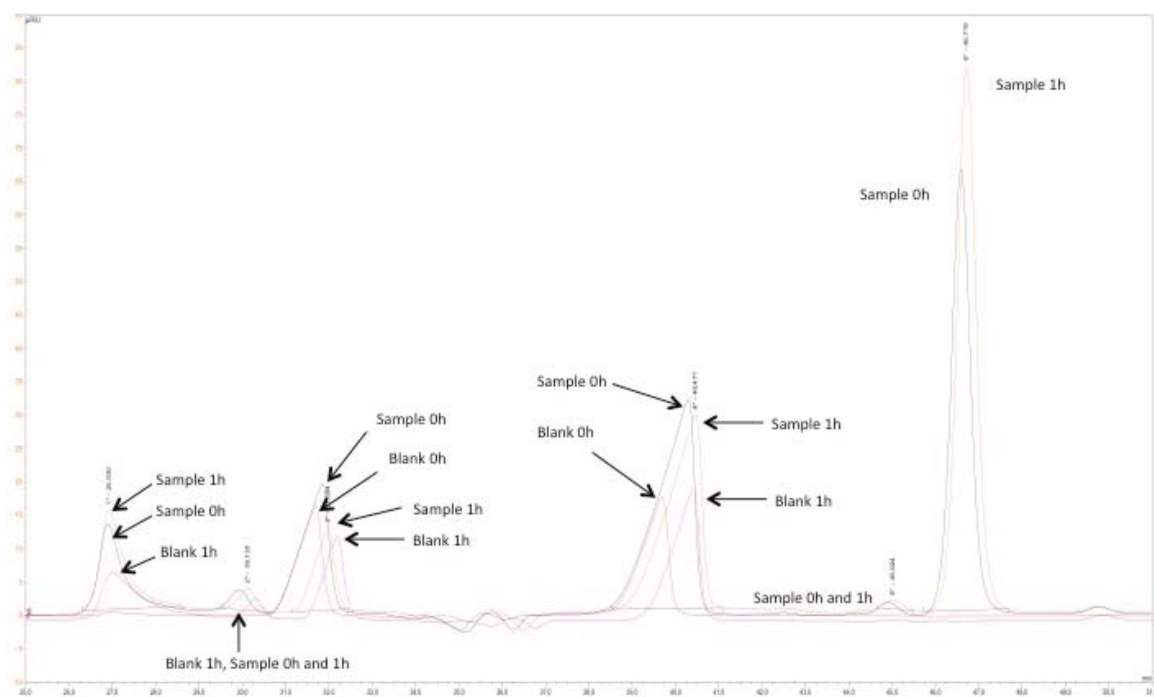

Figure 38 Hydrolysis of a dextran hydrogel with enclosed laccase from *Myceliophthora thermophila* from a dextran hydrogel using a α-amylase from *Bacillus amyloliquefaciens* (0.8 U/mL). The flow rate was 0.6 mL/min and the refraction index was detected. Samples and blanks after 0 and 1 hour were investigated.

For all the samples of this experiment the chromatograms looked similar so only one of them is included in this thesis. With the calibration curve the number of possible glucose units was estimated.

Table 10 List of peaks detected during HPLC size exclusion investigation of the hydrolysis of a dextran hydrogel with enclosed laccase from *Myceliophthora thermophila*. The hydrogels were hydrolyzed with α-amylase from *Bacillus amyloliquefaciens* (0.8 U/mL). Sizes of the separated molecules were estimated over a calibration curve and the possible number of glucose units was calculated

Retention time of Peak [min]	Number of calculated glucose units	Found in...
27	372	Samples and blank after 1h, higher in samples
30	174	Samples and blank after 1h
32	24	In sample and blank, decreases over time
40	15	Found in samples and blank, bigger in samples
45	5	Only in samples
47	5	Only in samples, increases over time

The peaks at 27 minutes and 40 min were increased in the sample chromatograms. The peaks at 31 and 40 min decreased in sample and blank over time.

The highlight of the chromatogram is the peak 47 min (5 calculated glucose units). It was only sample associated and was clearly increased after one hour.

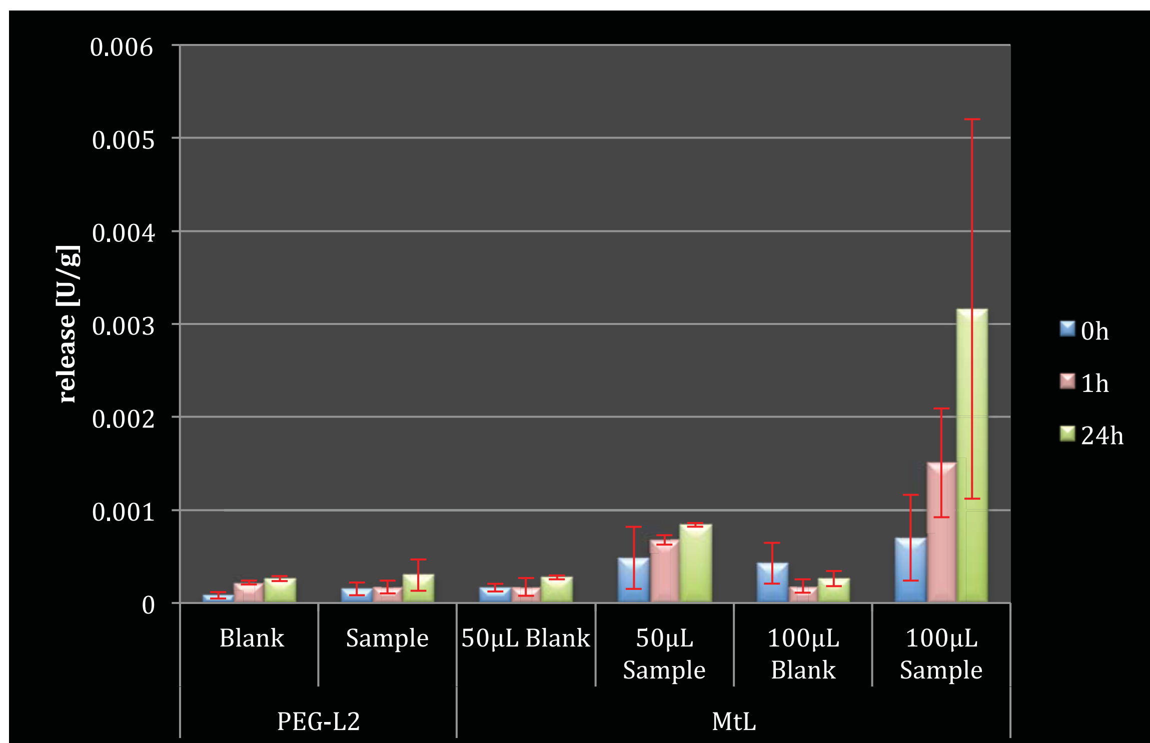

Figure 39 Hydrolysis of a dextran hydrogels with enclosed modified laccase from *Trametes hirsuta* and laccase from *Myceliophthora thermophila* using α-amylase from Bacillus amyloliquefaciens (0.8 U/mL). Laccase from *Trametes hirsuta* was modified with polyethyleneglycol (PEG-L2). Laccase from *Myceliophthora thermophila* was added in two concentrations (50µL and 100µL). The release was measured via an activity assay for laccases using ABTS as a substrate

The dextran hydrogel with the enclosed enzymes MtL and PEG-L2 was hydrolyzed using α-amylase from Bacillus amyloliquefaciens. In the blanks the volume of enzyme was replaced with buffer. With the supernatant of the enzymatic reaction the activity assay using ABTS, which served as a pro-dye was performed to determine how much MtL or PEG-L2 had been released due to hydrolysis of the hydrogel.

A minimal activity difference between the blank and sample with modified laccase from *Trametes hirsuta* PEG-L2 was found.

As expected the more laccase from *Myceliophthora thermophila* was enclosed in the polymer, the more was released over time. In both cases the release between blank and sample was much clearer than with the PEG modified enzyme. However the high standard deviations are not acceptable.

Mixed hydrogels based on a dextran and PVA blend

Hydrogels based on dyed dextran/PVA blend were successfully produced. Dextran T100 was modified with methylmethacrylate based on the work of (A. Yu. Men'shikova 2001) (Yasuhiko Onishi 2005) (Yasuhiko Onishi∗ 1978) . Two kinds of modified PVA were tested. In PVA (8.5 %)

the activation of the hydroxyl groups with methacrylic acid had worked for 8.5% and in PVA (11%) for 11%.

As a result PVA (11%) is able to form tighter networks. For hydrogels based on dyed dextran with Reactive Black and PVA (8.5%) mixtures with a PVA/dextran ratio from 8:2 to 1:9 were investigated to determine the most suitable ratio. Samples with a PVA:dextran ration 8:2 to 5:5 had already hardened after UV treatment. All the samples with a higher dextran ratio, however had not. For this reason the plate with all the samples was kept in the oven for 10-15min at 80°C to complete the process.

The samples at 0 h were taken directly from the enzyme dilutions of α-amylase from *Bacillus amyloliquefaciens*. The enzyme had a faint yellow color. The blanks stayed more or less colorless however after 4 hours Reactive Black had visibly diffused from samples containing more dextran than PVA.

Overall the more dextran was used, the more Reactive Black was released. This result was expected since α-amylase can only break down dextran, not PVA and only the dextran was dyed. Therefore the more dextran a sample contains the more color could theoretically be released. Looking at the photograph (Figure 40) one might have said that more dye was released after 4 hours via auto diffusion than with the enzyme dilution 1:15.4 (1.75 U/mL) of α-amylase from *Bacillus amyloliquefaciens*. However when the photometrical measurements were taken into account it became very clear that visual control is influenced by the color of the enzyme dilution. If an application were planned where only visual control was possible this should be considered.

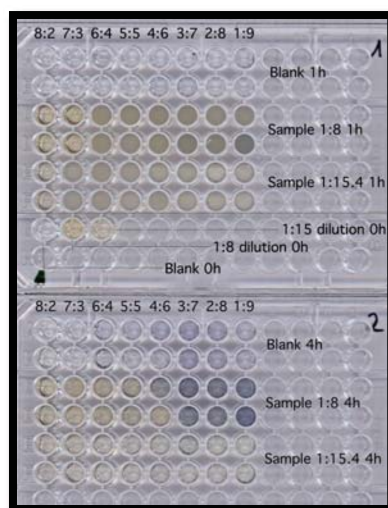

Figure 40 Hydrolysis of PVA (8.5%)/dextran blends (ratio: 8:2 to 1:9) with α-amylase from *Bacillus amyloliquefaciens* in two concentrations 1:8 (3 U/mL) and 1:15.4 (1.75 U/mL). The dextran was previously dyed with Reactive Black. The released color was detected photometrically at 580 nm.

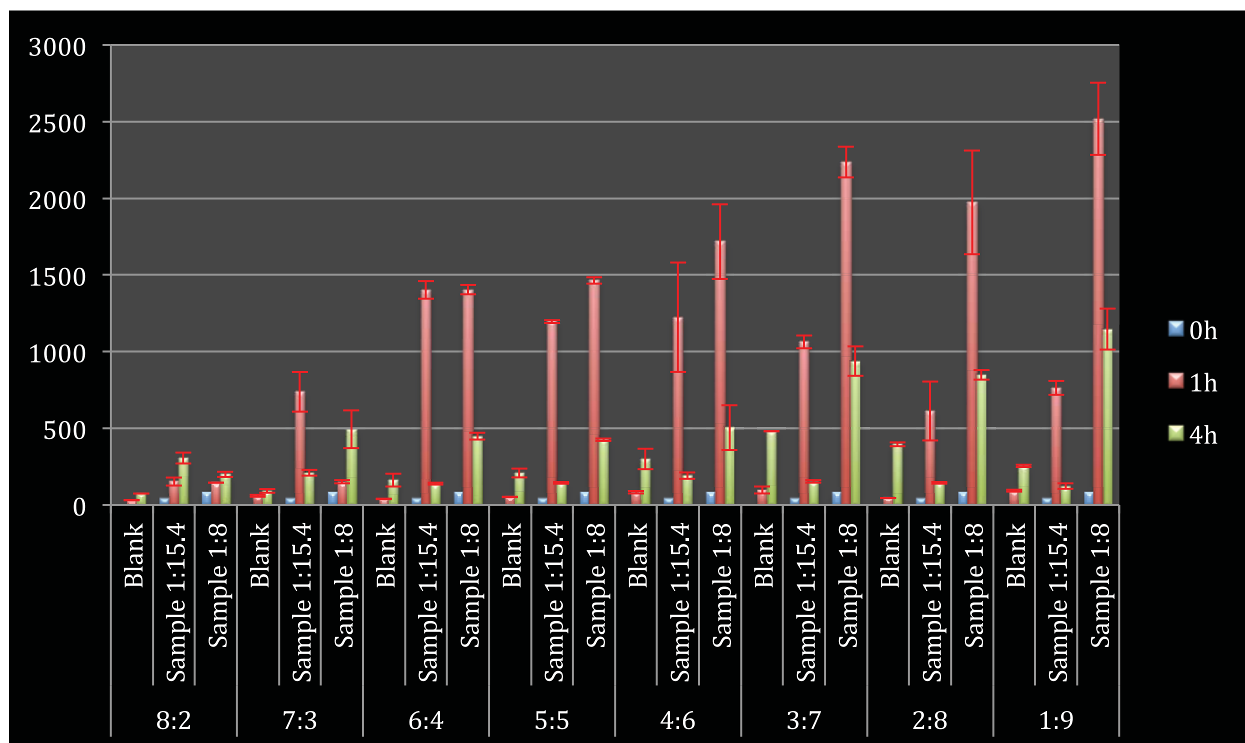

Figure 41 Hydrolysis with α-amylase from *Bacillus amyloliquefaciens* in two dilutions (1:15.4 (1.75 U/mL) and 1:8 (3 U/mL)) of PVA (8.5 %)/dextran blends (ratio 8:2 to 1:9). The dextran was previously dyed with Reactive Black and modified with methylmethacrylate. The color release was measured over time. The absorption maximum was found at 580nm and the release [%] was expressed in proportion to the blank at 0 hours.

For almost all of PVA/dextran ratios most of the dye was released by the lower enzyme dilution of α-amylase from *Bacillus amyloliquefaciens* 1:8 (3 U/mL) over time. However for samples made of 80 %, 70 % and 60 % PVA the higher enzyme dilution worked better.

After one hour most the maximum of Reactive Black had been released except for the best working enzyme dilution. The only exception were the samples with 80% PVA.

The standard deviations were in general higher the more dextran was used. After 24 hours some of the samples had dehydrated in the plate.

In the second experiment larger amounts of gel and supernatant were used to minimize the influence of evaporation. On top of that instead of an 8.5 % PVA solution an 11% PVA solution was used.

Only PVA:dextran ratios 8:2, 6:4 and 5:5 were used.

The samples at 0 h were taken directly from the enzyme dilutions of α-amylase from *Bacillus amyloliquefaciens*. The enzyme had a faint yellow color.

Again the higher the dextran ratio, the more dye got released. As with the samples with PVA (8.5 %) the higher enzyme dilution 1:15.4 (1.75 U/mL) of α-amylase from *Bacillus amyloliquefaciens* was able to degrade the gels best.

It was possible to prolong the release period. After 24 hours the maximum had not been reached.

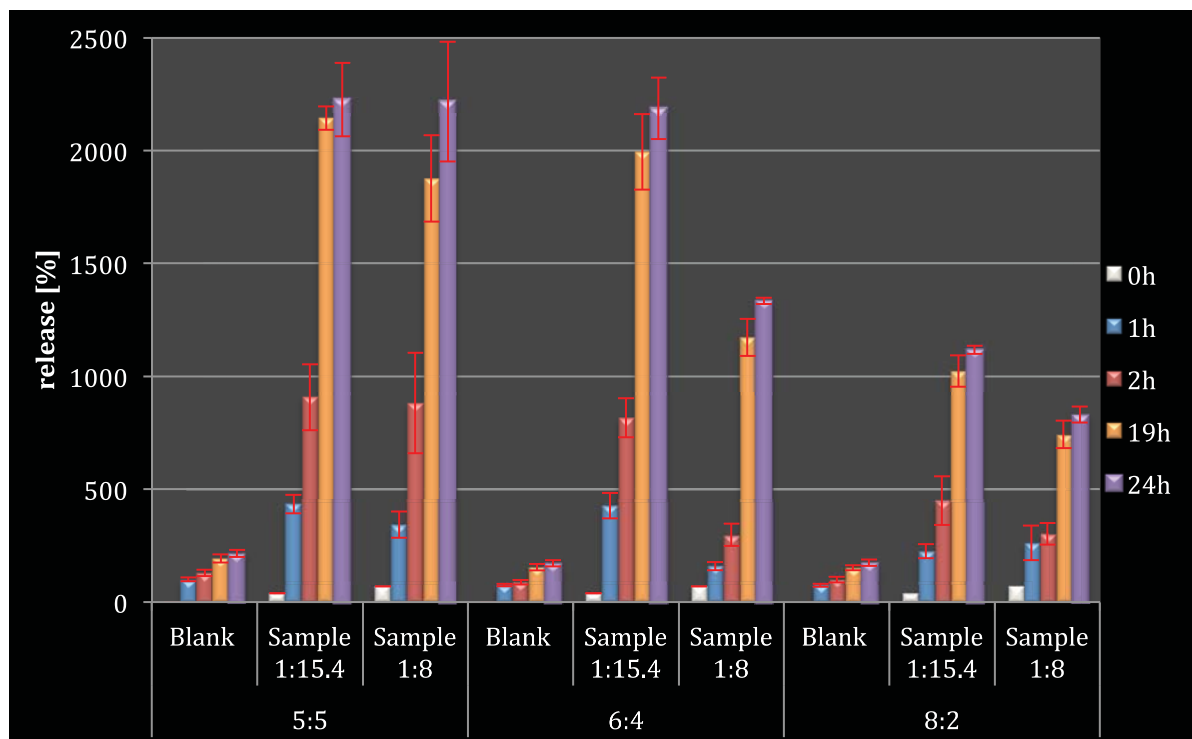

Figure 42 Enzymatic hydrolysis of PVA(11%)/dextran blends (6:4, 5:5, 8:2) with α−amylase from *Bacillus amyloliquefaciens* in two dilutions (1:15.4 (1.75 U/mL) and 1:8 (3 U/mL)). The dextran was previously dyed with Reactive Black and modified with methylmethacrylate. The color release was measured over time. The absorption maximum was found at 580nm and the release [%] was expressed in proportion to the blank at 0 hours.

All of the produced polymers were fragile except for the polymers made of the PVA/dextran blend that mostly consisted out of PVA. This made it very difficult to wash them or handle them all together. They often broke when being transferred to the glass vessels for the hydrolysis. This may be one of the reasons why we often had high standard deviations. On the one hand because of the unstable network and on the other hand if a polymer breaks the degrading enzyme has more working surface than with samples where the polymer is still in one piece.

Figure 43 Example of a dextran hydrogel modified upon enzymatic hydrolysis with α-amylase from *Bacillus amyloliquefaciens.* The blank is on the left and the sample on the right. This image clearly demonstrates the low stability of the dextran hydrogels

Carboxymethyl cellulose based, pectin based hydrogels and their blend

Dextran hydrogels had the disadvantage of being very unstable. For this reason alternative matrices were investigated Carboxymethylcellulose is a derivative of the glucose consisting cellulose. Pectin is a heteropolymer that is rich in galacturonic acid. (Robyt 1997) Methacrylated carboxymethylcellulose and pectin both can be interlinked to a sturdy network. Glycidylmetharcylate was chosen as donor of methacrylate groups as it easily reacts with esters based on previous work by (J.F.A.S. Maior 2008) (Konstantin P. Schneider and Armin Zankel 2011)

Dyed pure carboxymethylcellulose and pectin hydrogels were produced as well as gels of their blend.

The process of dyeing with alizarin was very convenient and reliable delivering a homogenous orange powder. (Figure 44) The color results were consistent.

Figure 44 Blend of carboxymethylcellulose and pectin dyed with alizarin

Filtration was much more convenient than with dextran as their were no problems with filter clogging.

The production of the sturdy orange gels was reproducible and they hardened quickly. Their stability was a big advantage during the cutting process prior to the enzymatic hydrolysis.

The results of the enzymatic hydrolysis were more reliable than with the dextran hydrogels as the polymer did not fall apart. A gel in pieces could provide additional contact surface for the enzymatic hydrolysis and cause distortion of the results.

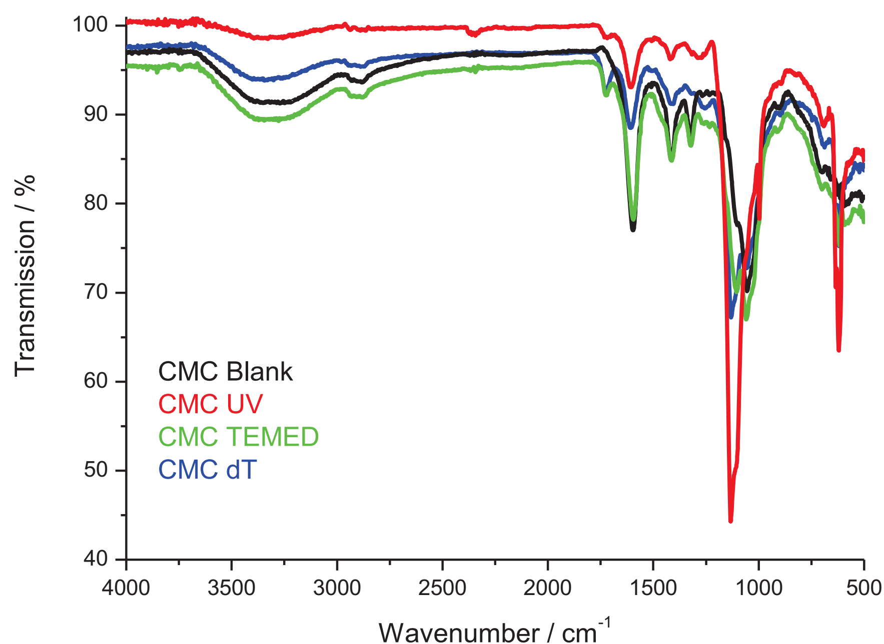

Figure 45 ATR-FTIR Spectrum of carboxymethylcellulose modified with glycidylmethacrylate. Polymerization was done by UV-irradiation, TEMED and elevated temperature. Also unmodified CMC was investigated.

An ATR-FTIR measurement was made of CMC hydrogels polymerized with UV-irradtion, TEMED and elevated temperature to determine if there is a visible difference between the different modification methods and unmodified CMC. The spectra can be found in Figure 45 and the interpretation of the peaks in Table.11 The peaks were interpreted with spectra and tables found in (Socrates 1994)

Table 11 Peak interpretation of the ATR-FTIR spectrum of carboxymethylcellulose modified with glycidylmethacrylate polymerized with UV irradiation

Peak	**Interpretation**
3432	OH-stretching (sugar)
3898	OH-stretching vib (sugar)
1596	COO^- group
1416 + 1323	CH_2-scissoring and OH-banding vibration

Enzymatic hydrolysis of carboxymethylcellulose hydrogels with different cross-linking grades with cellulase

To determine the most suitable cross-linking degree and enzyme concentration CMC was died with alizarin and various degrees of cross-linking where achieved by adding three different amounts of glycidylmethacrylate: 2,5mL (low degree of cross-linking, LD) , 5mL (medium degree of cross-linking, MD) and 7,5mL (high degree of cross-linking, HD).

In general the polymerization with UV-light delivered lower peaks than the polymers cross-linked under elevated temperature. The only exception is the LD gel.
However the grade of auto diffusion is much higher.
When the gels were polymerized by UV irradiation there was not such a drastic difference between the two highest cross-linking degrees.
LD and MD delivered best results, in our eyes. As MD had a better Blank/sample peak ratio, we decided to use 5mL glycidylmethacrylate for all further experiments.
Also two different concentrations of cellulase were used 0,5% (0.9 U/mL) and 2,9%(5 U/mL). As expected the higher enzyme concentration, the more alizarin gets released over time. Gels polymerized under elevated temperature delivered better results. Gels with a low crosslinking degree showed the highest alizarin release, however also the highest rate of auto diffusion. A crosslinking level that lies between LD and MD was chosen as the most effective in an attempt to get a compromise of the great degradability of LD gels and the stability of the MD gels. (Figure 46)

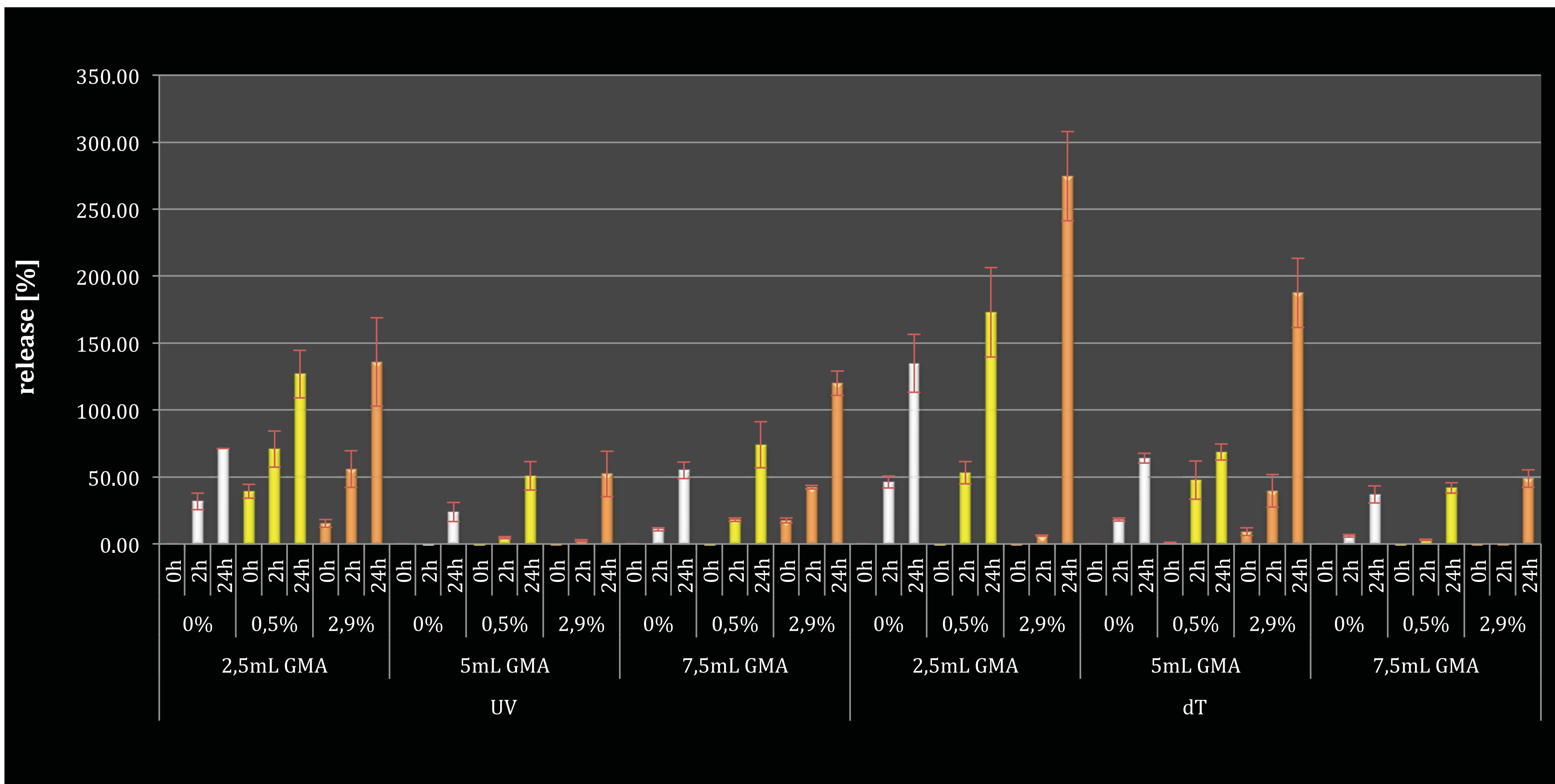

Figure 46 Hydrolysis of dyed CMC-hydrogels with different cross-linking grades and polymerization methods using cellulase from *Aspergillus sp.*. Carboxymethylcellulose was cross-linked with glycidylmethacrylate (GMA) in a low (2,5 mL), medium (5 mL) and high (7,5 mL) degree and then hydrolyzed with cellulase in 2 concentrations (0.5% (0.9 U/mL)and 2.9% (5 U/mL))

Enzymatic hydrolysis of hydrogels from carboxymethylcellulose, pectin and blends with glycidylmethacrylate with pectinase and cellulose

Carboxymethlcellulose and pectin based hydrogels were hydrolyzed with cellulase from *Aspergillus sp*. and pectinase from *Aspergillus aculeatus.*

	Cellulase from *Aspergillus sp* [mL]	Pectinase from *Aspergillus aculeatus* [mL]	Succinate buffer (50 mM, pH 4.5) [mL]
B	/	/	10.5
C	0.25 (4 U/mL)	/	10.25
P	/	0.25 (3 U/mL)	10.25
CP	0.25 (4 U/mL)	0.25 (3 U/mL)	10.0

The hydrolysis process could easily be visualized with the release of alizarin from the hydrogels. In general UV polymerization delivered gels that were not as easily degradable as gels produced with elevated temperature. A possible explanation could be that the backbone gets broken with elevated temperature, which makes it possible for the enzyme to reach the reaction sites more easily. All the measured values were put in relation to the weight of the hydrolyzed gel pieces and the release of alizarin was expressed in % compared to the blank.

In each case hydrolysis with a mixture of cellulase from *Aspergillus sp*. and pectinase from *Aspergillus aculeatus* worked best. In gels that contained more pectin, pectinase worked slightly better than cellulase and when the gels contained more carboxymethylcellulose cellulase worked better than pectinase.

Parts of the results of the carboxmethylcellulose , pectin based hydrogels and their blends were published in (Konstantin P. Schneider and Armin Zankel 2011) and was also a part of the doctoral thesis of Konstantin Schneider.

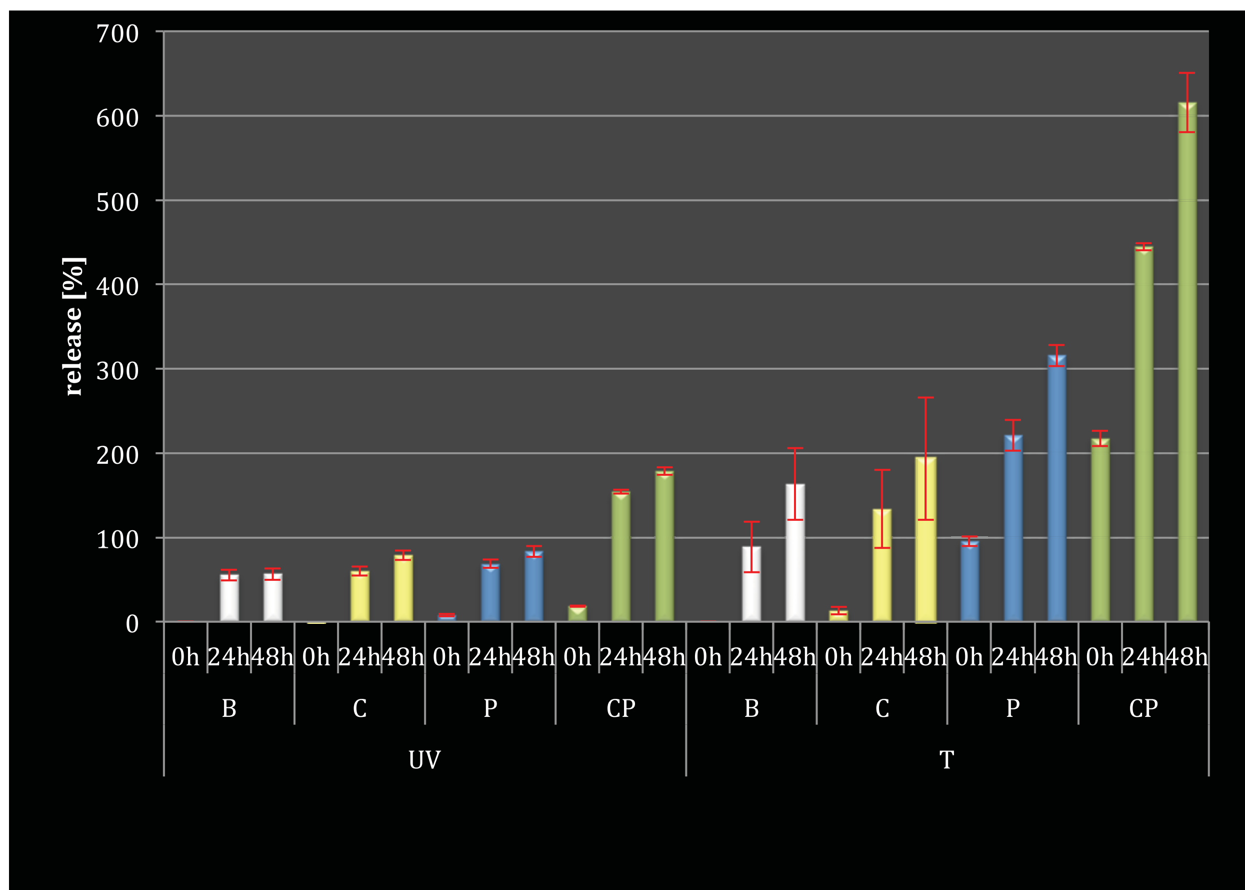

Figure 47 Release of enclosed alizarin from an enzymatically hydrolyzed pectin hydrogel with pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL)
B = Blank , C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

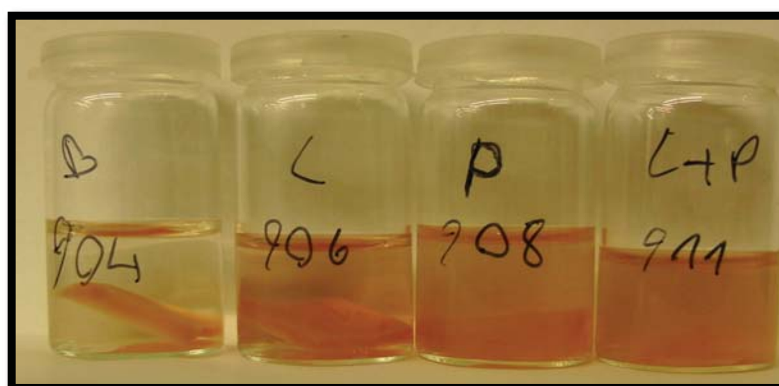

Figure 48 Release of enclosed alizarin from an enzymatically hydrolyzed pectin hydrogel with pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL)
B = Blank, C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

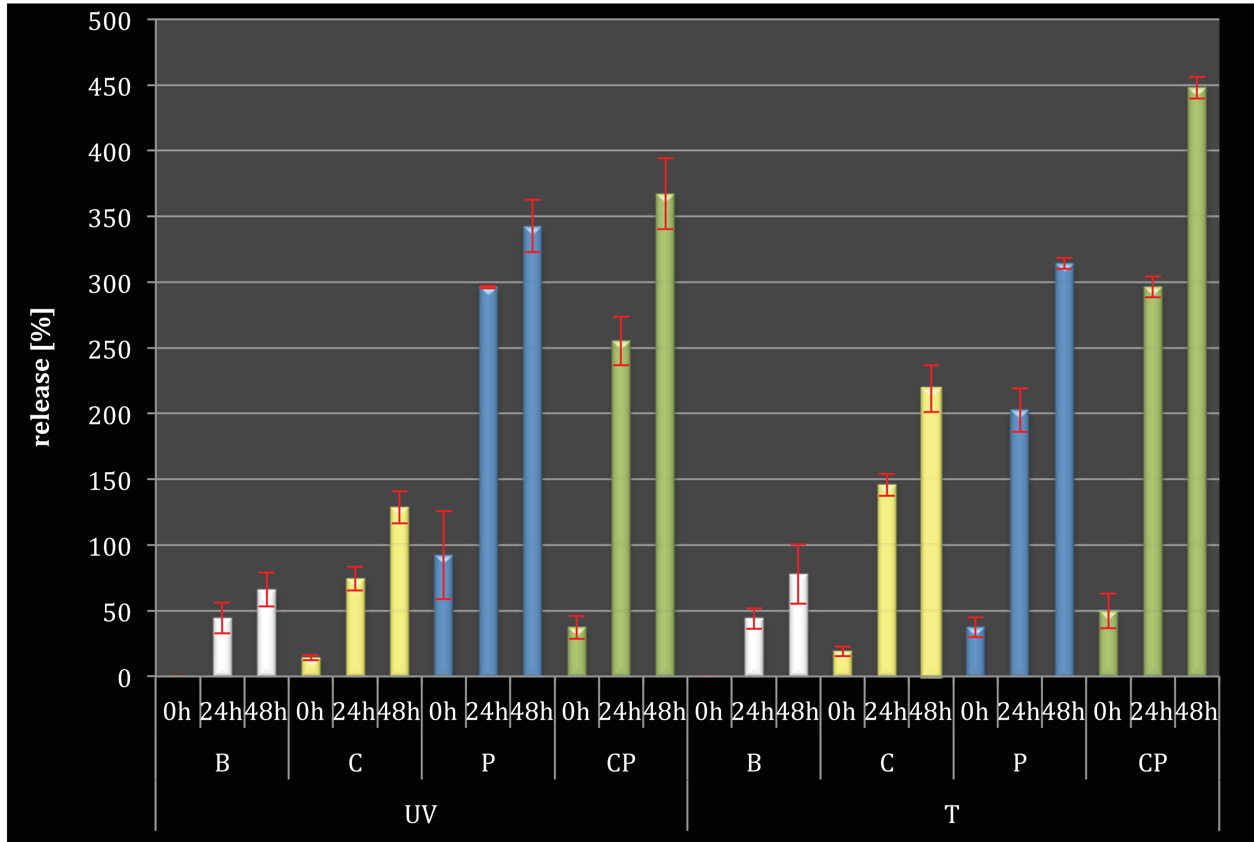

Figure 49 Release of enclosed alizarin from an enzymatically hydrolyzed CMC/pectin (1/2) hydrogel with pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL) B = Blank, C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

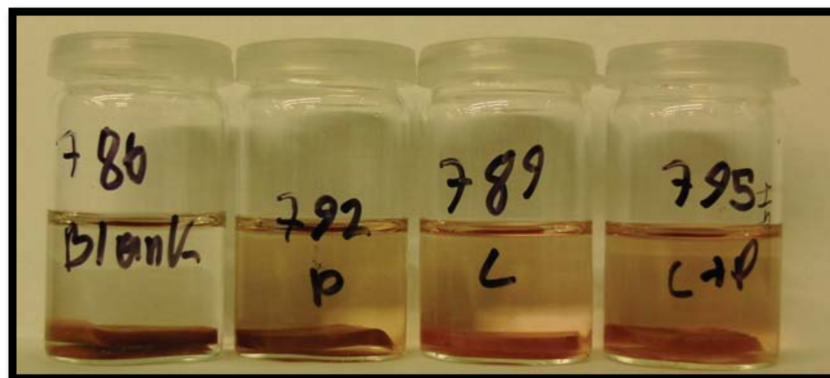

Figure 50 Release of enclosed alizarin from an enzymatically hydrolyzed CMC/pectin (1/2) hydrogel with pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL) B = Blank, C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

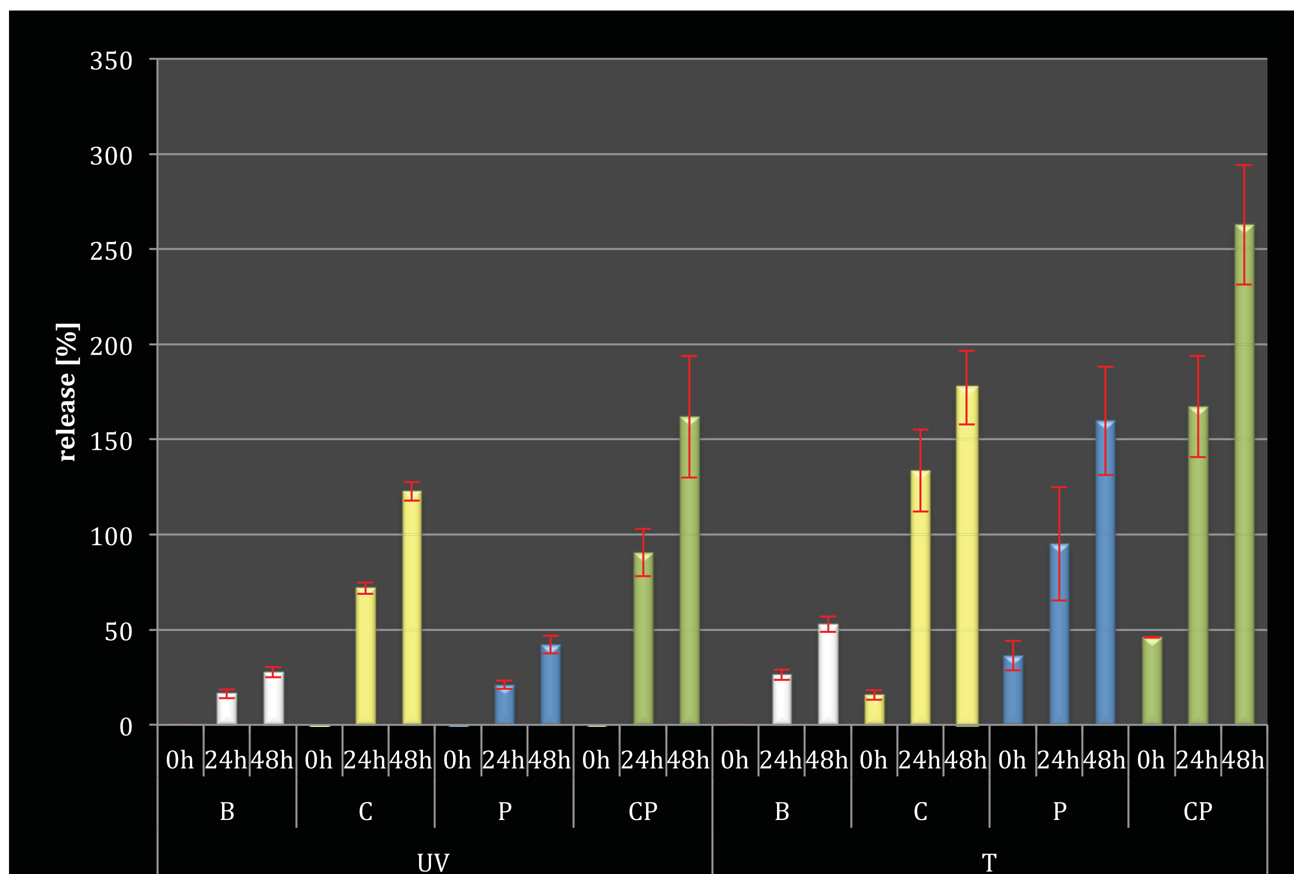

Figure 51 Release of enclosed alizarin from an enzymatically hydrolyzed CMC/pectin (1/1) hydrogel with pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL) B = Blank, C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

Figure 52 Release of enclosed alizarin from an enzymatically hydrolyzed CMC/pectin (1/1) hydrogel with pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL) B = Blank, C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

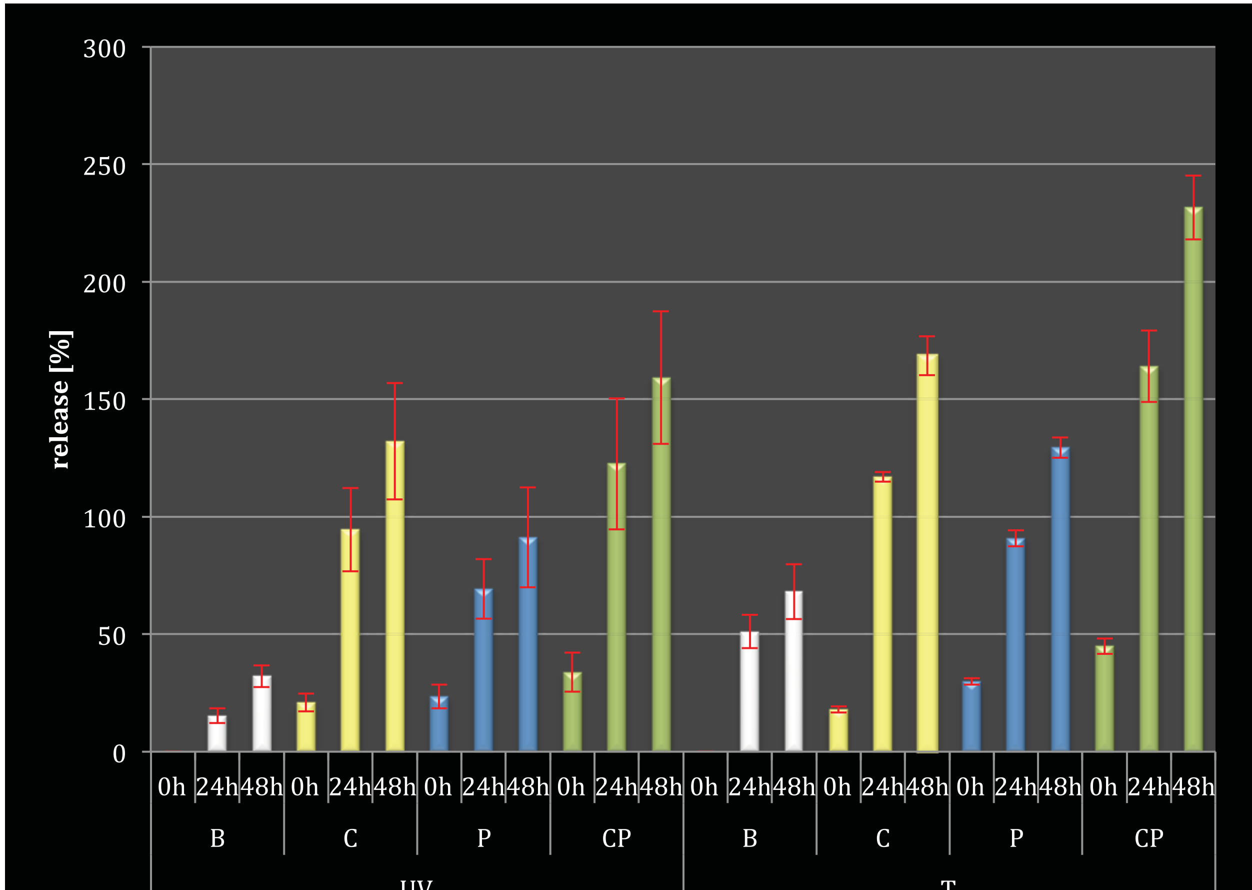

Figure 53 Release of enclosed alizarin from an enzymatically hydrolyzed CMC/pectin (2/1) hydrogel with pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL) B = Blank, C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

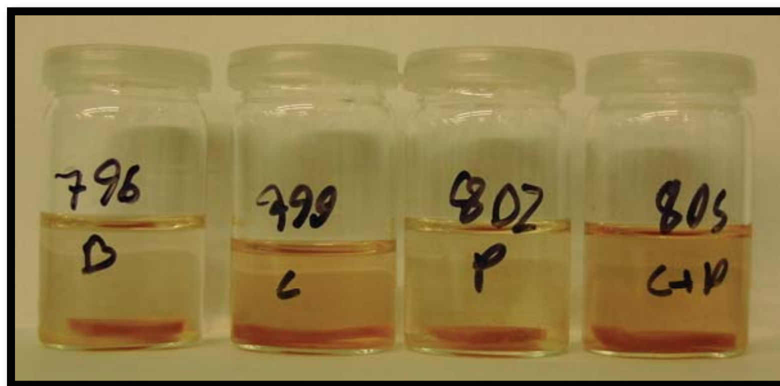

Figure 54 Release of enclosed alizarin from an enzymatically hydrolyzed CMC/pectin (2/1) hydrogel with pectinase and cellulase. Cellulase and the mixture of cellulase and pectinase clearly released more dye than pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL) B = Blank, C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

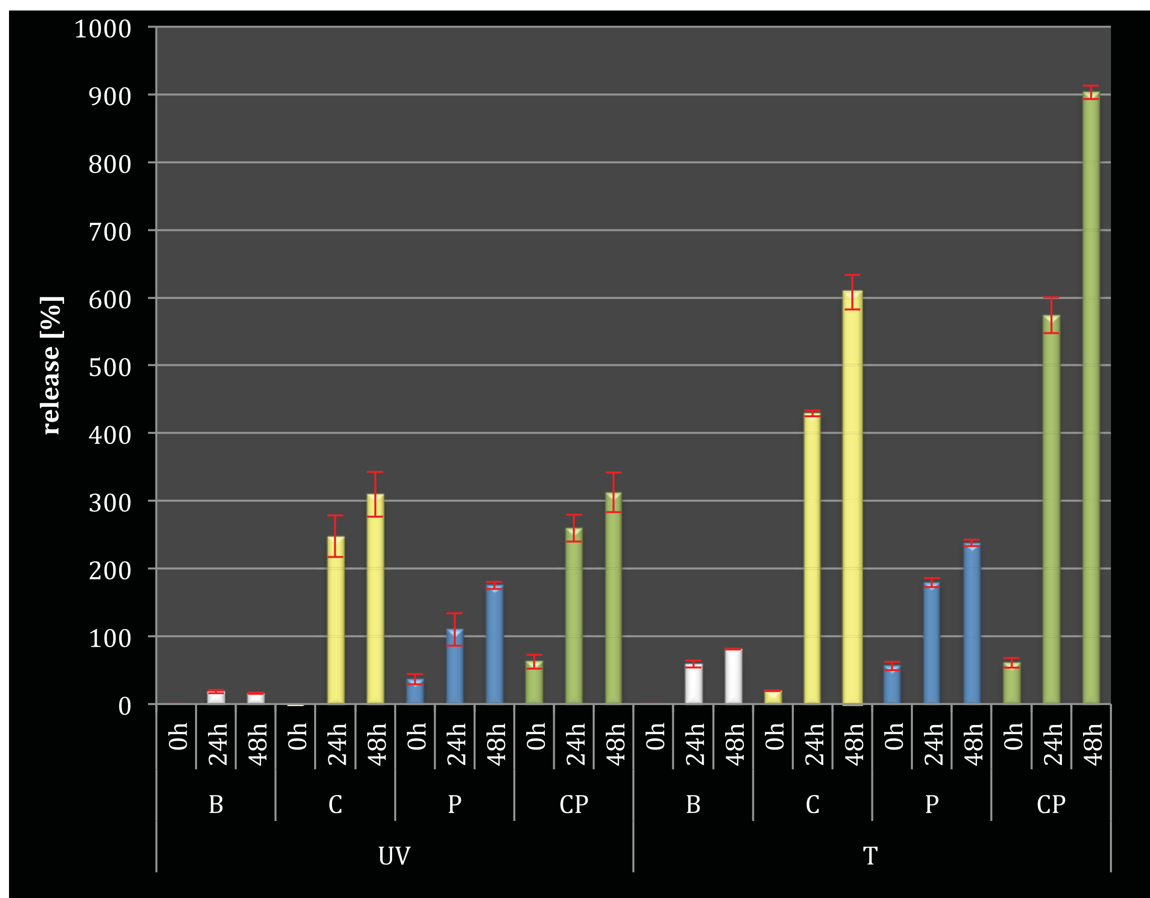

Figure 55 Release of enclosed alizarin from an enzymatically hydrolyzed carboxymethylcellulose hydrogel with pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL) B = Blank, C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

Figure 56 Release of enclosed alizarin from an enzymatically hydrolyzed carboxymethylcellulose hydrogel with pectinase from *Aspergillus aculeatus* (3U/mL) and cellulase from *Aspergillus sp..* (4U/mL) B = Blank, C = Samples hydrolyzed with cellulase, P = Samples hydrolyzed with pectinase, CP = samples hydrolyzed with cellulase and pectinase

Mixed and pure hydrogels on CMC and pectin base with enclosed laccase

In the methods above dye was used to visualize the enzymatic hydrolysis process by releasing dye molecules to the supernatant. to have an easily measurable and visible control of the polymer hydrolysis. The next step was to enclose enzymes in order to create a signal enhancement. The oxidoreductase Laccase from *Trametes hirsuta,* which is produced by fungi was chosen for this purpose. On the one hand because this enzyme does not need any cofactors and on the other hand because the activity of laccase could be measured with an assay where ABTS served as a substrate. ABTS gets oxidized to a dark green cation that can easily be detected.

Laccase was first enclosed without modification, which didn’t provide satisfactory results because of a high rate of auto diffusion. To inhibit this unwanted process two modification methods were used: increasing the size of the laccase and introducing methacrylate groups to the laccase

Modification of Laccase from *Trametes hirsuta*

Laccase from *Trametes hirsuta* was modified with 2 different buffers. Laccase modified with glycine buffer (0.1M, pH 4.5) was labeled as L4 laccase and laccase modified with borate buffer (0.1M, pH 9.3) was labeled as L3 laccase during further experiments.

To determine the activity loss of laccase during the modification, purification and polymerization, the enzyme activity was determined with an assay where ABTS served as substrate.

Table 12 Measured enzyme activities during the modification of laccase from *Trametes hirsuta* with glycidylmethacrylate in glycine (pH 9.3) and borate buffer (pH 4.5)

		Laccase activity [U/mL]	
	time [hours]	L 3 -laccase (pH 9.3)	L 4-laccase (pH 4.5)
Without GMA	Blank	28,742	25,289
With GMA	0	28,092	29,175
	0.5	29,421	27,451
	1	27,845	26,737
	2	26,664	24,522
	3	27,845	30,012
	24	20,752	26,688
	After purification	5,365	6,520

According to the activity measurements laccase lost more activity when modified in borate buffer. Therefore glycine buffer was chosen as buffer for modifications.

The activity loss may have been caused by methacrylate groups added near or in the reactive center during modification. (Schneider, Gewessler et al. 2012)

Modification of pectin and CMC with glycidylmethacrylate

Production of pectin and CMC hydrogels with enclosed enzyme

As enzymes could be sensitive towards elevated temperatures and UV irradiation, polymerisation was performed at room temperature with TEMED.

Enzymatic hydrolysis of pectin and CMC hydrogels with enclosed enzyme

Hydrogels on a carboxymethylcellulose and pectin base as well as their blend with enclosed unmodified laccase and laccase modified with methacrylate groups and modified with PEG were investigated,

In table 13 the used enzyme activities for the different gel matrices can be seen. For hydrolysis of pure pectin and CMC hydrogels cellulase from *Aspergillus sp.* and pectinase from *Aspergillus*

niger were used. Blends were were hydrolyzed from with cellulase from *Aspergillus sp.* (175 U/mL) and pectinase from *Aspergillus aculeatus* (130U/mL) instead of pectinase from *Aspergillus niger* (485 U/mL) as they had more similar activities.

Table 13 Table of used enzyme activity for the biotransformation of CMC and pectin hydrogels with cellulase from *Aspergillus sp.* and pectinase from *Aspergillus niger and Aspergillus aculeatus*

		Cellulase *Aspergillus sp.*		**Pectinase *Aspergillus niger***	
		Volume [µL]	**Activity [U/mL]**	**Volume [µL]**	**Activity [U/mL]**
CMC gels		75	0,88	-	-
		150	1,75	-	-
		450	5,25	-	-
Pectin gels		-	-	27,06	0,87
		-	-	54,12	1,75
		-	-	162,37	5,25
		Cellulase *Aspergillus sp.*		**Pectinase *Aspergillus aculeatus***	
		Volume [µL]	**Activity [U/mL]**	**Volume [µL]**	**Activity [U/mL]**
Pectin/CMC gels (1/1)	Blank	-	-	-	-
	CI	75	0,88	-	-
	CII	450	5,25	-	-
	PI	-	-	75	0,88
	PII	-	-	450	5,25
	CPI	75	0,88	75	0,88
	CP II	450	5,25	450	5,25

When laccase from Trametes hirsuta (Figure 59 and Figure 61) was enclosed in a carboxymethylcellulose hydrogel without modification, the rate of auto diffusion was quite high. There was a difference between gels that were enzymatically hydrolyzed and the blank but it could definitely be improved.

When the enclosed laccase had been modified with glycidyl methacrylate (Figure 60) and was cross-linked into the polymer during the polymerization reaction, the rate of auto diffusion could be decreased. However there was less activity released all together. Oligosaccharides that

are still covalently attached to laccase after its release from the gel could be a possible explanation) (Schneider, Gewessler et al. 2012)

When the enclosed laccase had been modified with PEG (Figure 62) not the initial but the total activity released due to auto diffusion could be lowered.

When carboxymethylcellulose was hydrolyzed with 75µL of cellulase an improvement of the released activity ratio from the blank to the sample could be improved. The measurements should be repeated, as there was a high standard as the high standard deviation made it difficult to draw conclusions.

When methacrylated laccase was used in pectin hydrogels the auto diffusion rate also improved however the released activity ratio between the blank and the sample was a lot worse than when native laccase from *Trametes hirsuta* was used. (Figure 57 and Firgure 58)

The same result goes for the blends of pectin and carboxymethylcellulose. (Figure 65 and Figure 66)

To improve the signal between blanks and samples, the effect of a longer washing time was tested on carboxymethylcellulose gels with unmodified laccase and laccase modified with glycidylmethacrylate. The auto diffusion was even lower and the ratio of signal and blank could indeed be improved by 44% in case of hydrolysis with 0,88 U/mL cellulase and by 17% in case of hydrolysis with 5,25 U/mL. (Figure 64 and Figure 64)

Pure CMC and pectin gels were best hydrolyzed with 150µL (1,75 U/mL)of enzyme.

The CMC/pectin blend with enclosed modified laccase was better hydrolyzed by the mixture of cellulase and pectinase with an activity of 0,88U/mL whereas the samples with unmodified enclosed laccase were hydrolyzed just slightly better by pure pectinase (5,25 U/mL)

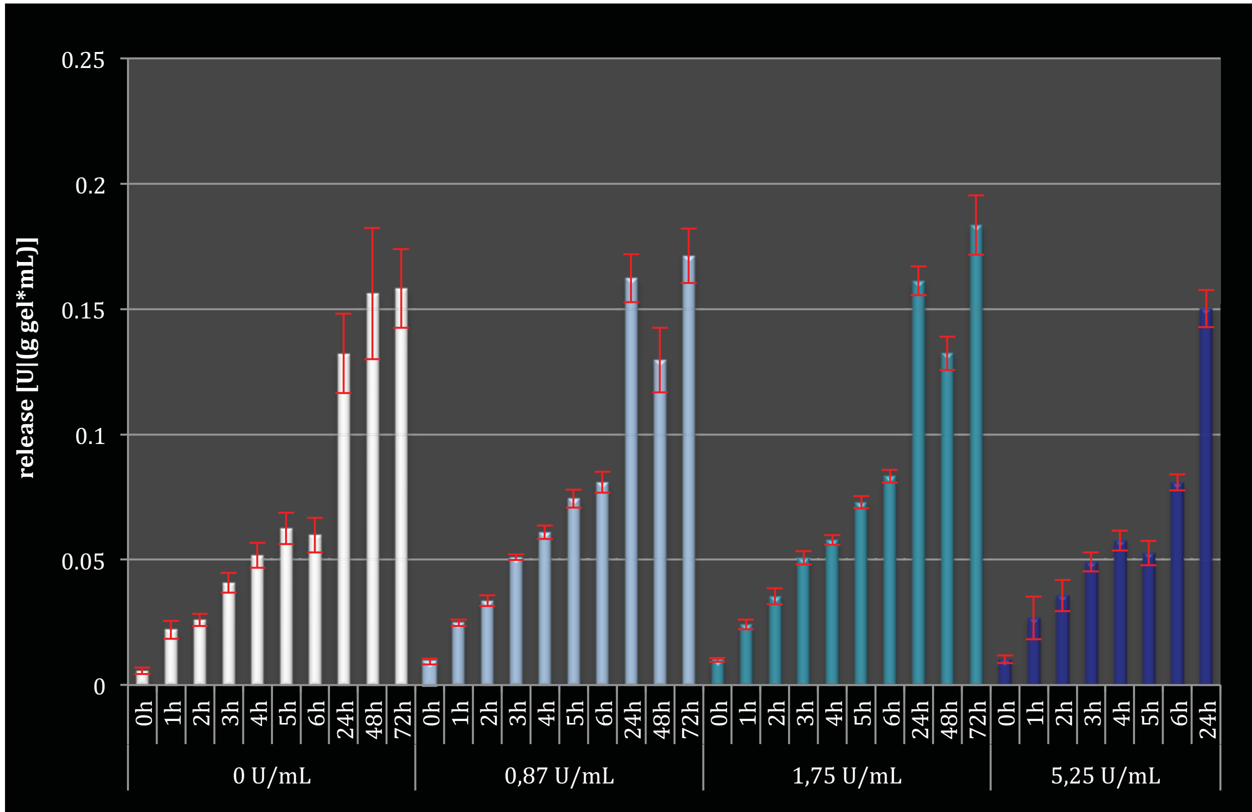

Figure 57 Release of enclosed *Laccase from Trametes hirsuta* upon hydrolysis with pectinase from *Aspergillus niger* in various concentrations from a pectin hydrogel polymerized at room temperature with TEMED

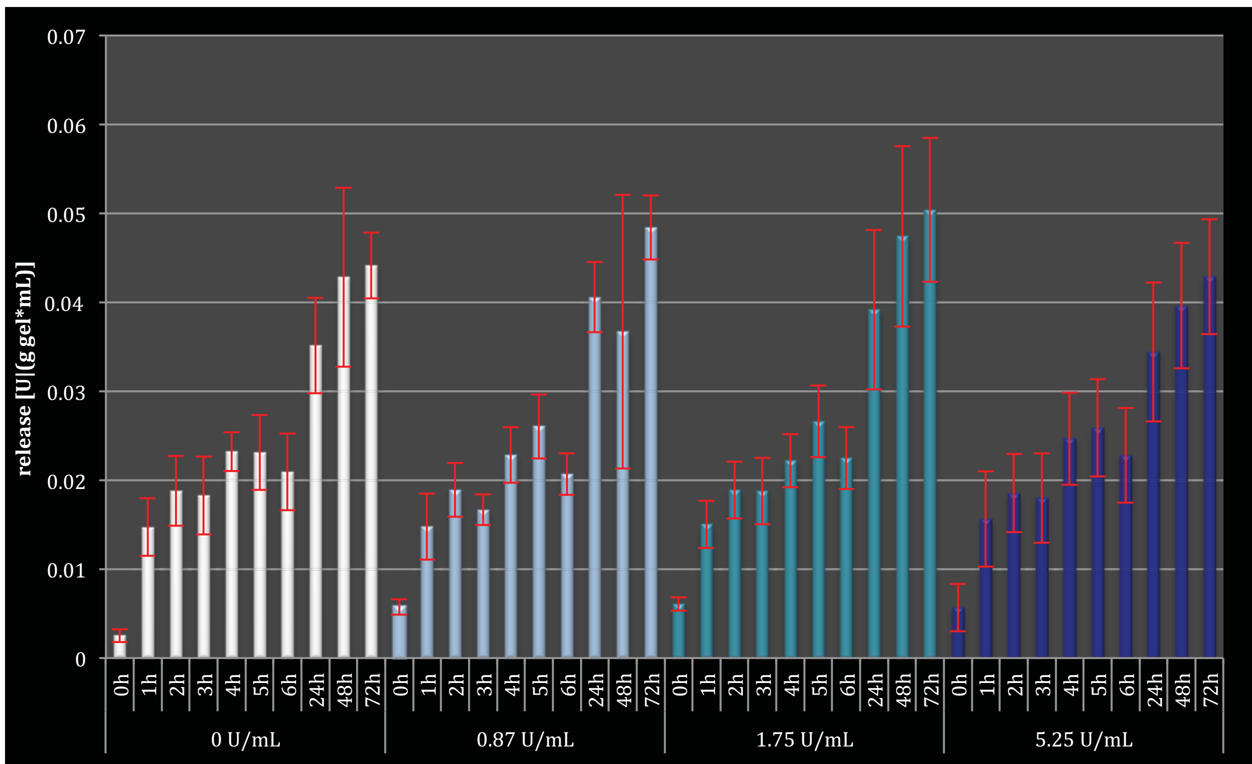

Figure 58 Release of enclosed *Laccase from Trametes hirsuta* modified with methacrylic groups upon hydrolysis with pectinase from *Aspergillus niger* in various concentrations from a pectin hydrogel polymerized at room temperature with TEMED

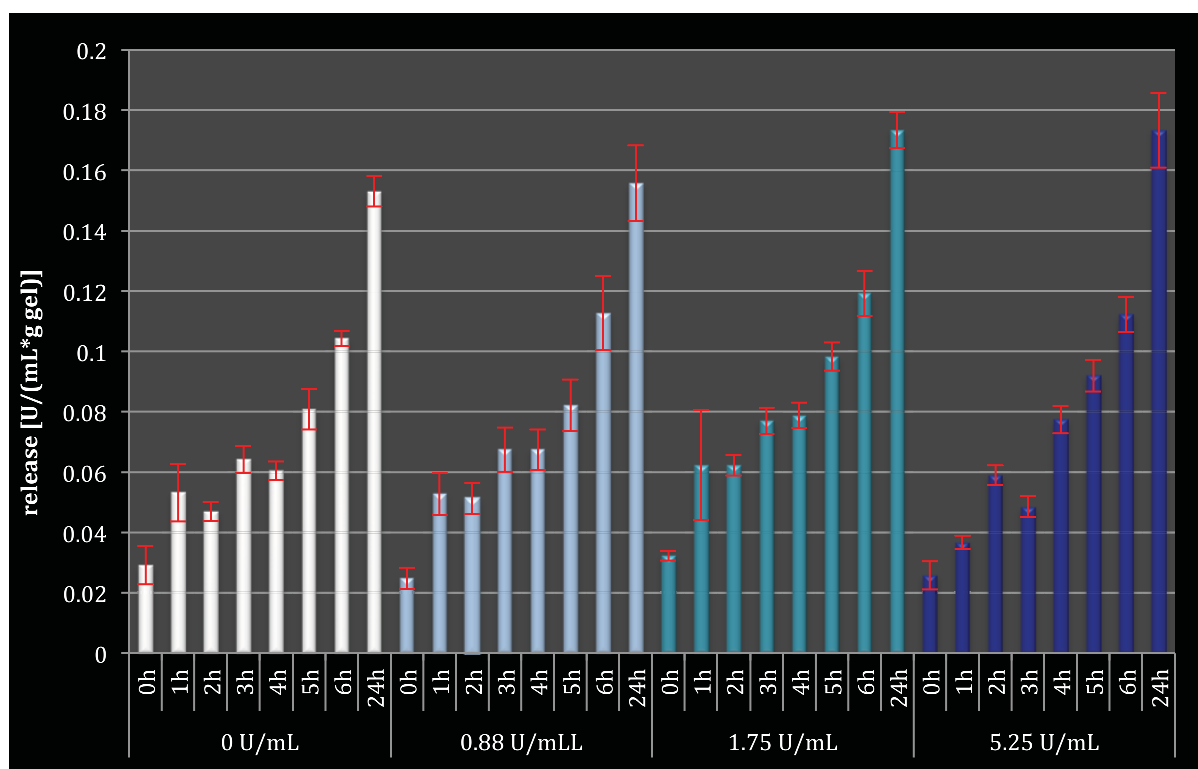

Figure 59 Release of *Laccase from Trametes hirsuta* upon hydrolysis with cellulase from *Aspergillus sp.* in various concentrations from a carboxymethylcellulose hydrogel polymerized at room temperature with TEMED

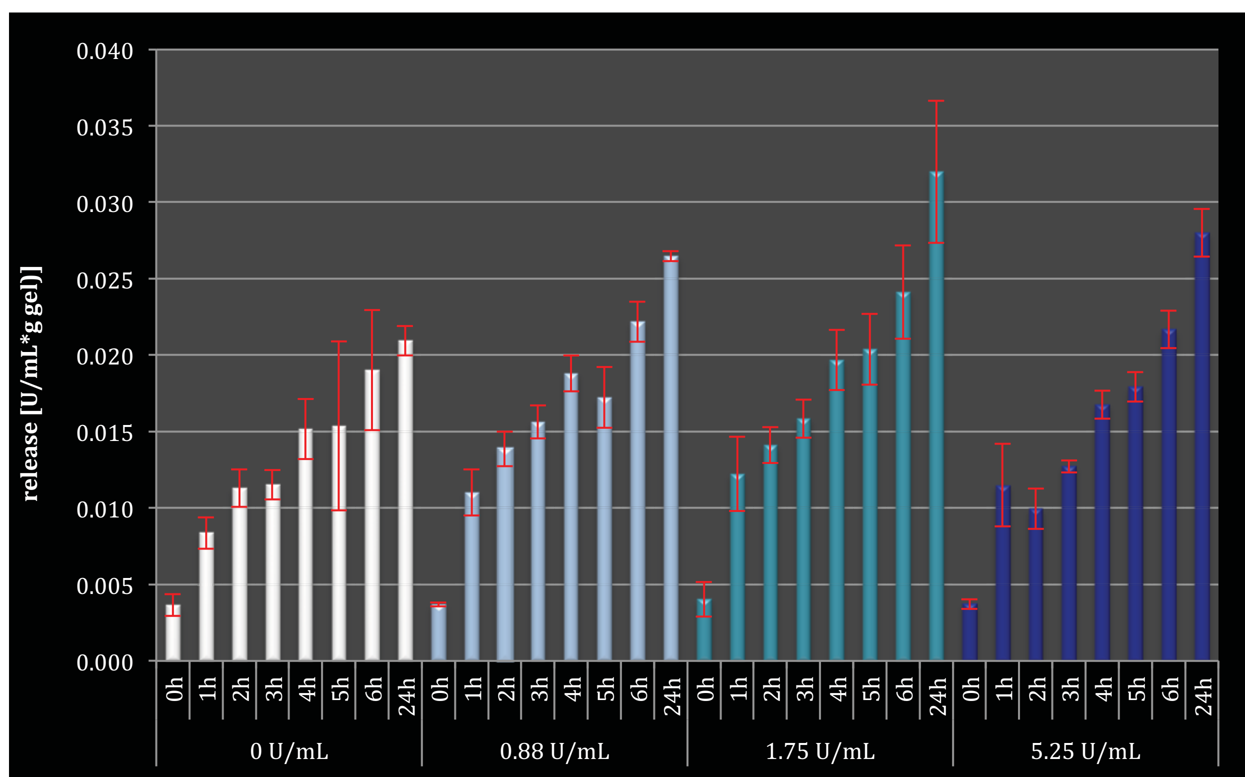

Figure 60 Release of Laccase from *Trametes hirsuta* modified with methacrylate groups upon hydrolysis with cellulase from *Aspergillus sp.* in various concentrations from a carboxymethylcellulose hydrogel polymerized at room temperature with TEMED

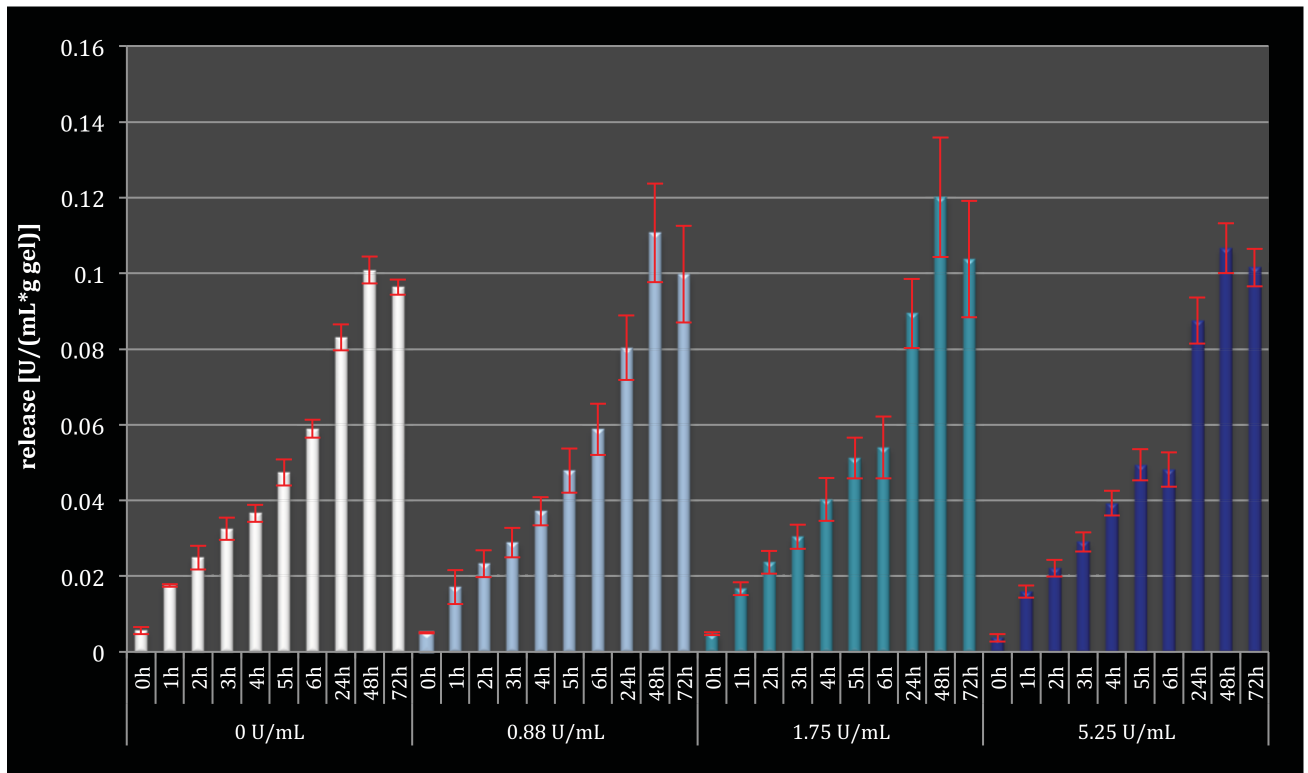

Figure 61 Release of *Laccase from Trametes hirsuta* upon hydrolysis with cellulase from *Aspergillus sp.* in various concentrations from a carboxymethylcellulose hydrogel polymerized at room temperature with TEMED

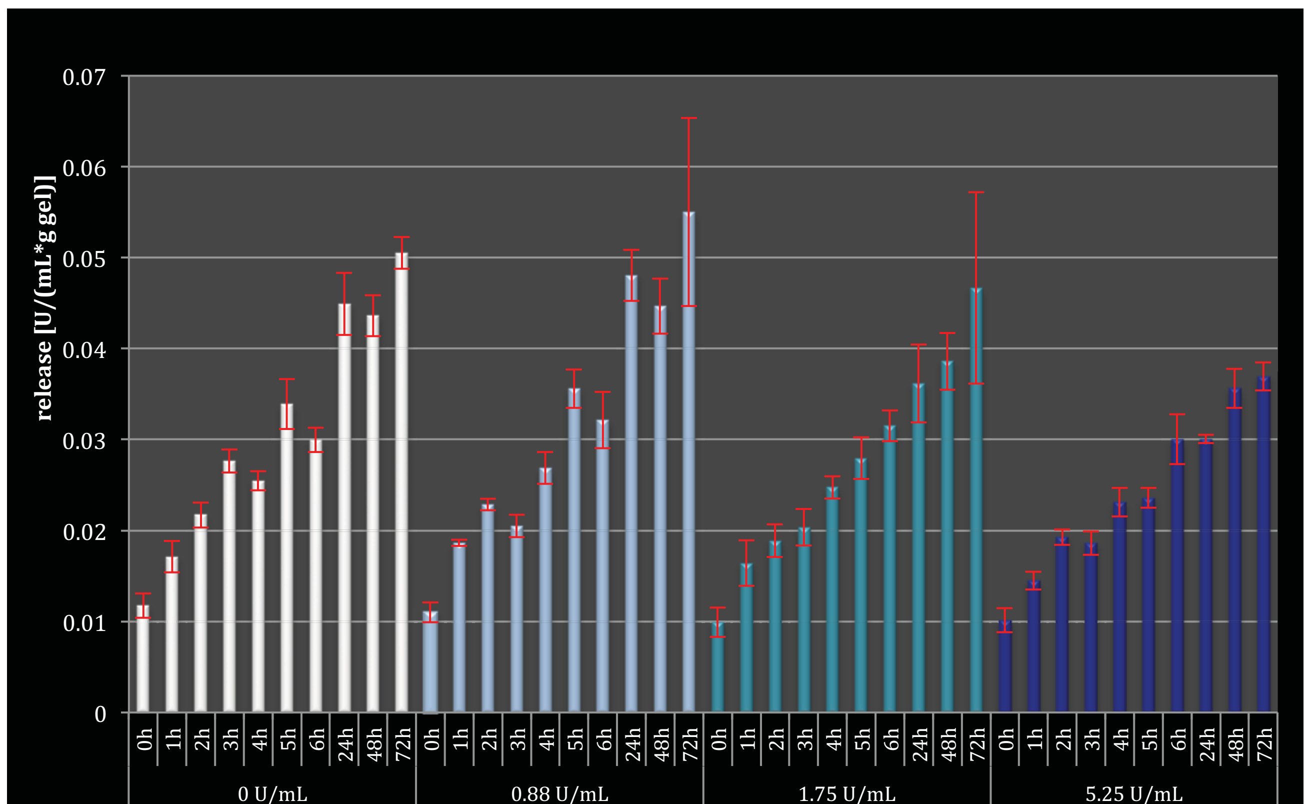

Figure 62 Release of *Laccase from Trametes hirsuta* modified with polyethylene glycol upon hydrolysis with cellulase from *Aspergillus sp.* in various concentrations from a carboxymethylcellulose hydrogel polymerized at room temperature with TEMED

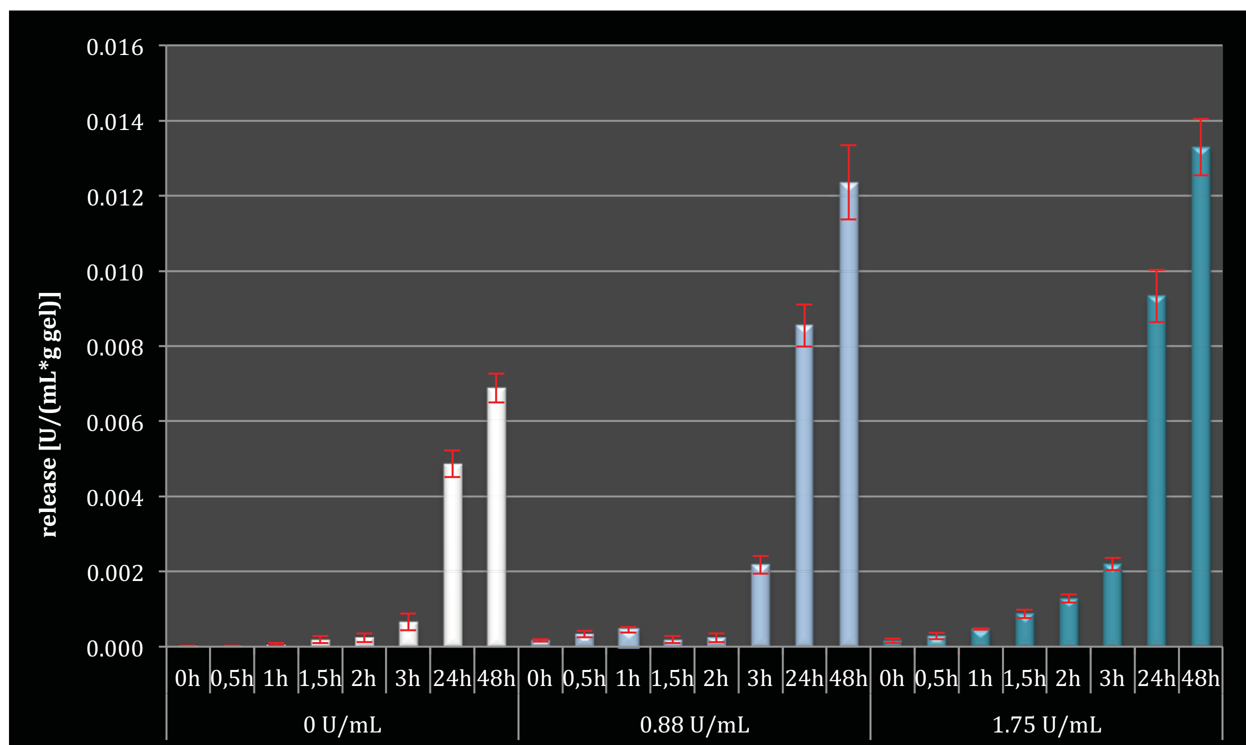

Figure 63 Release of enclosed *Laccase from Trametes hirsuta* f upon hydrolysis with cellulase from *Aspergillus sp.* in various concentrations from a carboxymethylcellulose hydrogel polymerized at room temperature with TEMED. The gels were washed over night before they were hydrolyzed.

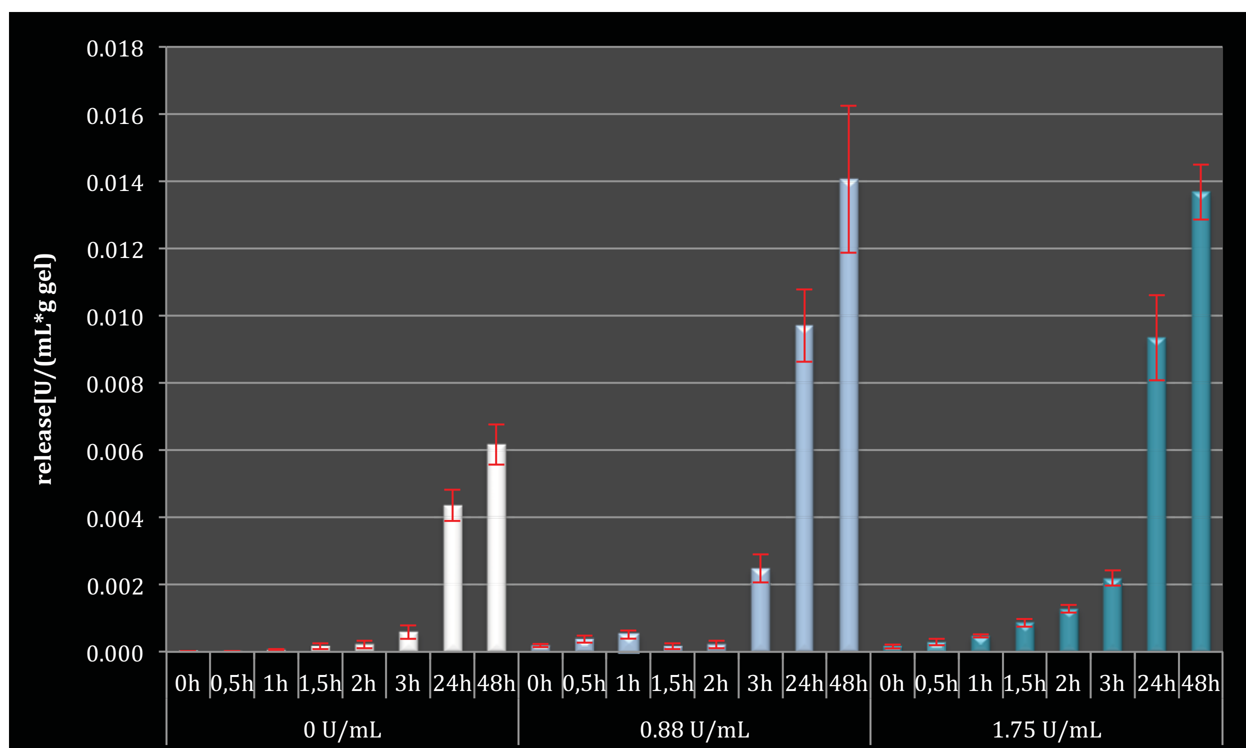

Figure 64 Release of enclosed *Laccase from Trametes hirsuta* modified with methacrylate groups upon hydrolysis with cellulase from *Aspergillus sp.* in various concentrations from a carboxymethylcellulose hydrogel polymerized at room temperature with TEMED. The gels were washed over night before they were hydrolyzed.

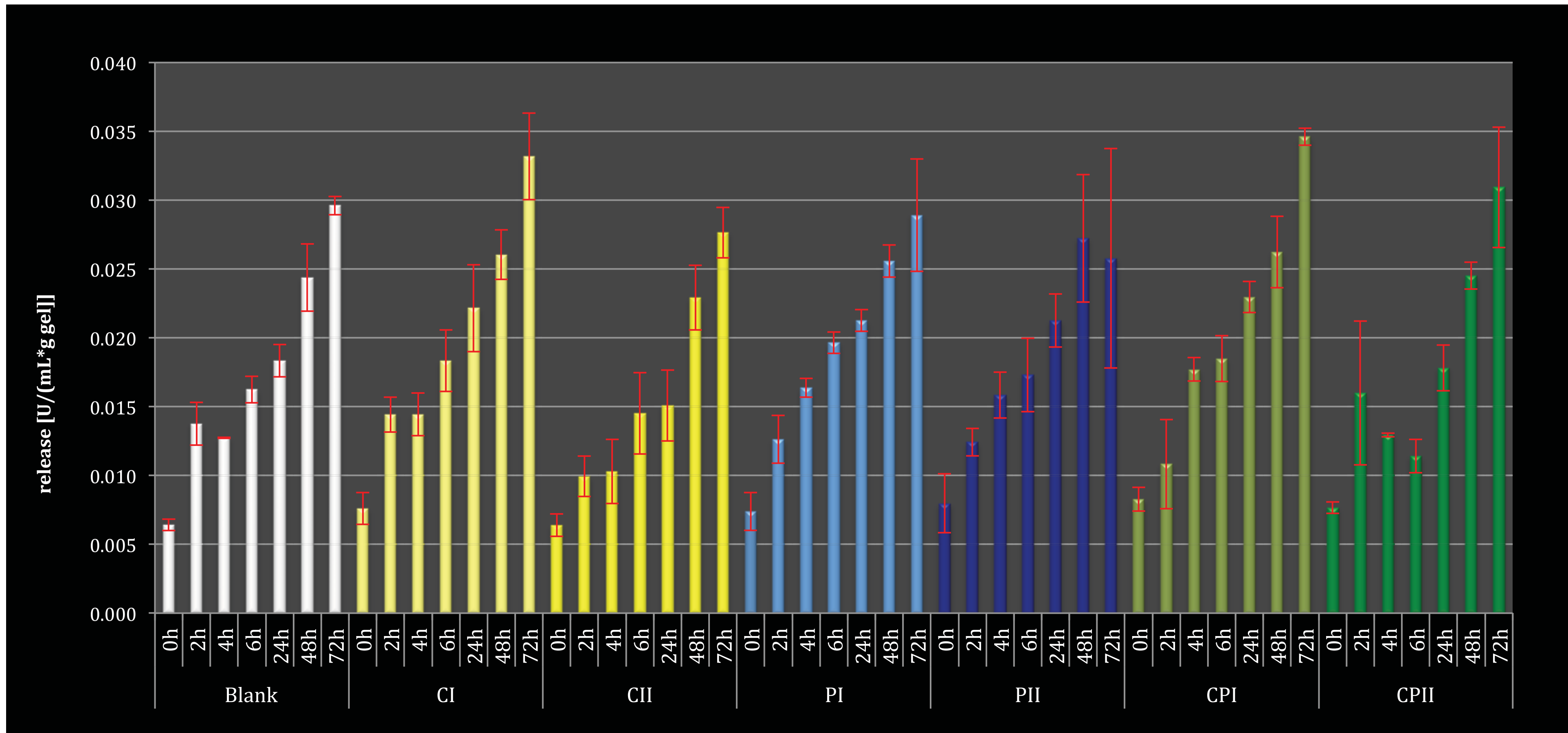

Figure 65 Release of enclosed laccase from *Trametes hirsuta* modified with methacrylic groups from a carboxymethylcellulose and pectin (1:1) hydrogel polymerized at room temperature with TEMED. The gel was enzymatically hydrolyzed with pectinase from *Aspergillus aculeatus* and cellulase from *Aspergillus sp.* in various concentrations CI and CII...hydrolysis with 0.88 U/mL and 5.25 U/mL cellulase, PI and PII...hydrolysis with 0.88 U/mL and 5.25 U/mL pectinase, CPI...hydrolysis with 0.88 U/mL cellulase and pectinase, CPII...hydrolysis with 5.25 U/mL cellulase and pectinase

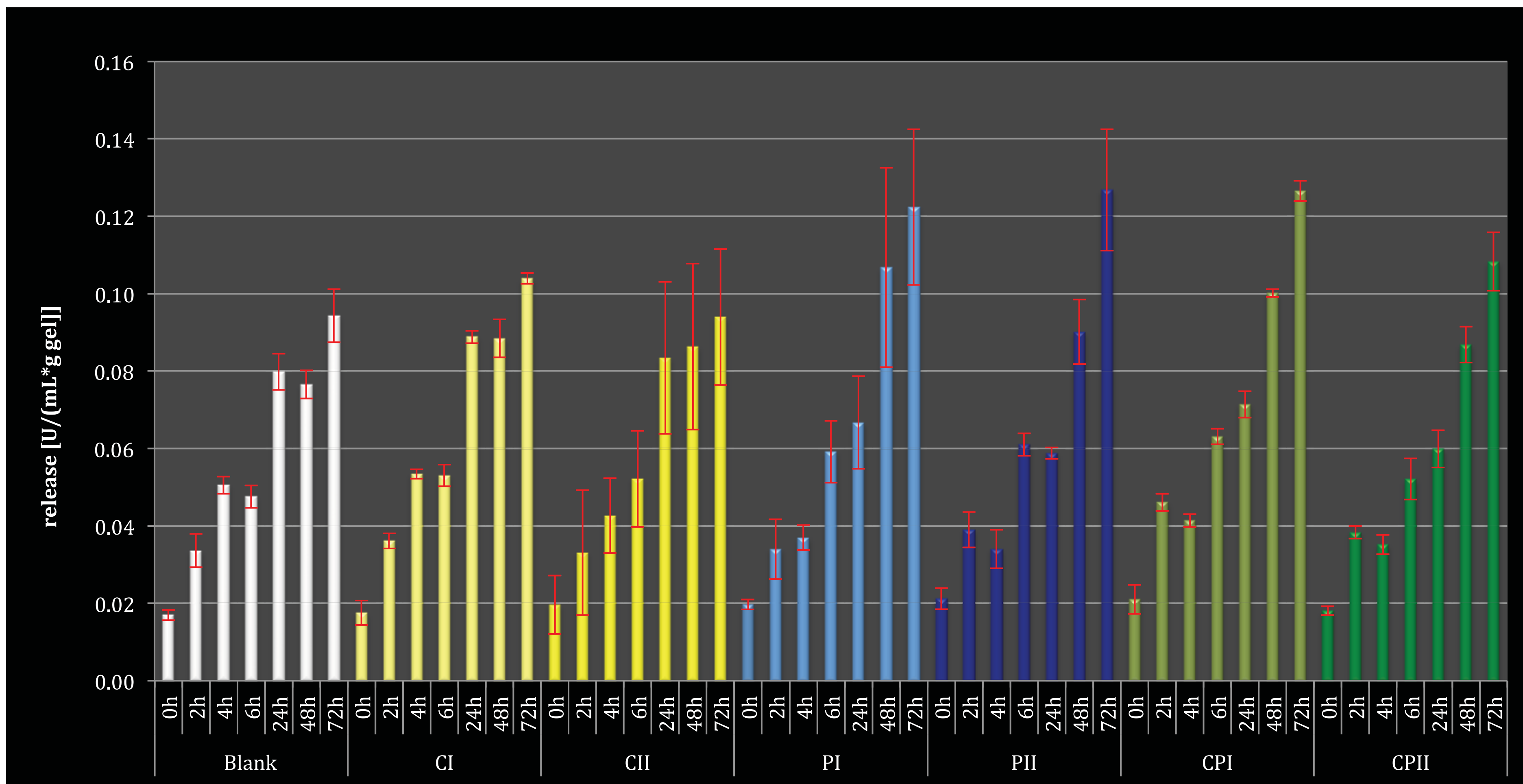

Figure 66 Release of enclosed laccase from *Trametes hirsuta* from a carboxymethylcellulose and pectin (1:1) hydrogel polymerized at room temperature with TEMED. The gel was enzymatically hydrolyzed with pectinase from *Aspergillus aculeatus* and cellulase from *Aspergillus sp.* in various concentrations

CI and CII…hydrolysis with 0.88 U/mL and 5.25 U/mL cellulase, PI and PII…hydrolysis with 0.88 U/mL and 5.25 U/mL pectinase, CPI…hydrolysis with 0.88 U/mL cellulase and pectinase, CPII…hydrolysis with 5.25 U/mL cellulase and pectinase

Final discussion

This work focused on the development of polysaccharide based bioresponsive devices for the detection of enzymes, of contaminating bacteria/fungi in e.g. packaging material of food. As a bioresponsive matrix, hydrogels of dextran, pectin and carboxymethylcellulose were modified (with methacrylate groups) and polymerized using three different methods, which was verified with FTIR analysis.

In this thesis dextran was hydrolyzed by α-amylase from *Bacillus amyloliquefaciens* as a side reaction (Y. Sakano 1985), pectin and carboxymethylcellulose by pectinases from *Aspergillus niger* and cellulases from *Aspergillus sp.*.

To visualize the hydrolyzation process of the hydrogels with enzymes various dyes and enzymes, which could create a visible color reaction with pro-dyes were enclosed in the hydrogel. The color release due to hydrolysis was detected photometrically. The hydrolysis of undyed hydrogels was demonstrated with SEM measurements and monitored with HPLC measurements in the case of dextran hydrogels.

It was possible to enclose dye, modified and unmodified enzymes within the polymer grid. Two different enzyme modification strategies for laccase from *Trametes hirsuta* were explored based on previous work of (Schroeder 2005): increase of the molecular weight with polyethylene glycol groups and the production of cross-linkable enzymes by modification with methacrylate groups.

Dextran was modified with glycidyl methacrylate (van Dijk-Wolthuis 1995), methylmethacrylate (Yasuhiko Onishi∗ 1978) (Yasuhiko Onishi 2005) (A. Yu. Men'shikova 2001) and methacrylic anhydride (Kim 2000). All these modification methods were successfully carried out when performed as described in the literature.

However when the methods were modified e.g. by using dyed dextran it was not always possible to produce a polymer.

On dextran the use of Bismarck Brown R, methyl red, carminic acid and Reactive Black 5 was explored. As Bismarck Brown R did not deliver the wanted results experiments with this dye were stopped. Carminic acid was physically enclosed in the polymer, whereas methacrylated methyl red and Reactive Black covalently bound to the dextran backbone, which can reduce auto diffusion. However using methyl red most of the dye was already released within the first hour. Possibly the dye molecule was too small. The release due to hydrolysis of the dextran hydrogel with α-amylase from *Bacillus amyloliquefaciens* of carminic acid and Reactive Black delivered better results than with methyl red, however the low stability of the gels and the signal ratio between blank and sample was not satisfactory.

Dyed dextran was often impossible to dissolve. To avoid this, a one-pot reaction was tried, however it was usually not possible to produce hydrogels. Possibly there is a concurrence between the reactive dye and the methacrylate group donor (glycidyl methacarylate, methacrylic anhydride, methyl methacrylate), which could lead to a too low cross-linking degree.

Modified dextran can be hygroscopic which caused problems during the purification process because it became lumpy and clogged the filter. Furthermore, dextran did not deliver polymers with a sufficiently high stability. During the hydrolysis process the results were inconsistent. However, when enclosing enzymes instead of dye, the results improved. As the enzymes have a higher molecular weight than the dye it was more difficult for them to escape the unstable hydrogel.

We tried to improve the stability of dyed dextran by mixing it with photoactive PVA produced by A.Mühlebach. (Mühlebach 1997). The produced blends with different dextran/PVA ratios were also successfully hydrolyzed. Higher PVA ratios increased the stability but the release signal was reduced.

In table 14 the ratio [%] between blank at 0h and the highest sample peak is listed for each hydrolysis reaction of dextran hydrogels depicted in this thesis. The improvement factor of each reaction was calculated in reference to the lowest ratio [%]:

Table 14 Calculated ratio [%] between the blank signal at 0h and the highest sample peak of all the hydrolysis experiments of the various dextran hydrogels in this thesis using α-amylase from *Bacillus amyloliquefaciens*. The improvement factor [%] of each reaction was calculated in reference to the lowest ratio$_{\text{sample to blank}}$ [%]:

Hydrolysis with α-amylase from Bacillus amyloliquefaciens of a...	ratio$_{\text{sample to blank}}$ [%]	Improvement factor [%]
Dextran hydrogel dyed with 100mg of carminic acid	186	1
Dextran hydrogel dyed with methyl red	440	3
Dextran hydrogel dyed with Reactive Black and precipitated in acetone	165	1
Dextran hydrogel with enclosed laccase from *Myceliophthora thermophila*	800	5
Dextran/(8%)PVA hydrogel (1:9) dyed with Reactive Black 5	2500	15
Dextran/(11%)PVA hydrogel (5:5) dyed with Reactive Black 5	2250	14

Clearly the PVA/dextran blends delivered the best ratio of blank to signal. Dextran/(8%)PVA hydrogel (1:9) dyed with Reactive Black 5 has a higher improvement factor [%] than Dextran/(11%)PVA hydrogel (5:5) dyed with Reactive Black 5. However, the maximum release

was already found after 24h. For this reason, the dextran/PVA(11%) delivered the best results in our eyes.

For our purposes however, the focus was moved to carboxymethylcellulose and pectin based polymers.

CMC and pectin were successfully modified with glycidymethacrylate based on previous work by (J.F.A.S. Maior 2008) and (Konstantin P. Schneider and Armin Zankel 2011). Not only were the results more consistent, these modified sugars also provided better handling. They were not hygroscopic, which made the purification process swifter and easier. The modification and dyeing process of CMC and pectin with alizarin is simpler than the dyeing of dextran. Dyed dextran needs to be recovered by precipitation or tedious filtration due to clogged filters. Dyed CMC and pectin can be recovered by a quick filtration and removal of the residual solvent under reduced pressure delivering a homogenously colored powder. After the modification dextran needed to be precipitated and dissolved again for the polymerization reaction, with modified CMC and pectin, however, the modification solution could immediately be used for the polymerization reaction. The CMC and pectin hydrogel could easily be washed, cut and transferred into the suitable vessels for the hydrolysis, whereas the dextran hydrogels often broke to pieces already during the washing process.

CMC and pectin hydrogels were hydrolyzed with cellulase from *Aspergillus sp.* or/and pectinase from *Aspergillus niger*.

Blends of pectin and carboxymethylcellulose provide the opportunity to multiple enzymatic hydrolysis. As both biomatrices were dyed with alizarin we were able to dye the blends in one go. Because of the high stability of the gels they are easier to handle and auto diffusion can be easily reduced.

It was possible to enclose laccase from *Trametes hirsuta* into CMC and pectin based hydrogels. To reduce the effects of auto diffusion the laccase was also modified with methacrylate groups based on the previous work of (Schroeder 2005) This opened up the opportunity to crosslink the sugars with the laccase.

The second modification strategy of laccase involved the increase of its molecular weight by introducing polyethylene glycol groups into the enzyme previously described by (Schroeder 2005)

The crosslinking of enzyme delivered better results than the increase of size. With this new approach and further improvements e.g. improved washing steps, optimization of cross-linking degree,... an enhancement of the signal with a factor 800 could be reached. (Schneider, Gewessler et al. 2012)

During analysis of the hydrolysis processes of all of the hydrogels the weight of the hydrolyzed gel piece was taken into account. However during the analysis of the results and the calculation of the release in relation to the weight it became clear that this was not the best option. As the degrading enzymes worked on the surface of the gel, the surface was more important than the weight. These two parameters are not in direct relation to each other. A heavy gel piece would deliver a small release compared to its weight and a light gel piece a big release compared to its molecular weight although the enzymes had almost the same surface to work on. In future either gel pieces with exactly the same surface should be used or the surface could be measured or calculated.

An improvement of dextran modification techniques or other modification/polymerization methods could be explored. If improvement is impossible, adding miniscule amounts of modified dextran to a polymer blend of carboxymethylcellulose and pectin, that do not diminish the stability of the network, could be beneficial if the polymer is used in a function were α-amylases should be able to degrade it. Maybe pectin and CMC would provide enough stability if only small amounts of dextran were included. However it would be beneficial if all biomatrices would be able to release the same molecules e.g. all biomatrices release the same dye or enclosed enhancer enzymes.
In the future, modifying other poly- or oligosaccharides or even peptides and introducing them into the blend could be further investigated. The hydrogel would then be able to respond to a broader band of enzymes and microorganisms.

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