Norwegian University
of Life Sciences

Master's Thesis 2020 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

An Improved Method for the Synthesis of Polyurethane Enables the Efficient Immobilization of HRP

Siri Heggheim
Masters in Chemistry

Acknowledgments

The work described in this thesis was done at the department of Chemistry, the Faculty of Chemistry, Biotechnology and Food Science, at the Norwegian University of Life Sciences.

I wish to express my gratitude towards my supervisors Professor Yngve H. Stenstrøm, Professor Morten Sørlie, Dr. Simen Antonsen and Lucas Rieder for an interesting and exciting topic. Thank you for all the great help and encouragement with both practical and theoretical work. I also wish to thank Anne Grethe Hamre for the help with cultivating ChiA, you have all inspired me tremendously.

Thank you to Professor Reidar Barfod Schüller for performing the particle size analyses, and Professor Vincent Eijsink for the use of lab-facilities.

I particularly wish to thank Anniken Løvig, with whom I worked together on part of this project, for great cooperation and for being a terrific companion. And to the rest of the chemistry department, both students and staff, for participating in making me feel right at home and for making each day at the lab a real joy; it would not have been the same without you.

Last but not least, I wish to thank my family and friends for your ever-present love and support. Especially I want to thank Camilla, Nina, and the rest of “Rappkjefta Ryper” for making these past five years amazing, you are the best!

Abstract

Immobilization of enzymes are used to optimize the use of enzymes in industrial settings. In this thesis a method for immobilization of Horseradish peroxidase (HRP), using polyurethane as support material, was developed.

The synthesis of polyurethane was optimized using both conventional heating and microwave assisted organic synthesis. Poly (vinyl-alcohol) (PVA) and hexamethylene diisocyanate (HMDI) were used to synthesize polyurethane. Different mole ratios between PVA and HMDI were used for the synthesis of polyurethane and tested for the immobilization of HRP.

A variation in size distribution of polyurethane, gives varying immobilization results when the same conditions were used. However, polyurethane with a mole ratio of 1:5, 1:10, 1:15 and 1:20 show good immobilization results (between 50 - 80 % of added HRP was immobilized).

Stored PU (up to 90 days) were also used to successfully immobilize HRP.
The activity of the immobilized HRP was tested, and all gave positive results.

Sammendrag

Immobilisering av enzym ble brukt til å effektivisere bruken av enzymer i industrien. I dette masterprosjektet ble det utviklet en metode for å immobilisere peroxidase fra pepperrot (HRP), ved bruk av polyuretan som bærer.

Syntesen av polyuretan ble optimalisert ved å bruke både konvensjonell oppvarming og mikrobølge syntese. Poly (vinyl-alkohol) (PVA) og hexametylen diisocyanat (HMDI) ble brukt til syntesen av polyuretan. Forskjellige mol-forhold mellom PVA og HMDI ble brukt til å syntetisere polyuretan og testet for immobilisering av HRP

En variasjon i partikkelstørrelse av polyuretan, ga varierende immobilisering resultater når samme betingelser er brukt i syntesen av polyuretan. Allikevel viser polyuretan med mol-forhold 1:5, 1:10, 1:15 og 1:20 gode immobilisering resultater (mellom 50 – 80 % av tilsatt HRP er immobiliser).

Tidligere lagret polyuretan (opp til 90 dager) ble også brukt til å immobilisere HRP. Aktiviteten på immobilisert HRP ble testet, og alle ga positive resultater.

Abbreviations

AA	Auxiliary activities
CAZy	Carbohydrate-Active EnZYmes
EDTA	Ethylenediaminetetraacetic acid
ES	Enzyme-substrate intermediate
His (H)	Histidine
HMDI	Hexamethylene diisocyanate
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LPMO	lytic polysaccharide monooxygenase
MAOS	Microwave-assisted organic synthesis
MOPS	3-Morpholinepropane-1-sulfonic acid
OD	Cell density
PU	Polyurethane
PVA	Poly (vinyl-alcohol)
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TG	Transglycosylation

General remarks

Due to the outbreak of Covid-19 lab work was pushed back, and some experiments were not possible to finish.

Table of Contents

Acknowledgments I

Abstract II

Sammendrag III

Abbreviations IV

General remarks V

Table of Contents VI

1. Introduction ..... - 1 -

1.1 Enzymes ..... - 1 -

1.1.1. Peroxidase ..... - 5 -

1.1.2 Horseradish peroxidase ..... - 5 -

1.1.3 Hydrolases ..... - 7 -

1.1.4* Chitinases from *Serratia marcescens ..... - 8 -

1.1.5 Lytic Polysaccharide (mono)oxygenases ..... - 10 -

1.2 Immobilization of enzymes ..... - 12 -

1.2.2 Covalent immobilization ..... - 13 -

1.2.3 Adsorption ..... - 14 -

1.3 Chemical background ..... - 15 -

1.3.1 Polymer chemistry ..... - 15 -

1.3.2 Polyurethane ..... - 16 -

1.4 Methods ..... - 18 -

1.4.1 Microwave assisted organic synthesis ..... - 18 -

1.4.2 SDS-PAGE ..... - 21 -

1.4.3 Enzymatic Assays ..... - 21 -

1.4.4 Affinity chromatography ..... - 22 -

Aim of study ..... - 23 -

2. Results and discussion ..... - 24 -

2.1 Synthesis of polyurethane using 1:2 mole ratio ..... - 24 -

2.1.1 Immobilization results when PU (1:2 mole ratio) was used ..... - 24 -

2.2 Synthesis of polyurethane using 1:5 mole ratio ..... - 26 -

2.2.1 Immobilization results using homogenized PU ..... - 29 -

2.2.2 Immobilization results using different buffers ..... - 31 -

2.3 Detecting immobilization of HRP using SDS-PAGE - 32 -
2.3.1 Immobilization of HRP onto PU from conventional heating - 32 -
2.3.2 Immobilization of HRP onto PU synthesized in the microwave - 33 -
2.3.3 Immobilization of HRP using PU synthesized from different mole ratios - 35 -
2.3.4 Immobilization results using stored PU - 36 -
2.4 Enzymatic assay results - 37 -
2.4.1 Enzymatic assay using Pyrogallol - 37 -
2.4.2 Enzymatic assay using amplex red - 38 -
2.4.3 Enzymatic assay using ABTS - 39 -
2.4.4 Reuse of immobilized HRP - 40 -
2.5 Particle size - 43 -
2.6 Cultivation of ChiA - 46 -
3. Conclusion - 47 -
Future work - 47 -
4. Experimental - 48 -
4.1 General information - 48 -
4.2 General synthesis of polyurethane - 48 -
4.2.1 Synthesis using conventional heating - 48 -
4.2.2 Synthesis using microwave - 50 -
4.3 Synthesis of silver nanoparticles - 52 -
4.4 Synthesis of gold nanoparticles - 53 -
4.5 Formation of PU-nanoparticles conjugates - 53 -
4.6 Immobilization method - 54 -
4.6.1 Immobilization of HRP without stirring - 54 -
4.6.2 Immobilization of HRP with stirring - 54 -
4.7 Testing Immobilization - 55 -
4.7.1 Using UV - 55 -
4.7.2 Using SDS-PAGE - 56 -
4.7.3 Measuring concentration using Nanodrop - 57 -
4.8 Enzymatic assay of HRP - 57 -
4.8.1 Solutions used for enzymatic assay - 57 -
4.8.2 Enzymatic assay using Pyrogallol - 57 -
4.8.3 Enzymatic assay using Amplex Red - 58 -

4.8.4 Enzymatic assay using ABTS ..... - 59 -
4.8.5 Reuse of immobilized HRP ..... - 59 -
4.9 Cultivation of His10-ChiA-D313N-F296W ..... - 60 -
4.9.1 Solutions used for cultivation of His10-ChiA-D313N-F296W ..... - 60 -
4.9.2 Method for cultivation of His10-ChiA-D313N-F396W ..... - 62 -
5. References ..... - 64 -
Appendix A – Immobilization results using PU with 1:2 mole ratio i
Appendix B – Immobilization results using PU synthesized from new method iv
Appendix C – Immobilization results using homogenized PU (1:5 mole ratio) vi
Appendix D – Immobilization results using different buffers vii
Appendix E – Detecting immobilization using SDS-PAGE ix
Appendix F – Saturation of PU from conventional heating xi
Appendix G – Saturation of PU from microwave heating xii
Appendix H – Immobilization results using PU with different mole ratios xiii
Appendix I – Immobilization results using stored PU xv
Appendix J – Particle size xvi
Appendix K – NMR and IR spectra xix

1. Introduction

1.1 Enzymes

Enzymes catalyze biochemical reactions in cells and are therefore important for living organisms. Enzymes act as catalysts and as such they do not change the equilibrium of a reaction, but rather increase the rate of a reaction by lowering the activation energy. Enzymes are both reaction- and substrate-specific; meaning that enzymes catalyze only one single, or few closely related chemical reactions. Most enzymes are proteins, but they can also be RNA-molecules, known as ribozymes[1-2].

The structure and three-dimensional arrangement determines the properties of the enzyme[1-2]. The structures are described by the primary, secondary, tertiary, and quaternary structure (Figure 1). In short, the primary structure describes the sequence of amino acids in the polypeptide chain. The secondary structure describes the different three-dimensional structure that occurs due to different patterns of hydrogen bonds between amino acids in the polypeptide chain. The tertiary structure describes the three-dimensional folding of the entire protein. The quaternary structure describes the arrangement and number of multiple polypeptide chains in a protein, the presence of cofactors is also described by the quaternary structure[1, 3].

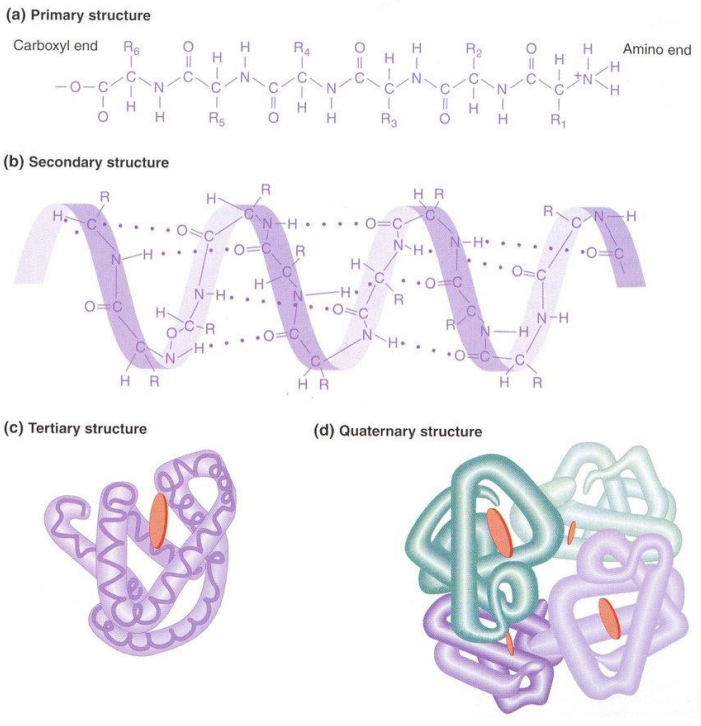


Figure 1: Visualize the primary, secondary, tertiary, and quaternary structure of proteins[4]

Cofactors are organic molecules, metal ions, or metal organic complexes attached to an enzyme; these cofactors are part of determining the property of enzymes[5]. Bonds between enzyme and cofactors can either be permanent or temporay[1].

Metalloenzymes are enzymes that contain metal ions as cofactors. These metal ions contribute to the catalytical reactions by donation or attraction of electrons[6]. Hemoproteins are one type of metalloenzyme that has both an organic and inorganic component, where iron coordinates a porphyrin ring (see Figure 2). This heme-group is essential for the activity, and is called the active site[7].

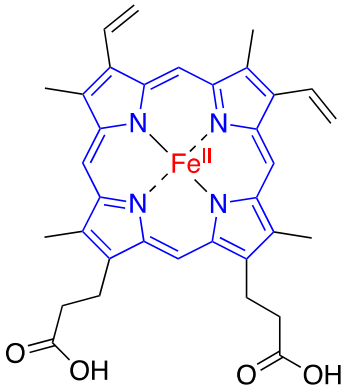


Figure 2: Structure of heme B. Porphyrin part shown in blue, iron in red.

The active site is the section of the enzyme where the catalytic reaction occurs. A substrate binds to the active site, and an enzyme-substrate complex is formed (ES). The catalytic reaction occurs, the product is released, and the enzyme is back in its natural form and can catalyze another reaction (Eq 1.1)[6].

Eq 1.1 $$E + S \rightarrow ES - intermediate \rightarrow P + E$$

The active site is typically only 10-20 % of the total volume of an enzyme. Amino acids that are not part of the active site determine and maintain the tertiary structure of the enzyme. Both the structure of the active site and the tertiary structure of the enzyme makes the enzyme substrate and reaction specific[7].

Enzymes are classified by the reaction they catalyze. This classification system was made in 1964, and enzymes are divided into six main groups[6].

1. Oxidoreductases: catalyze oxidation/reduction reactions[8].
2. Transferases: catalyze the transfer of a (a) specific functional group(s) from one molecule to another molecule[6].

3. Hydrolases: use water to divide molecules into smaller molecules by breaking chemical bonds[6].

4. Lyases: catalyze addition and elimination reactions[9].

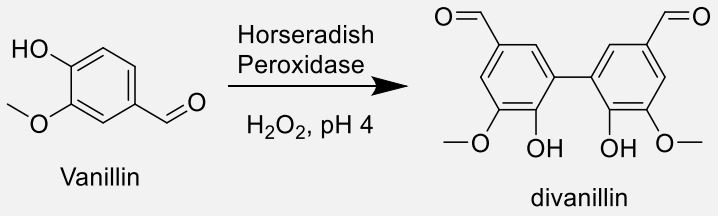
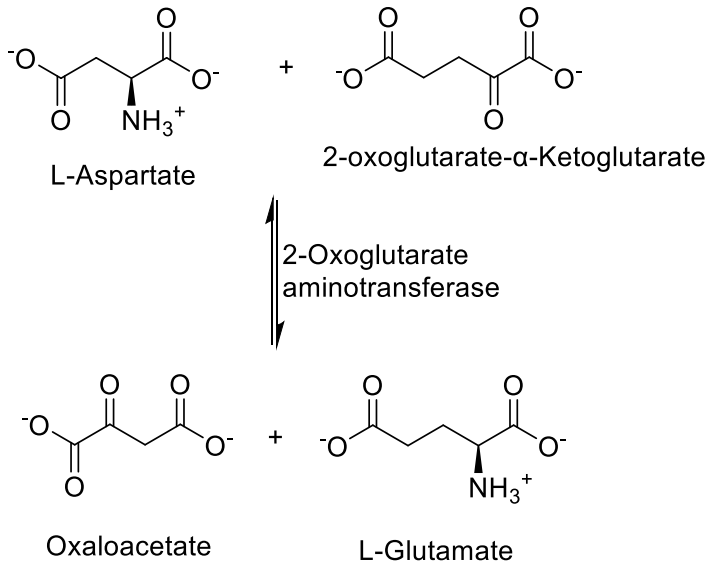
5. Isomerases: catalyze the conversion of one isomer to another[6].

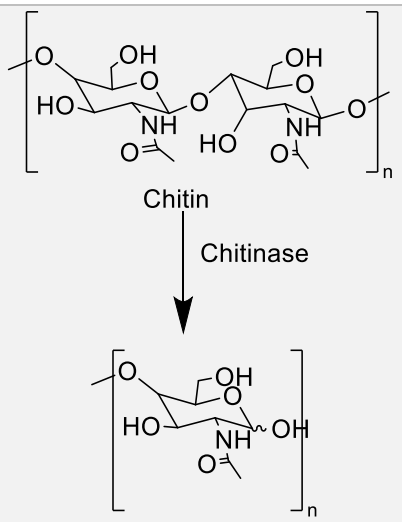
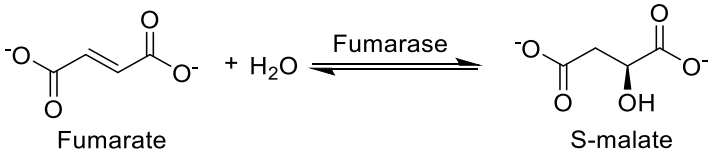
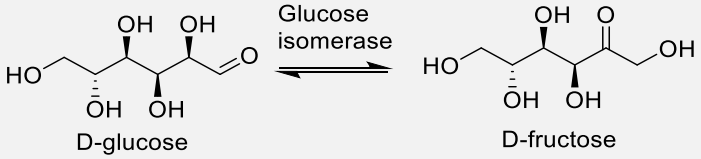
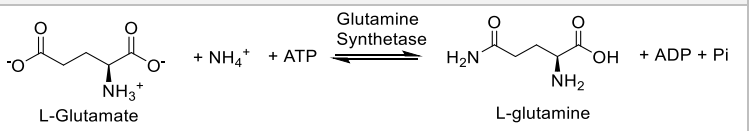
6. Ligases: catalyze the formation of a new chemical bond between two large molecules[6].

These six main groups are further divided into subclasses, and every enzyme has a four-number code: EC A.B.C.D. Where A is the number of the main group, B indicate the first subclass, C tells which sub-subclass the enzyme belongs to, and D indicates which serial number the enzymes has in the sub-subclass. For example, horseradish peroxidase (HRP) has the code EC 1.11.1.7[1]. This indicate that HRP is in the first main group, oxidoreductases. In subclass 11 which is described as an enzyme that act with peroxide as acceptor, 1 is the sub-subclass and is a peroxidase, and lastly HRP is number 7 in the peroxidases sub-subclass.

An example of a catalytic reactions for each main group are described in Table 1.

Table 1: Examples of enzymes and a catalytic reaction in the different main groups.

Main group	**Example enzyme**	**Catalytic reaction**
Oxidoreductases	Horseradish peroxidase	
Transferases	2-oxoglutarate aminotransferase[6]	

Hydrolases	Chitinase[10]	
Lysases	Fumarase[9]	
Isomerases	Glucose isomerase[9]	
Ligases	Glutamine synthetase[6]	

Peroxidases and hydrolases will be used in this study and their structure and function will be described in more detail in the next section.

1.1.1. Peroxidase

Peroxidases are a group of oxidoreductases and can be divided into three groups: animal peroxidases, plant peroxidase, and catalases. Research has shown that plant peroxidases are the best candidate for the industrial use of peroxidase. Plant peroxidases are divided into three classes: Class 1 prokaryotic, class 2 fungal, and class 3 plant peroxidases[11-12].

The active site for peroxidases is a heme group, which is a planar coordination complex with an iron ion firmly structured in the middle of a porphyrin ring (Figure 3). Thus peroxidases are metalloenzymes, and the iron ion are essential for the catalytic activity[13-14].

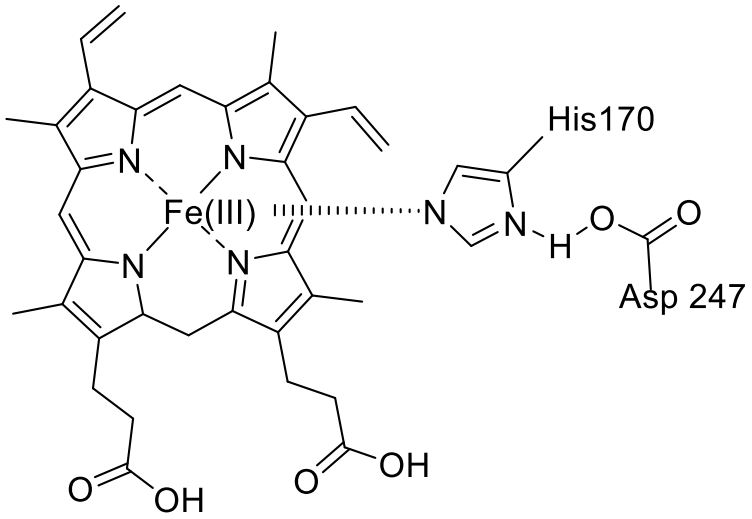


Figure 3: Heme group of peroxidases[15]

One plant peroxidase that has been wildly studied for more than a century is the horseradish peroxidase, and will be further described[15].

1.1.2 Horseradish peroxidase

Horseradish peroxidase (HRP) are found in the roots of horseradish *(Amoracia Rusticana*). HRP is a single chain polypeptide, containing a heme group, two calcium ions, and about 18-22 % carbohydrates. Figure 4 shows the three-dimensional structure of an HRP enzyme; the heme group is in the center, with the iron atom as a red sphere, and the two black spheres are calcium atoms[15].

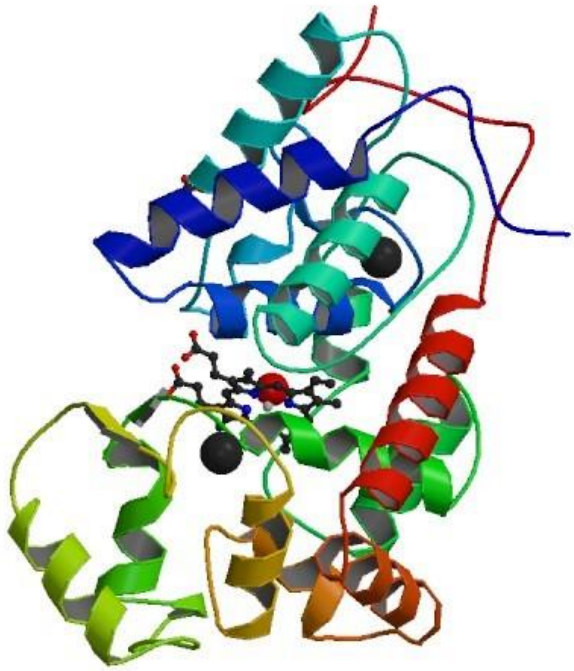
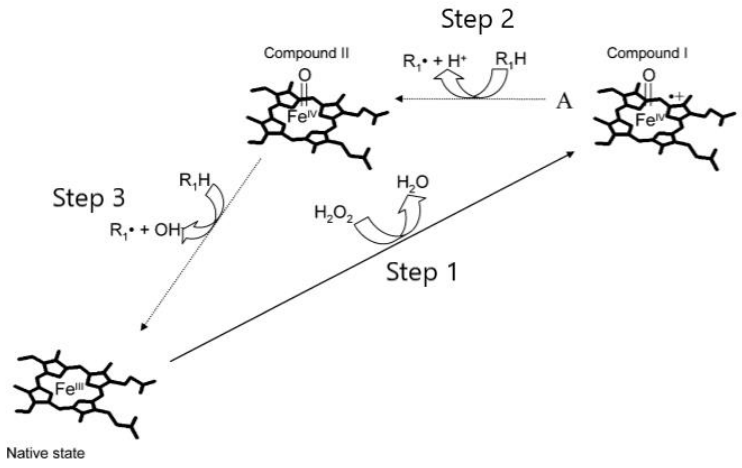


Figure 4: The three dimensional structure of Horseradish peroxidase[16]

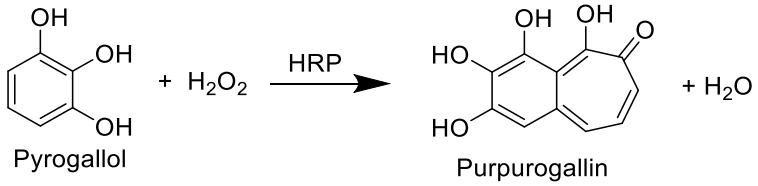
Both the calcium atoms and the carbohydrates are essential for the structure and the catalytic activity of HRP. The overall catalytic reaction by the heme group in peroxidase occurs in three steps shown in Scheme 1[11-12].



Scheme 1: The three steps of the catalytic reaction between a peroxidase and substrate[12].

HRP catalyze the oxidation of pyrogallol to purpurogallin (Scheme 2). This catalytical reaction with HRP occurs through the three steps shown in scheme 1. In the first step, the hydrogen peroxide (H_2O_2) reacts with iron in the active site and produce compound 1 and water. In step 2, compound 1 oxidizes pyrogallol and compound 2 and purpurogallin is

formed. In the third step compound 2 is reduced, and purpurogallin is formed, while Fe^{3+} is reformed[12, 17].



Scheme 2: Oxidation of Pyrogallol to purpurogallin with horseradish peroxidase and hydrogen peroxide[17]

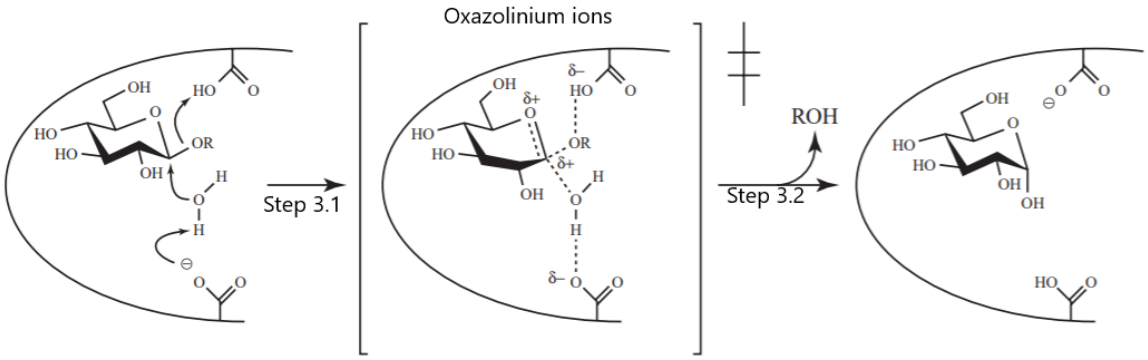
1.1.3 Hydrolases

Hydrolases are enzymes that catalyze the cleavage of a chemical bond by addition of water[9].

As of today, there are 13 known subgroups of hydrolases. These subgroups are categorized by the types of compounds and functional groups they hydrolyze e.g. esterases, catalyze the hydrolysis of ester bonds. Glycosidases are another subgroup, that catalyze the hydrolysis of glycoside bonds and will be focused on in this thesis[9].

Hydrolysis reaction can occur through two different mechanism: inversion or retention[18-19].

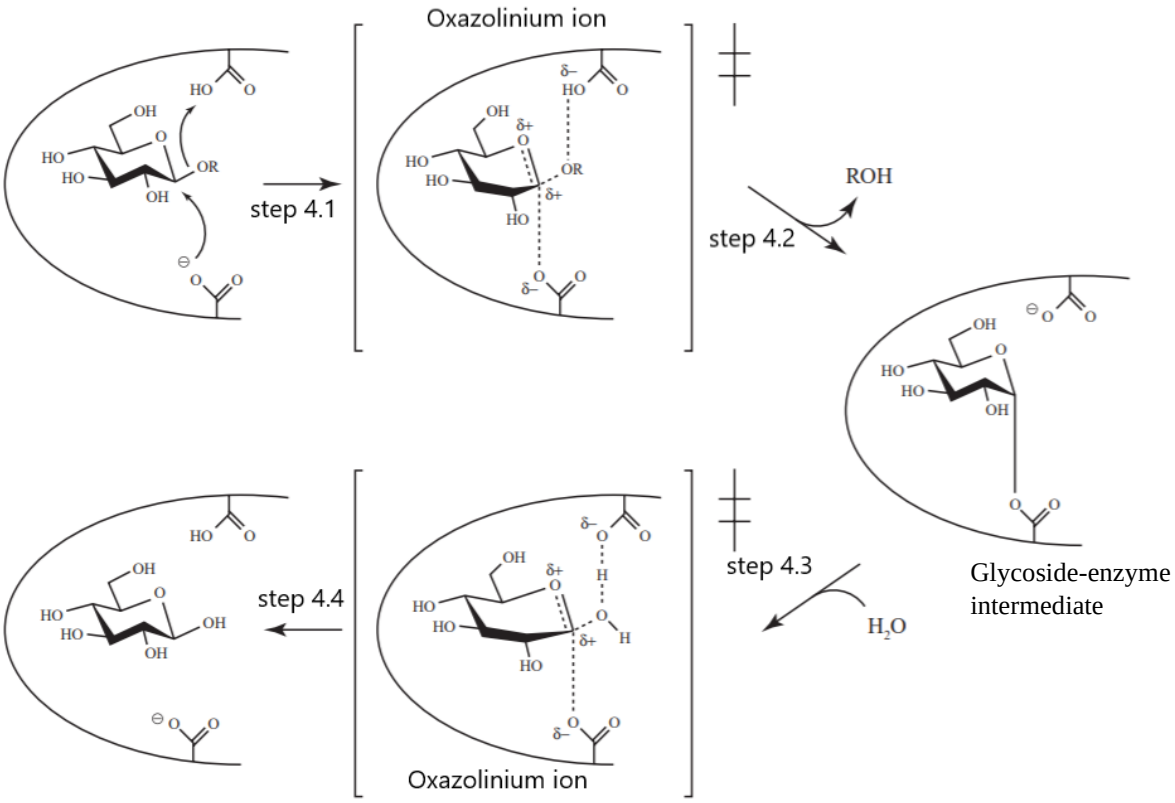
Inversion happens through a direct displacement; this is a two steps reaction where an oxazolinium ion is formed (Scheme 3). The catalytic reaction occurs with the help of two carboxylic acids in the enzymes active site. Where one acid provides general-acid-catalytic assistance for the cleavage of the glycosidic bond and the second provides a general base assistance for the attack of water[18-19].



Scheme 3: Inversion, direct displacement[18]

Retention happens through a double displacement mechanism; through four steps the R group is replaced with a hydrogen atom (Scheme 4). In step 4.1 an oxazolinium ions is formed, and after cleaving of ROH a covalent glycoside-enzyme intermediate is formed. This intermediate is then hydrolyzed through a second oxazolinium ion. The two carboxylic acids from the

enzymes active site has different roles than in the inversion mechanism. One act as a nucleophile by attacking the anomeric center form the covalent glycoside-enzyme intermediate. The second carboxyl acid act as both acid- and base- catalysts, first it protonate the glycosidic oxygen (step 4.2) and then deprotonate the water molecule (step 4.4)[18].



Scheme 4: Retention mechanisms through a double displacement[18].

Some glycosidases catalyze transglycosylation (TG) reactions. In a TG reaction new glycoside bonds are formed between carbohydrates.

TG reaction occurs through a double-displacement mechanism. For a TG reaction to occur the active site in the enzyme must disfavor the positioning of water and/or favor the binding of a saccharide[18, 20].

1.1.4 Chitinases from *Serratia marcescens*

Family 18 and 19 of glycoside hydrolases are called chitinases, the difference between family 18 and 19 is the origin of the chitinases. Family 18 consist of chitinases from various prokaryotic and eukaryotic organisms, and family 19 chitinases are found in higher plants and in gram positive bacteria[10].

Chitin is found in the exoskeleton of insects and in the cell walls of different fungi, enzymes that plays an essential role in the normal cell cycle of organism containing chitin are

chitinases. Chitinases catalyze the decomposition of chitin (Figure 5), which is a 1,4-β-linked polymer of N-acetyl-β-D-glucosamine[10].

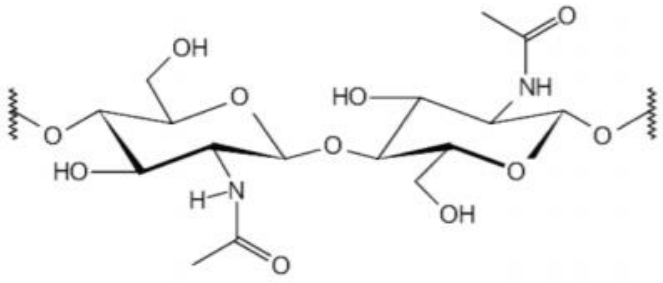
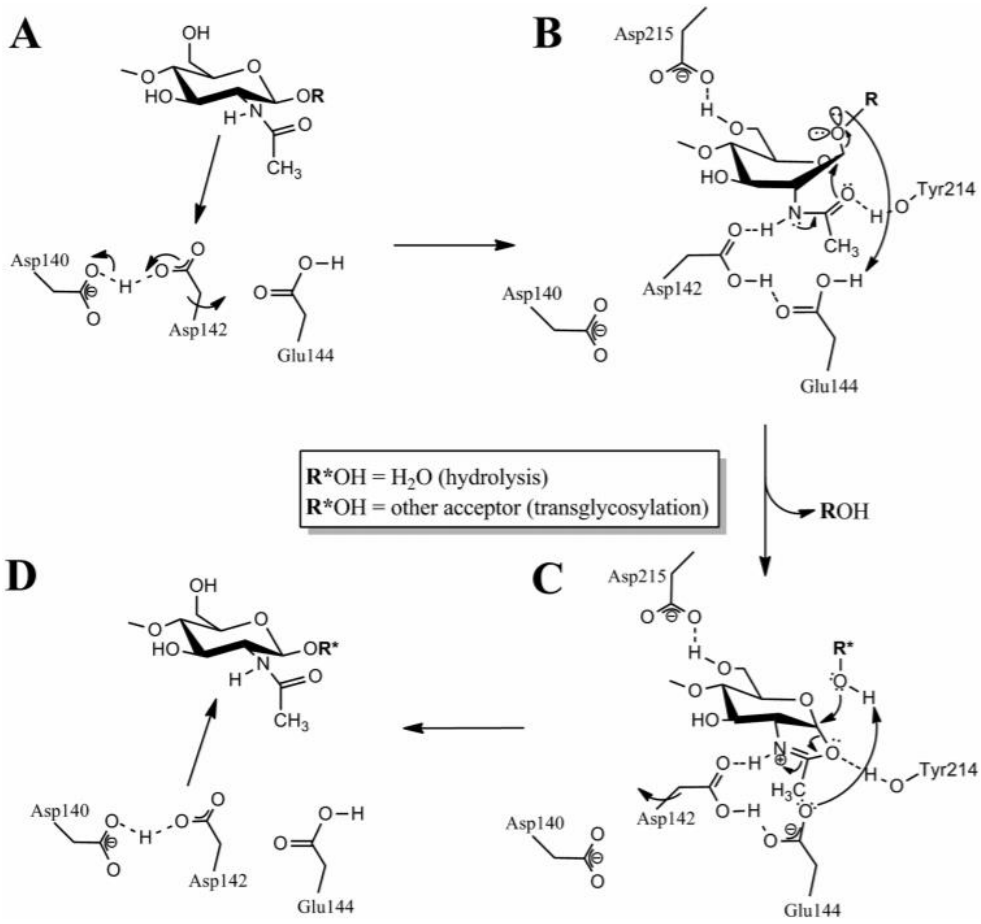


Figure 5: The structure of chitin[21]

Serratia marcescens is a gram-negative bacterium that secreted chitinase[22]. Three wildly studied chitinases found in *S. marcescens* are ChiA, ChiB and ChiC[10]. ChiA, ChiB, and ChiC are some of the glycosidases that can both catalyze a hydrolysis and TG reaction. Scheme 5 shows the mechanism of these catalytic reaction with ChiB as the enzyme. ChiB does not form a glycoside-enzyme intermediate (as shown in Scheme 4), but a oxazolinium-ion intermediate is formed (C in Scheme 5)[20, 23].

The active site of chitinases are a deep substrate binding groove, with aromatic surfaces. Studies have shown that these aromatic residues in the active site is essential for the TG activity of the enzyme[24-25]. Mutations to disfavor the correct position of water or favor the binding of carbohydrates has been attempted to increase the TG activity of the chitinases[20, 23, 26].



Scheme 5: shows the hydrolysis and TG mechanism of ChiB[20]

1.1.5 Lytic Polysaccharide (mono)oxygenases

For a long time, it was a mystery how chitinases could degrade crystalline chitin or cellulose on itself[27]. In 2010 Vaaje-Kolstad *et al.* identified an enzyme that acts on crystalline chitin, this enzyme also promotes further degradation by chitinases[28]. This enzyme was classified by CAZy, a database that describe and classify the families of enzymes that are active on carbohydrates, in the family 33 of carbohydrate-binding modules (CBM33)[29]. Vaaje-Kolstad *et.al* showed that a chitin-binding protein, CBP21 (from *Serratia marcescens*) catalyze the cleavage of crystalline chitin and making it accessible for hydrolysis by chitinases[28]. Similar enzymes that are active on cellulose was shortly after identified[30].

Today these enzymes are known as lytic polysaccharide monooxygenase (LPMOs), and the structure and mechanism has been widely studied. LPMOs are today classified by CAZy as auxiliary activities (AA), which cover redox enzymes that work in conjugation with carbohydrate-active enzymes (CAZymes)[31]. LPMOs are divided into family 9-11 and 13-16 of the AA. LPMOs are metalloenzyme, and the activity are dependent on copper (Cu(II)),

which is coordinated by an N-methylated-N-terminal histidine, known as histidine brace (see Figure 6)[32-33].

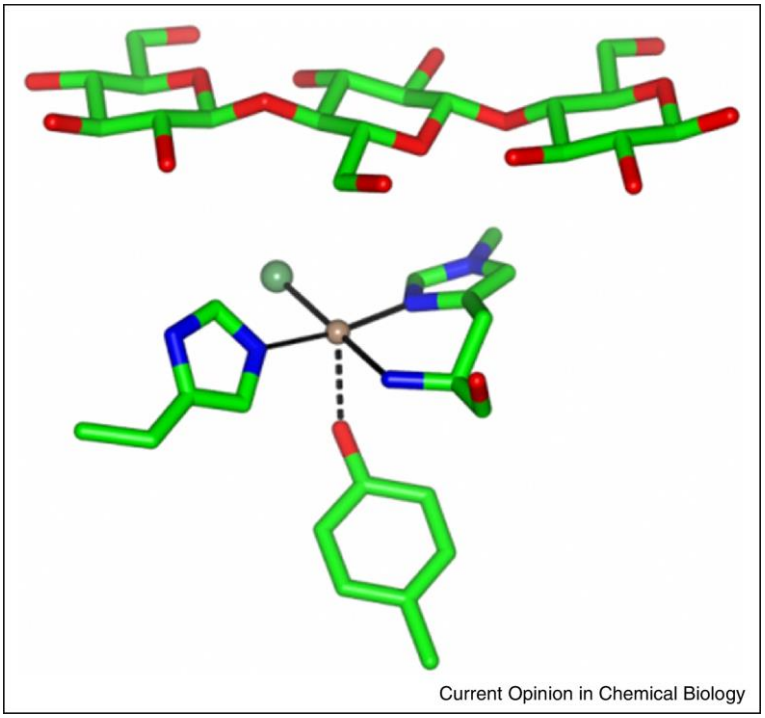


Figure 6: Histidine brace with tyrosine in the active site from ref. 31. Cupper is shown as a grey sphere, the green sphere is a chloride ligand/substrate in the equatorial position[33].

The mechanism of LPMOs was first thought to be a monooxygenase reaction, which is a reaction that is dependent on molecular oxygen (O_2) and a reductant that delivers two electrons. Where LPMOs with the presence of O_2 break glycosidic bonds and form oligosaccharides (Eq 1.2), these oligosaccharides are further degraded by glycoside hydrolases[28].

Eq 1.2 $$R - H + O_2 + 2e^- + 2H^+ \rightarrow R - OH + H_2O$$

However, in a study by Bissaro *et al*. in 2017, it was proposed that LPMOs might be dependent on hydrogen peroxide (H_2O_2) as an oxidant instead of O_2 (Eq 1.3). The same study also showed that a high amount of H_2O_2 inactivate the catalytic reaction, it is therefore important to control the levels of H_2O_2[34].

Eq 1.3 $$R - H + H_2O_2 \rightarrow R - OH + H_2O$$

New studies have shown that it is possible that LPMO reactions can also be driven by light by using photocatalysts or photosynthetic pigments[35-37]. Bissaro *et al*. recently discovered that light driven LPMOs produce H_2O_2, which is necessary for the activity. They also reported that

LPMOs might catalyze a reaction with only visible light - no other reducing power is necessary. But this mechanism is still not known[35].

Because of these new studies, classification and naming of LPMOs are difficult. LPMO might not be a suitable name since the enzymes might not be dependent on O_2, as the name indicate.

1.2 Immobilization of enzymes

Enzymes are of industrial interest because they speed up and control reactions, and they have been used in food processing long before recorded history. In the production of barley malt the enzyme malt diastase was first identified by Payen & Pensozok in 1833[38]. Today, enzymes are vastly integrated in modern food processes, for example in food fermentation such as in production of cheese, bread, beer, and wine[39]. A few problems have been reported when free enzymes are used in industry. Reusability and stability are some of these problems.

Immobilization of enzymes has shown to fix problems with reusability and stability[40]. Immobilization of enzymes means that enzymes are localized in one spot during the catalytic reaction, which leads to optimized operational performance in industrial processes[41].

Nelson and Griffin first reported immobilization in 1916[42]. But it was not until the 1960s that immobilization of enzymes were studied in more detail[14].

Several positive characteristics has been identified by immobilizing enzymes. Some examples are better stability against harsh reaction conditions, such as resistance towards higher pH and temperature changes[13, 40], better product and enzyme separation[40, 43] and easier enzyme reusability[44]. Some negative effects have also been shown, such as, decrease on the catalytic activity and the high price of immobilization[40].

Different types of interactions between enzymes and support material depend on the surface properties of both. Polar groups on the amino acids, sugar groups, or apolar surface areas are properties that influence the surface properties of the enzyme[44]. When enzymes are immobilized, the interaction between support material and enzyme can happen through one-point or multipoint interaction. Often the number of interactions between enzyme and support material increases after time. In a multipoint immobilization system, the enzyme is attached to the support material in more than one point, and this may enhance activity, reusability, and stability[40].

The difference in surface properties of enzymes and support material determines how they interact with each other. These different interactions can be divided into different methods of

immobilization. One way of characterizing is by dividing it into chemical immobilization and physical immobilization (Figure 7).

Chemical immobilization can again be divided into two different methods, covalent and cross-linking. In covalent immobilization, the enzyme forms a covalent bond with support material. Cross-linking occurs when enzymes form covalent bonds between each other[40].

Physical immobilization is divided into three different methods: encapsulation, entrapment, and adsorption. Encapsulation is when enzymes are enclosed inside a semipermeable membrane. Entrapment occurs when enzymes physically get trapped inside a porous matrix, covalent and non-covalent bindings between matrix and enzymes occurs. Adsorption happens with physical surface interactions between the enzyme and the support matrix.

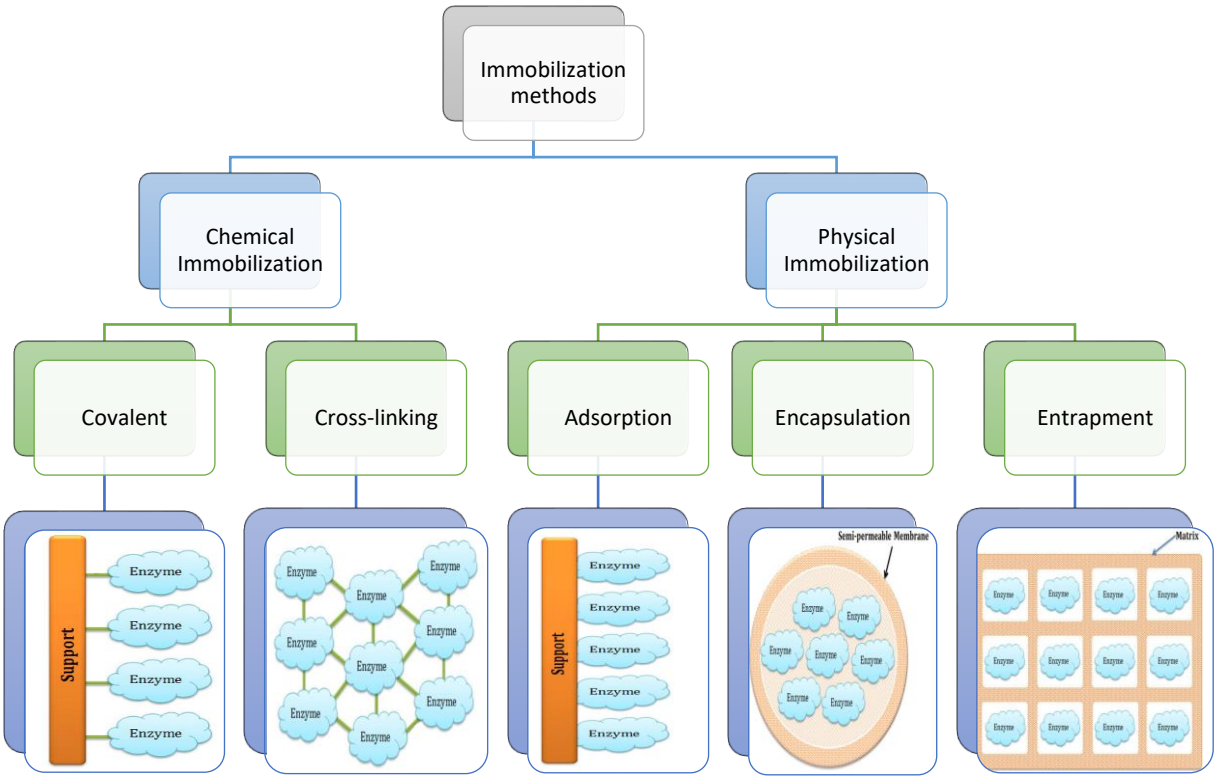


Figure 7: Different methods for immobilization[45]

In this thesis, immobilization via covalent binding and adsorption are being tested. Therefore, these methods will be described in more detail.

1.2.2 Covalent immobilization

The covalent bond often occurs between nucleophilic groups on the enzyme and reactive chemical groups on the support. Amino groups, carboxyl groups, phenol ring, thiol group, and

hydroxyl groups are some functional groups that are important for enzymes to form covalent bonds with support material. The advantages of covalent bonding include no leakage of the enzyme because of the strong linkage to support material. A simple immobilization method because of the varieties of different functional groups that can take part in the binding and a covalently immobilized enzyme can be used in any medium. There are also negative sides to the covalent bonding, and one of these disadvantages is the chemical modification of the enzyme. This can cause a change in the conformation of the enzyme and can also lead to a change in enzyme activity[44]. Covalent immobilization often occurs through multipoint immobilization[40].

1.2.3 Adsorption

Adsorption is the oldest method used for immobilization; this is the method Nelson and Griffin used back in 1916[42]. During immobilization via adsorption, weak bonds are involved in stabilizing the enzyme on the support matrix. These bonds are mainly hydrogen bonds, ionic interactions, and Van der Waals forces. No permanent bonds are formed with this immobilization method. The advantage of adsorption is that it is an easy method, and not many activation steps are involved. It is also a cheap method for immobilization, there are no requirements for reagents. But the most common disadvantage is the desorption of enzyme from the carrier[42, 45].

The support material can be made to fit the surface properties of the enzymes. We can divide the different materials into three big groups: Natural polymers, synthetic polymers, and inorganic materials.

1.3 Chemical background

1.3.1 Polymer chemistry

Polymers are also called macromolecules, which are large molecules built by many repeated subunits, monomers. Polymers can either be natural or synthetic, and both play an essential role in everyday life. Carbohydrates, proteins, DNA, and RNA are some of the natural polymers (also called biopolymers) that are essential for life. Synthetic polymers are present in everyday items such as paints, clothing, toys, vehicles, and tires[46-47].

There are different ways to classify polymers; the four main ways to classify are shown in Figure 8.

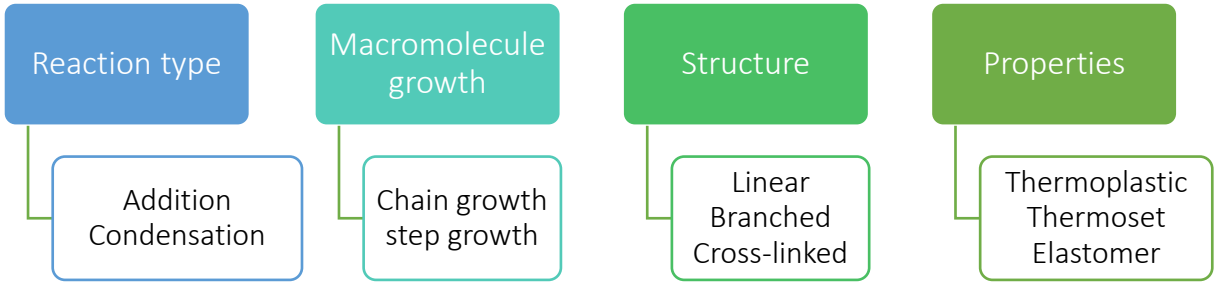


Figure 8: Show the different ways to classify polymers[47]

The two different reaction types to form polymers are addition reaction and condensation reactions. In an addition reaction, two monomers react with each other, and no atoms are lost. Eq. 1.4 shows an addition reaction, A and B are monomers[47].

Eq. 1.4 $$A + B \rightarrow A - B$$

Condensation is the second reaction type and is the formation of a covalent bond between monomers, with the loss of a small molecule. A simplified example is shown in equation 1.5. X-Y is a small molecule, such as water or alcohol[47].

Eq. 1.5 $$A - X + Y - B \rightarrow A - B + X - Y$$

As shown in figure 8, the two different ways of synthesizing polymers are chain growth and step growth. Chain-growth polymers are synthesized by the repetitive addition of a monomer unit to the same end of the polymer. Step-growth polymers are often synthesized through two steps. First the monomers form an oligomer, which is a mixture of compounds of intermediate molecular weight, then secondly the oligomers react with each other and form a polymer[47].

Synthetic polymers are made up of one or more different types of monomers. The selection and quantity of monomers will determine the properties and structure of the polymer. Selection of monomers is the biggest advantage of synthesizing polymers. A copolymer is formed from two or more different monomers, in this case the monomers are usually not present in equal amount in the polymer.

Polymers synthesized from one monomer is called a homopolymers. The composition of the monomer in a homopolymer can vary and the overall structure and properties of the polymer are different. Polymer can be synthesized from toxic monomers, and toxic chemicals can therefore, be released during synthesis, use or disposal of the polymer[48].

Different functional groups in polymers, like carbonyl, hydroxyl and amine-groups plays a huge role in immobilization. One type of polymer that has favorable functional groups for immobilization is polyurethane.

1.3.2 Polyurethane

Polyurethanes (PU) are synthetic polymers that was first synthesized in the 1930's by Otto Bayer[49]. It was developed as a replacement for rubber, but also used as coating to protect wood and metal. Today polyurethanes are found all around us; in cars, chairs, clothes, in pacemakers, and in the insulation in walls[50-52].

Several different structures of PU exist, but the common backbone for all PU is the urethane linkage, also called carbamate (Figure 9)[49].

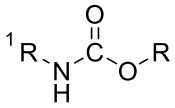


Figure 9: Generic urethane structure.

Polyurethanes are copolymers, they are synthesized by two monomers: Isocyanates and diols/polyols, with the presence of suitable catalysts and additives[53]. A variety of diol/polyol and isocyanate exists, and these can be used to produce polyurethanes with different characteristics. These types of PUs include foam (flexible and rigid), coatings and elastomers, thermoplastic PU, and microspheres[53-55].

The synthesizing method of polyurethane is step-growth polymerization[56]. Linear polyurethanes are synthesized when diols and diisocyanates are used as monomers. Branched,

or networked, polyurethane is synthesized when isocyanates reacts with polyfunctional alcohols[53]. Isocyanates and diols/polyols forms different segments of PUs, isocyanates forms the hard segments whereas diols/polyols forms soft segments[51].

It has been shown that PUs is applicable for immobilization of enzymes, both PU foams and microspheres (10-100 µm) have been used[55]. The free isocyanate groups in PU can bind enzymes through a covalent bond. While synthesizing an excess amount of isocyanates are used, this is to ensure that polyurethanes are end terminated with isocyanate groups[50].

By covering PU microspheres with nanoparticles of gold or silver, immobilization of enzymes via adsorption is also possible[57-58]. Nanoparticles have a large surface area for binding of enzymes, which can lead to increased immobilization yield[59].

1.4 Methods

1.4.1 Microwave assisted organic synthesis

Microwave irradiation are electromagnetic waves with the frequency range between 0,3 – 300 GHz, this corresponds to wavelengths between 1 mm – 1 m[60-61]. The use of microwave irradiation in synthetic chemistry was first reported 1986 by Gedye *et al.* and Majestic *et al*[62-63]. Microwave-assisted organic synthesis (MAOS) have many positive effects on reactions, such as reduced reaction times, increased product yields and reducing of unwanted side reactions causing enhanced product purity.

Open vessel reaction will be limited by the boiling point of the solvents both in a microwave and during a conventional heating process. In MAOS closed vessels are normally used, these reactions will occur under high pressure and solvents can be heated above their boiling points[60].

A conventionally heating process is time consuming, because the sample in contact with the vessel wall are heated first making a heat gradtient. Whereas in microwave irradiation the entire sample is heated simultaneously.

Dielectric heating effect describe the mechanisms behind the rapid heating of samples. The two main mechanisms behind this effect are dipolar polarization and ionic conduction. Molecules that can form a dipole moment will align itself according to the electromagnetic field. The electromagnetic field will alternate between positive and negative charge, and the dipoles will oscillate with the field. In all commercially available microwave reactors for MAOS a frequency of 2.45 GHz is used, this frequency gives the dipoles time to align in the electromagnetic field. The dipoles will release energy in the form of heat through molecular friction and dielectric loss[60-61].

Ions will also oscillate with the electromagnetic field and release energy in the form of heat. Figure 10 show how ions and dipoles act in an electric field.

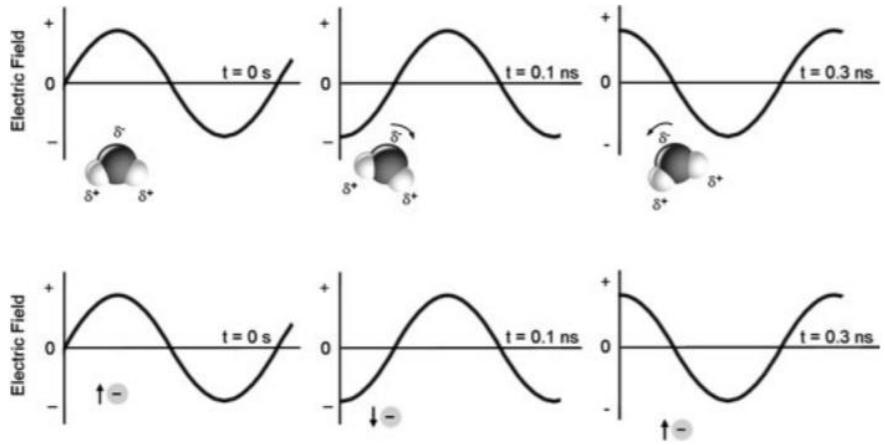


Figure 10: Dipoles and ions in an electric field[60]

The dielectric properties are dependent on the solvents/reagents ability to absorb and convert microwave energy to heat. Loss tangent, tan δ (Eq. 1.6) determine the ability of a solvent to convert electromagnetic energy to heat at a given frequency and temperature[60].

Eq. 1.6 $\tan \delta = \varepsilon'' / \varepsilon'$

ε' is the dielectric constant and it describes the polarizability in the electric field, which is the amount of energy the solvent can absorb from the electromagnetic field.

ε'' is the dielectric loss and describes how much of the electromagnetic radiation is converted into heat.

Higher tan δ results in a more efficient absorption and therefore rapid heating.

Specific microwave effects only occur in a microwave assisted heating process. One of these effects are super-heating, which describe that solvents boiling point is higher than the normal boiling point under atmospheric pressure. Another specific microwave effect is hot spots, which occur when some areas of a sample contain more energy than others, the temperature in these areas will for a moment be higher. Selective heating is also considered a specific microwave effect, which describes that only the component that couples with microwaves will be heated[64].

Changes to the time and temperature by using Arrhenius equation, can optimize a synthesis in a microwave. Figure 11 summarize a time and temperature chart, this can be used to transfer reaction settings from conventional heating to MAOS[60].

T (°C)	Times – change in field color represents change in unit (h/min/s)									
20	1	2	4	6	8	12	24	48	96	172
30	30	1	2	3	4	6	12	24	48	86
40	15	30	1	1.5	2	3	6	12	24	43
50	8	15	30	45	1	1.5	3	6	12	22
60	4	8	15	23	30	45	1.5	3	6	11
70	2	4	8	11	15	23	45	1.5	3	5
80	56	2	4	**6**	8	11	23	45	1.5	3
90	28	56	2	3	4	6	11	23	45	1
100	14	28	56	1	2	3	6	11	23	40
110	7	14	28	42	56	1	3	6	11	20
120	4	7	14	21	28	42	1	3	6	10
130	2	4	7	11	14	21	42	1	3	5
140	53	2	4	**5**	7	11	21	42	1	3
150	26	53	2	3	4	5	11	21	42	1
160	13	26	53	1	2	3	5	11	21	38
170	7	13	26	40	53	1	3	5	11	19
180	3	7	13	20	26	40	1	3	5	9
190	2	3	7	10	13	20	40	1	3	5
200	1	2	3	5	7	10	20	40	1	2
210		1	2	2	3	5	10	20	40	1
220			1	1	2	2	5	10	20	35
230					1	1	2	5	10	18
240						1	1	2	5	9
250								1	2	4

Figure 11: Time and temperature chart based on Arrhenius equation, direct from ref. 56.

Microwave-assisted polymer chemistry has shown advantages when synthesizing polymers, including improved product quality and properties, greater flexibility and better speed and energy saving reactions[65].

1.4.2 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) use sodium dodecyl sulfate (SDS) to separate proteins by molecular masses between 5 and 250 kDa. SDS is a negatively loaded detergent, which binds to hydrophobic parts of the enzyme and break the three-dimensional structure of the enzyme, to give it a negative charge. When an electric field is added, the proteins will migrate towards the anode (positively charged). Larger proteins migrate slower due to resistance in the gel, and the enzymes are separated by mass[66]. An SDS-PAGE is visualized in Figure 12.

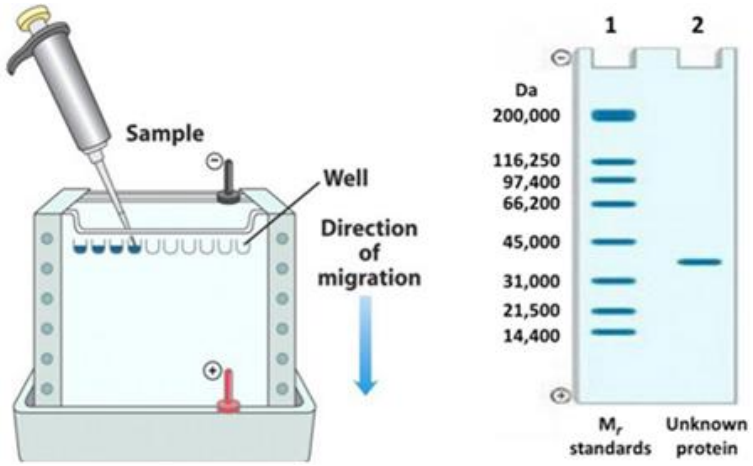


Figure 12: Visualization of an SDS-PAGE[67]

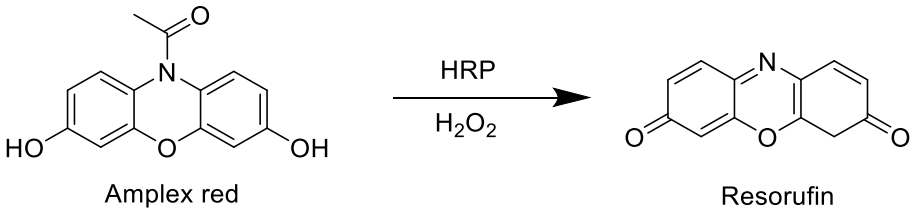
1.4.3 Enzymatic Assays

Enzymatic assay using pyrogallol

Pyrogallol is used to test the activity of HRP, with the presence of H_2O_2 purpurgallin is formed (Scheme 2, page 7). The amount of purpurgallin can be detected by measuring the absorption at 420 nm[68].

Enzymatic assay using Amplex® Red

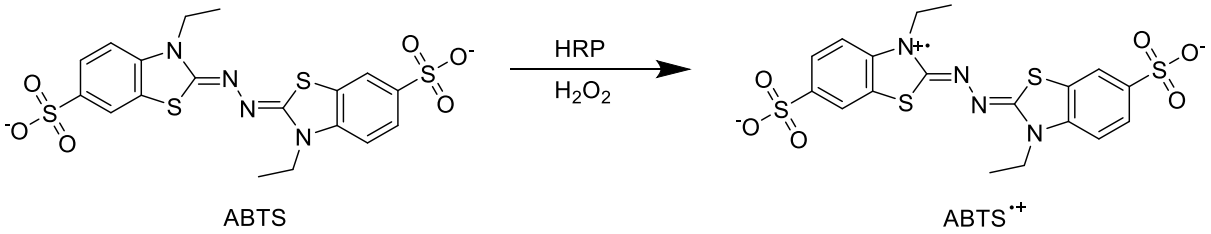
Amplex® Red is a substrate for HRP and can be used to test the activity. With the presence of H_2O_2, HRP catalyze the oxidation of Amplex® Red to resorufin (Scheme 6). Which is a fluorescent molecule with pink color, this color can be detected by the absorption at 580 nm[69].



Scheme 6: reaction of Amplex red to Resorufin

Enzymatic Assay using ABTS

The activity of HRP is also detected using ABTS. HRP with H_2O_2 catalyze the oxidation of ABTS (Scheme 7), oxidized ABTS is a dark blue/black color and can be detected by measuring absorption at 405 nm[70].



Scheme 7: Reaction of the oxidation of ABTS

Bradford Assay

Bradford assay is used for quantification of protein content in samples. It uses Coomassie Brilliant Blue, which makes complexes with amino and carboxyl groups in proteins. When the dye binds to protein a shift in color occurs, and this can be detected by measuring the absorption at 595 nm[71-72].

1.4.4 Affinity chromatography

Affinity chromatography is used to purify enzymes , the wanted enzymes is mutated to have histidine residues (see Figure 13). These residues have affinity to bind to metal ions (Ni^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}) on the column. When extracts are added to the column, enzymes that are mutated with histidine residues will contain a higher binding affinity to metal ions on the column. A buffer with a low imidazole concentration, will wash away unwanted proteins that

are weakly bonded to the column. To elute the wanted enzyme, a buffer with a higher concentration of imidazole is used[73].

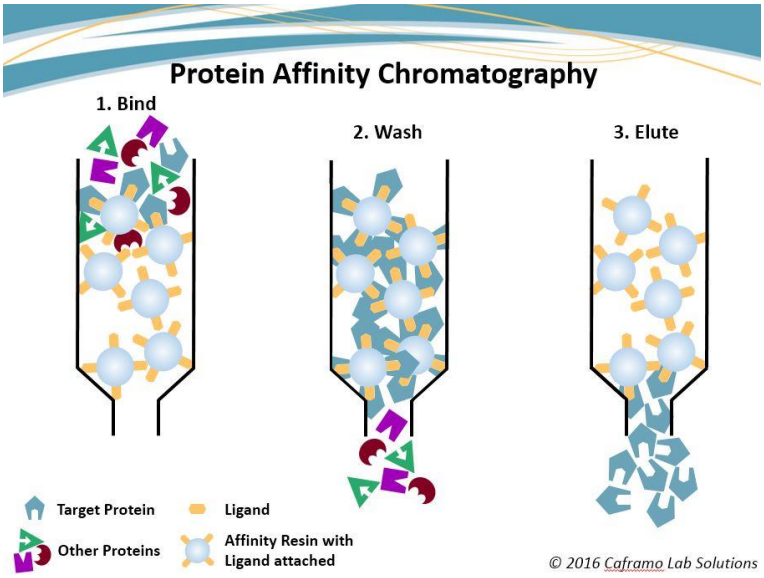


Figure 13: Visualization of Affinity Chromatography[74]

Aim of study

The aim is to synthesize polyurethanes both by conventional and by microwave heating and compare these two techniques.

Then a suitable test-system for the immobilization of HRP should be developed, in order to eventually make a suitable immobilization technique for chitinases (ChiA) and LPMOs.

Polyurethanes modified with nanoparticles of gold and silver, will be compared to non-modified polyurethane for the immobilization of HRP.

The activity of the immobilized enzymes will be compared to that of the free ones.

2. Results and discussion

2.1 Synthesis of polyurethane using 1:2 mole ratio

Synthesis of polyurethane (PU) was attempted by using the method described by Budriene *et al*[43]. In this paper the authors use the mole ratio 1:1 and 1:2 when DMSO was used as solvent. Therefore, the starting point was using 1:2 mole ratio between PVA and HMDI.

Poly (vinyl-alcohol) (PVA), Hexamethylene diisocyanate (HMDI) (1:2 mole ratio), and DMSO (≥ 99,7%) was used as solvent. PU was successfully synthesized when the solution was carefully heated to 120 °C. PU precipitated when a mixture of acetone and diethyl ether (1:1 V) was added. A folded paper filter was used to filter the sample, PU was dried in a desiccator. Some PU was modified with both silver- and gold-nanoparticles. Non-modified PU, PU/Ag-Nps and PU/Au-Nps were used to immobilize horseradish peroxidase (HRP).

Absorption at 403 nm were measured using UV/Vis and used to estimate the percentage immobilized HRP. Results are presented in Figure 14 and 15.

2.1.1 Immobilization results when PU (1:2 mole ratio) was used

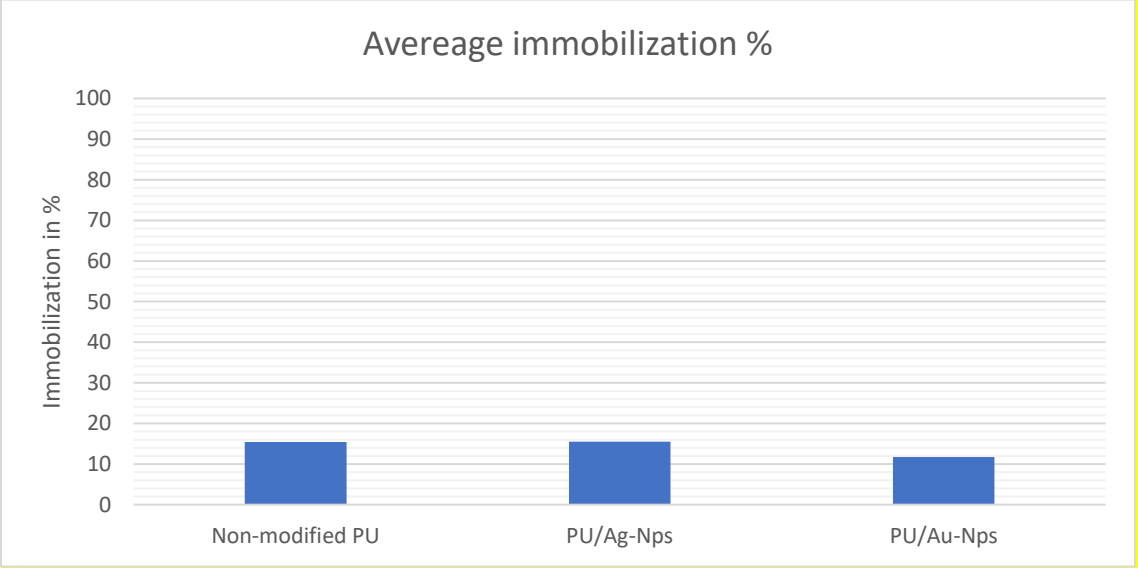


Figure 14: average immobilization % when PU were synthesized with a mole ratio of 1:2 of PVA and HMDI, average was calculated using all values above 5 %.

Average immobilization in percent is presented in Figure 14, these results show that the immobilization of HRP was low. All the percentages are presented in appendix 1 and show no correlation between amount of carrier and HRP and the percentages were non-cohesive. Different ratios of HRP and PU were compared, to find a trend in immobilization percentage, and to increase immobilization (Figure 15).

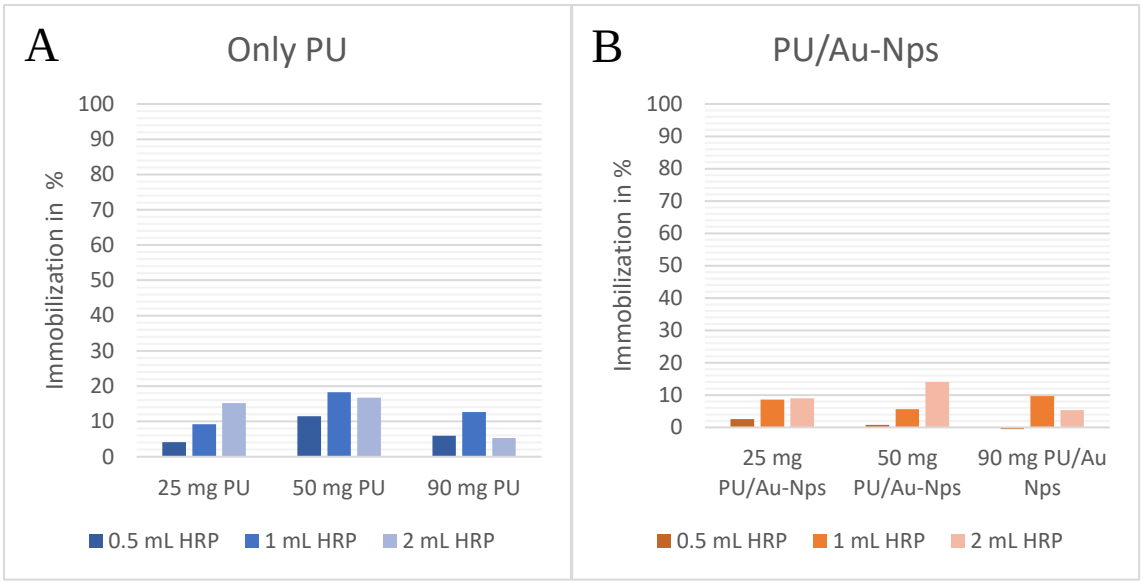


Figure 15: Immobilization results with different amount of HRP were used. A) Immobilization through covalent binding with non-modified PU. B) Immobilization through adsorption using PU/Au-Nps.

The percentages of immobilized HRP was still low, and Figure 15 does not show a trend between PU and HRP.

In synthesized PU, it was expected that three different types of units were formed.

1. Non-reacted PVA,
2. Units with one urethane group
3. Two urethane groups that have been crosslinked.

The ratio between these group changes when the ratio between PVA and HMDI is changed. Excess HMDI reportedly gives more of units 2 and 3, and will give more sites for immobilization[75]. Number of OH groups in PVA will impact how many of the different units formed, the molecular weight of PVA used was 31,00 – 50,00 M_w. Therefore, more of unit 1 might be present when PU was synthesized from PVA and HMDI (1:2 mole ratio). This might be the reason for the low immobilization of HRP

A 1:5 mole ratio of PVA and HMDI have previously been reported by Kochane *et al.* However, the authors do not list the molecular weight of the PVA they use[57]. Investigations must be carried out, and after a mole ratio of 1:2 gave low immobilization percentages, we felt that 1:5 should be tested.

2.2 Synthesis of polyurethane using 1:5 mole ratio

The same method, as described by Budriene *et al.*[43], was attempted using PVA (98-99% hydrolyzed, M_w 31,00-50,00), HMDI (1:5 mole ratio), and DMSO (≥ 99.9 %, freshly opened bottle). The mixture was carefully heated to 120 °C, cooled to room temperature then a mixture of acetone and diethyl ether (200 mL, 1:1 V) was added, no PU was observed.

Water was expected to be a contaminant, therefore, both DMSO (≥ 99.9 %, freshly opened) and DMSO (≥ 99.9%, anhydrous) was tested in the synthesis of PU. But both gave negative results.

Different changes to the method were attempted to synthesize PU successfully, these attempts are summarized in Table 2.

Table 2: Different attempts for the synthesis of PU. All attempts were tested multiple times, to make sure there were no human errors.

Isocyanate	Diol	Solvent	Comments	Observed results
Hexamethylene diisocyanate (HMDI)	Poly(vinyl-alcohol) (PVA)	DMSO (≥ 99.9 %, freshly opened)	1:5 mole ratio	No PU was observed, PVA crystals were still observed in the mixture.
Hexamethylene diisocyanate (HMDI)	Poly(vinyl-alcohol) (PVA)	DMSO (≥ 99.9%, freshly opened) and (≥ 99.9 %, anhydrous)	1:5 mole ratio PVA was dissolved in DMSO prior to addition of HMDI.	No PU was observed.
4,4'-methylenebis (cyclohexyl isocyanate)	Poly(vinyl-alcohol) (PVA)	DMSO (≥ 99.9%, freshly opened) and (≥ 99.9 %, anhydrous)	1:5 mole ratio HMDI might have been contaminated.	No PU was observed.
4,4'-methylenebis (cyclohexyl isocyanate)	Poly(vinyl-alcohol) (PVA)	Chlorobenzene	Using the method provided by Budriene *et al*.[43] In short, the mixture of PVA, 4,4'-methylenebis (cyclohexyl isocyanate) (1:5 mole ratio) and chlorobenzene was heated to 131 °C for 30 minutes.	No PU was observed,
4,4'-methylenebis (cyclohexyl isocyanate)	1,6 hexanediol	DMSO (≥ 99.9 %, anhydrous)	1:2 mole ratio. Maybe PVA was contaminated.	No PU was observed.

4,4'-methylenebis (cyclohexyl isocyanate)	Poly(vinyl-alcohol) (PVA)	DMSO (≥ 99.9%, anhydrous)	1:5 mole ratio. Added two drops of base (triethylamine), for it to work as a good leaving group.	No PU was observed.
Hexamethylene diisocyanate (HMDI)	Poly(vinyl-alcohol) (PVA)	DMSO (≥ 99.9%)	1:2 mole ratio. The original method that had previously shown positive results was used.	Some PU was observed, but also PVA crystals.
Hexamethylene diisocyanate (HMDI)	Poly(vinyl-alcohol) (PVA)	DMSO (99.9%) with 1% dH_2O	1:2 mole ratio.	PU was observed.

From the attempts shown in table 2, the general method of synthesizing polyurethane was developed and optimized. This method was used for further syntheses of polyurethanes. PU contains very reactive cyanate functional groups. Therefore, it was reported that PU needs to be freshly synthesized before being used for immobilization[43, 57, 75]. Both HMDI and 4,4'-methylenebis- (cyclohexyl isocyanate) was used for synthesizing PU, different mole ratios between PVA and the isocyanate was also used for synthesizing PU.

PU was successfully synthesized with a mole ratio of 1:2 and 1:5, HRP was immobilized using non-modified PU, PU/Ag-Nps and PU/Au-Nps.

Absorption at 403 nm were measured and used to estimate immobilization percentages, the results were non-cohesive and still low (see Appendix B). PU/Ag-Nps show negative results, which can be caused by interference of particles when absorption was measured. There was no obvious correlation between amount of carrier (PU, PU/Ag-Nps or PU/Au-Nps) and immobilized HRP. One reason for these observations might be the non-homogenized PU that were used, the binding cites for HRP can therefore vary between every sample. Homogenization of PU by using pestle and mortar before immobilization of HRP should be tested in an attempt of getting cohesive immobilization of HRP.

PVA and 4,4'-methylenebis (cyclohexyl isocyanate) (1:5 mole ratio) was used to synthesize PU and used for immobilization of HRP. The results were higher than before (see Appendix B), but because of time limitations only HMDI were used further.

It was also decided to only use non-modified PU for further analysis, due to limited time.

2.2.1 Immobilization results using homogenized PU

PU was synthesized from PVA and HMDI with a mole ratio of 1:5. During synthesis lumps were formed when the mixture became approx. 90°C. For one sample more DMSO (20 mL) was added to dissolve the lumps, but in the second sample the synthesis was performed without addition of more DMSO.

Both samples of PU were homogenized using pestle and mortar before immobilization of HRP. Absorption at 403 nm was measured and used to estimate immobilization of HRP (see Figure 16).

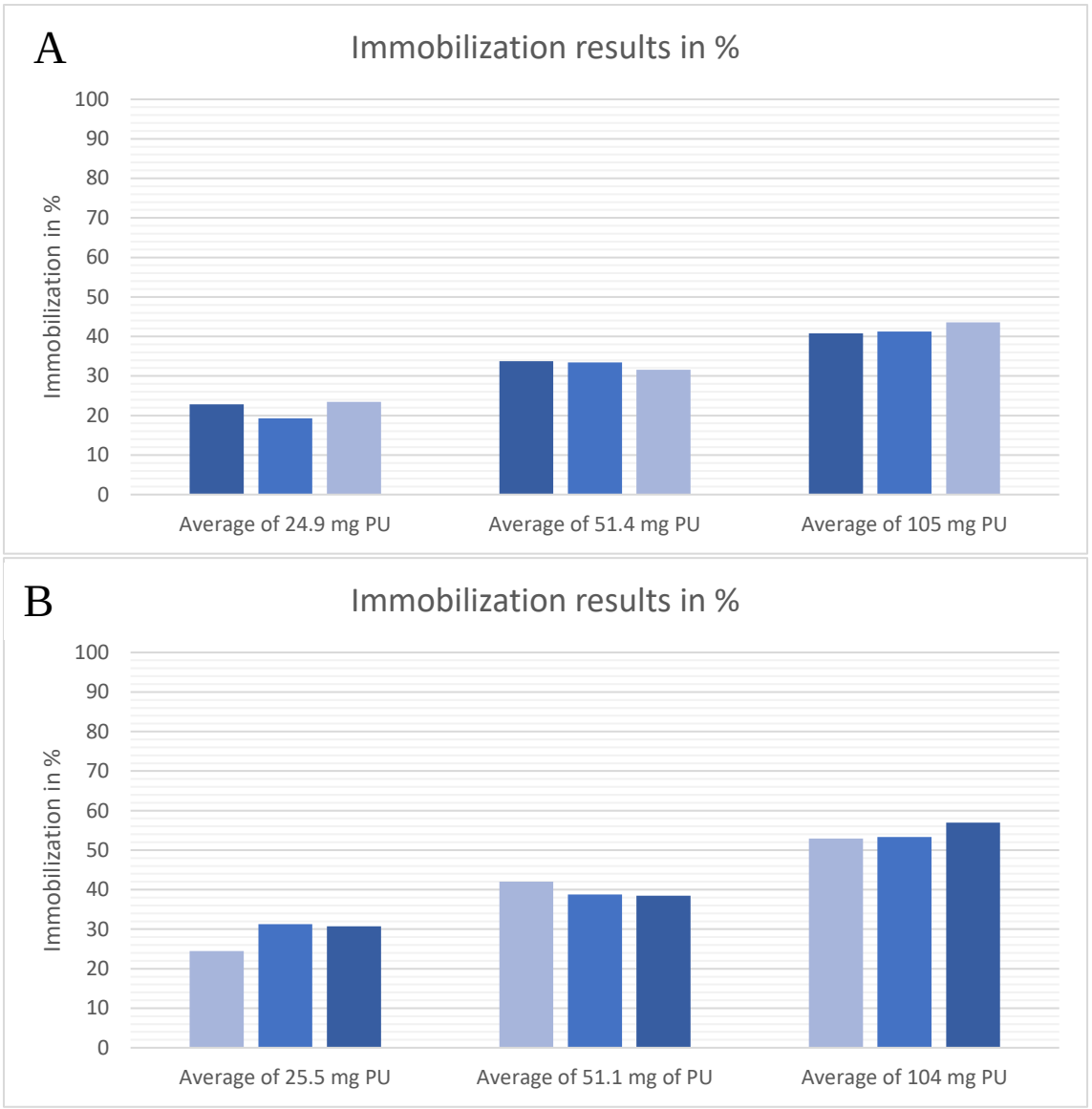


Figure 16: immobilization results A) addition of more DMSO, B) without addition of DMSO.

All results in figure 16 show more cohesive results, indicating that homogenization of PU was better. There was also an increase in immobilization of HRP when more PU (in mg) were used.

When the synthesis of PU was carried out without addition of more DMSO (Figure 16, B) the percentage of immobilization was higher, compared to when more DMSO was added (Figure 16, A). Therefore, in further synthesis of PU, the polymerization reaction was carried out without addition of more solvent.

2.2.2 Immobilization results using different buffers

In enzymatic assays of HRP 0.1 M phosphate buffer (pH 6) are often used[68, 70], while in immobilization using PU, 0.1 M citrate buffer (pH 5) are used[57].

PU was synthesized using microwave assisted synthesis and using conventional heating, all synthesized PU were tested for immobilization of HRP with both buffers.

All samples of PU were homogenized using pestle and mortar. Absorption at 403 nm was measured and used to estimate immobilization of HRP (see Figure 17).

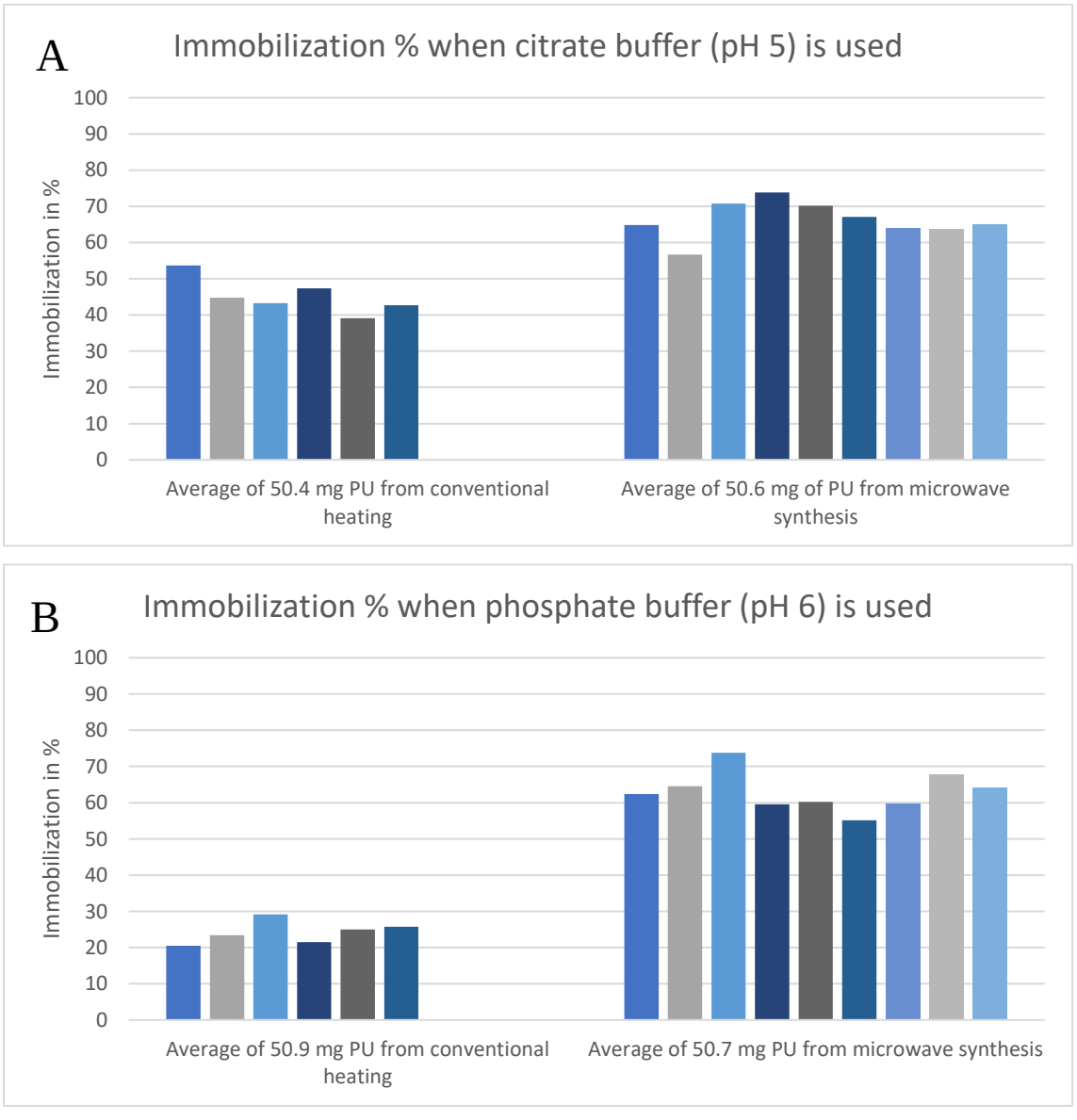


Figure 17: Immobilization resulst when different buffers were tested using samples, C.6, C.7 and M1-M.3. A) 0.1 M citrate buffer (pH 5), B) 0.1 M phosphate buffer (pH 6)

Both citrate and phosphate buffer show higher immobilization when PU were synthesized in the microwave, than synthesized by conventional heating. The biggest variation between the buffers were when the conventional heating process was used. The average percentage when citrate buffer was used (PU from conventional heating) was 45.14 % but when phosphate buffer was used the average was 24.21 %. The difference when PU samples from the microwave were used was small, but still the citrate buffer (average 66.23 %) was a bit higher than phosphate buffer (average 63.03 %). Therefore, the citrate buffer was used further.

The reason for this might be because it was suspected that at pH 5 all NH_2 groups that were formed was protonated[57], and therefore give better immobilization.

2.3 Detecting immobilization of HRP using SDS-PAGE

A new method for detecting immobilization of HRP were used. Protein content in the flow through (**FT**), the first wash (**W**) and immobilized onto PU (**C**) were detected using SDS-PAGE. For every sample FT, W and C were plotted respectively, arrows indicating the pattern for one sample is shown on every gel.

0.5 mg/mL HRP and 75 mg of PU were used unless something else is mentioned.

2.3.1 Immobilization of HRP onto PU from conventional heating

On the first attempt of detecting immobilization using SDS-PAGE, PU sample synthesized from conventional heating and microwave were used. 0.5 mg/mL HRP was added to different amounts of PU (0.25 mg, 0.5 mg, 1 mg, 2 mg, and 10 mg).

Results from SDS-PAGE gel when 10 mg PU from conventional and microwave are shown in Figure 18, the rest of the SDS-gels are presented in Appendix E. Sample number and amount of PU are presented on the gel.

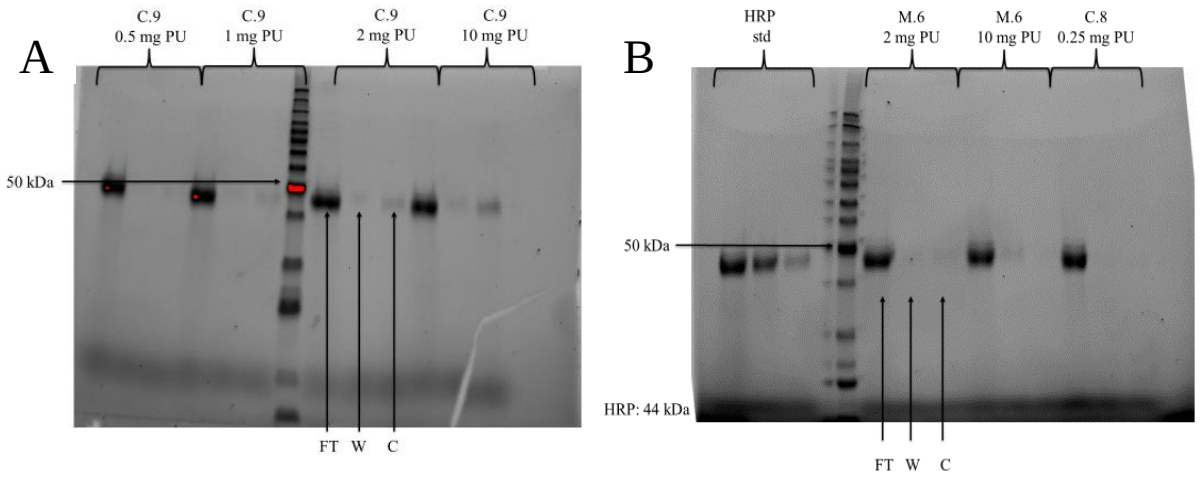


Figure 18: SDS-PAGE of FT, W, and C when samples 10 mg PU were used.

When 10 mg of PU from conventional heating (C.9) were used (Figure 18, A) the SDS gel indicate that some HRP were immobilized. But when 10 mg PU from the microwave (M.6) (Figure 18, B) there was no indication of immobilized HRP.

One reason for this might be the particle size when PU were synthesized in the microwave (120 °C, 15 minutes). After homogenization of PU, the particles from microwave looks larger, compared to particles from conventional heating. Changes to the synthesis of PU using microwave was therefore needed, in order to successfully immobilize HRP.

Saturation of PU synthesized from conventional heating was attempted. The same amount of HRP (0.5 mg/mL) were added to different amounts of PU (10 mg, 25 mg, 50 mg, and 75 mg). An SDS-PAGE were used to detect the best immobilization (see Appendix F), 75 mg showed the best results and were used further for all samples.

2.3.2 Immobilization of HRP onto PU synthesized in the microwave

PU was synthesized using microwave assisted synthesis, first different times (2, 5 and 7 minutes) at 120 °C were attempted, after homogenization these particles looked larger than PUs from conventional heating. Therefore, the time and temperature chart based on Arrhenius equation (Figure 11) was used to calculate at which settings the reaction should take place to get the same results as in conventional heating. The conclusion was 170 °C for 2-3 minutes, therefore two samples of PU were made one for 3 minutes and one for 2 minutes. Both PUs was synthesized using PVA and HMDI (1:5 mole ratio).

Protein content in FT, W, and C was detected on an SDS-PAGE (Figure 19), FT was the flow through (HRP in solution), W was the first wash and C indicate HRP that was immobilized

onto PU. Sample number, minutes and arrows indicating the pattern of FT, W and C are presented on the gel.

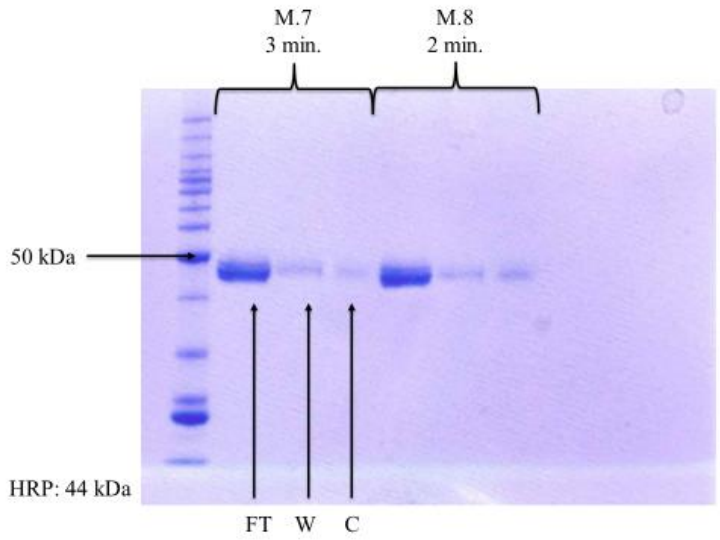


Figure 19: SDS-PAGE of FT, W, and C when samples M.7 and M.8 were used.

From these results HRP was successfully immobilized onto PU synthesized in the microwave. It looks like PU synthesized for 2 minutes at 170 °C has more HRP immobilized, these settings were used further to synthesize this PU.

Saturation of PU was attempted using PU synthesized from the microwave. Different concentrations of HRP was added to 75 mg of PU. Estimations show that when less HRP was used, more of the added HRP was bound. This indicate that there might be saturation between PU and HRP, but because of limited time, excess amount of HRP was used further.

At the same time changes to the immobilization method was tested to increase immobilization. After incubation at 40 °C one set of samples were placed overnight in the fridge (4°C), the other set of samples was placed overnight with stirring (4 °C and 1000 rpm).

Nanodrop one was used to measure absorbance at 403 nm and used to estimate immobilization percentages. Results are presented in Appendix G, and clearly show that immobilization was improved by stirring the samples overnight.

2.3.3 Immobilization of HRP using PU synthesized from different mole ratios

Different mole ratios between PVA and HMDI were used to synthesize PU samples. This was done in an attempt to increase possible binding sites for HRP, by increasing cyanate groups on the PU-particles. All samples were immobilized using the immobilization method with stirring.

SDS-PAGE were used to detect immobilization (see Appendix H). The absorption of HRP (403 nm) was measured using nanodrop one. The absorption of FT and W was used to estimate the amount of immobilized HRP in percentage (Figure 20, A), amount immobilized HRP (in mg) per mg PU was also estimated (Figure 20, B).

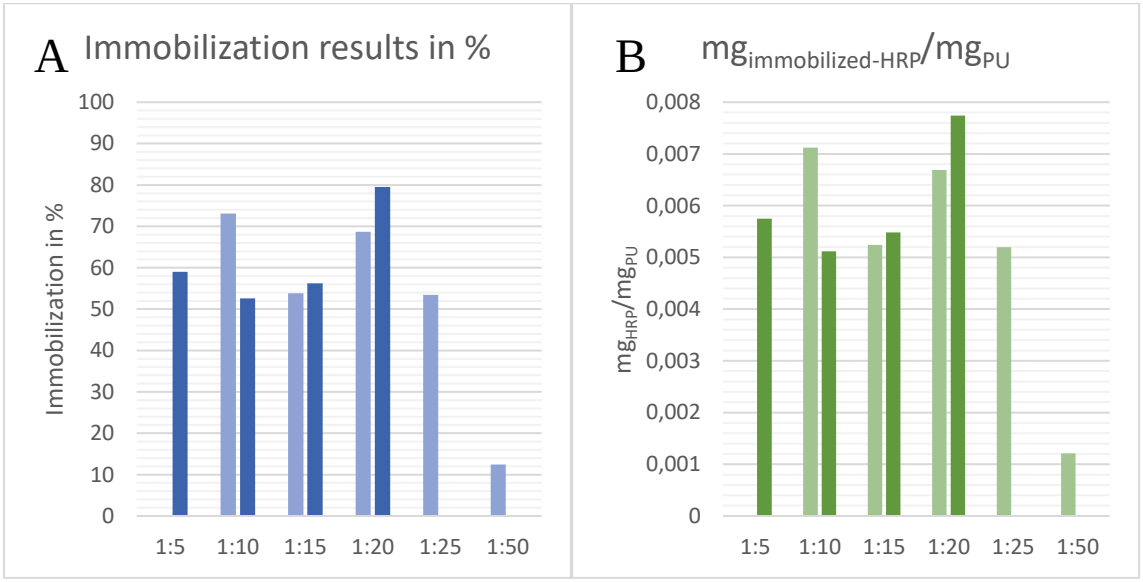


Figure 20: Results from nanodrop, A) shows immobilization in %, B) show the amount of immobilized HRP (mg) per mg of PU.

The immobilization percentage was over 50 % for every sample (except 1:50). When a mole ratio of 1:15 was used the two samples show cohesive results, while when 1:10 and 1:20 were used there was a bigger variation. Still the percentages were overall higher.

Variation in percentages when different batches of PU, synthesized under the same condition, indicate that the particles were different, or the number of binding sites for HRP vary between each sample.

2.3.4 Immobilization results using stored PU

In studies on immobilization using PU, it is mention that due to the reactive cyanate group, PU needs to be freshly synthesized before being used for immobilization.[43, 57, 75] To test this statement, PU that had been stored in a desiccator were tested for immobilization of HRP. PU samples had been stored for 1 week, 90 days, 4 weeks, and for 2 weeks before immobilization.

The SDS-PAGE of FT, W, and C for each sample are presented in Appendix I, sample number and storage time is presented on the gel.

All samples show that HRP have been immobilized, these results indicate that immobilization can occur after PU have been stored in a desiccator for up to 90 days. Concentrations and immobilization percentages were estimated from nanodrop results (see Figure 21).

Nanodrop results

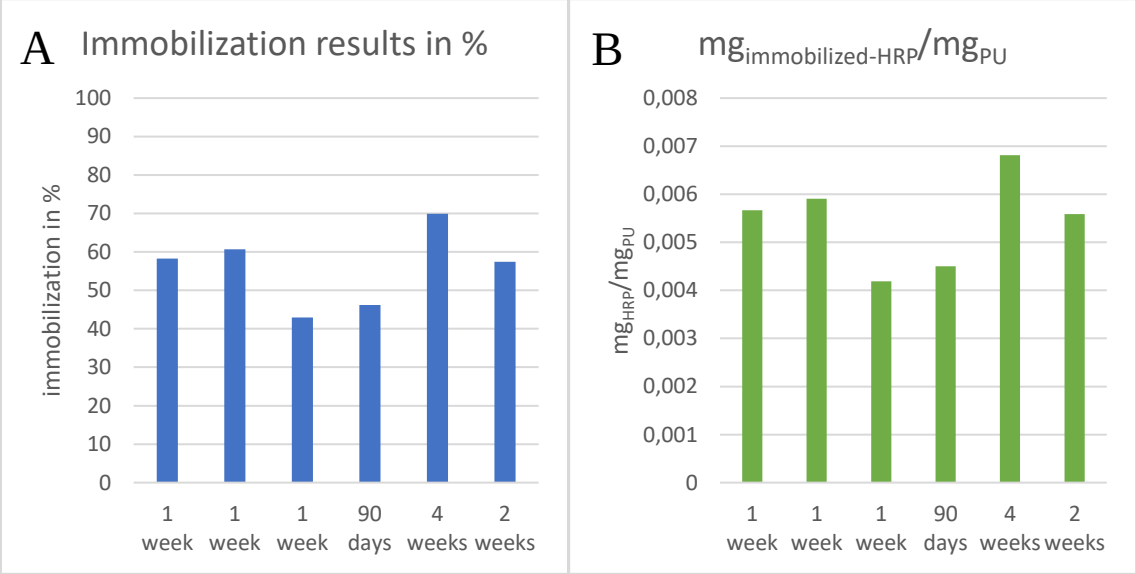


Figure 21: Results from nanodrop, A) shows immobilization in %, B) show the amount of immobilized HRP (mg) per mg of PU.

The results presented in Figure 21 confirm that stored PU (up to 90 days) gives higher than 40 % immobilization of HRP.

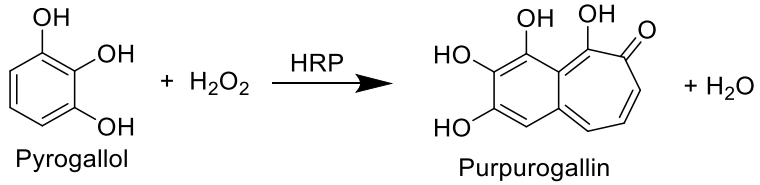
Samples that have been stored for 1 week were from the same batch of PU. Results presented in Figure 21 indicate that there was a variation of immobilization within a batch of PU. The reason for this can be that there was a variation of sizes of PU in each batch, or the number of binding sites vary in each sample.

2.4 Enzymatic assay results

Previous results (section 2.1 – 2.3) show that immobilization of HRP onto PU was possible, but it was also important to know if HRP was still active after immobilization. Different enzymatic assays are described in the sections below.

All samples were washed with buffer 3-4 times before being used in an assay.

2.4.1 Enzymatic assay using Pyrogallol



Enzymatic assay using pyrogallol was attempted, measuring the absorption at 420 nm. Due to the presence of particles, there was a lot of noise and the absorption was not detectable. The samples did change color, see Figure 22.

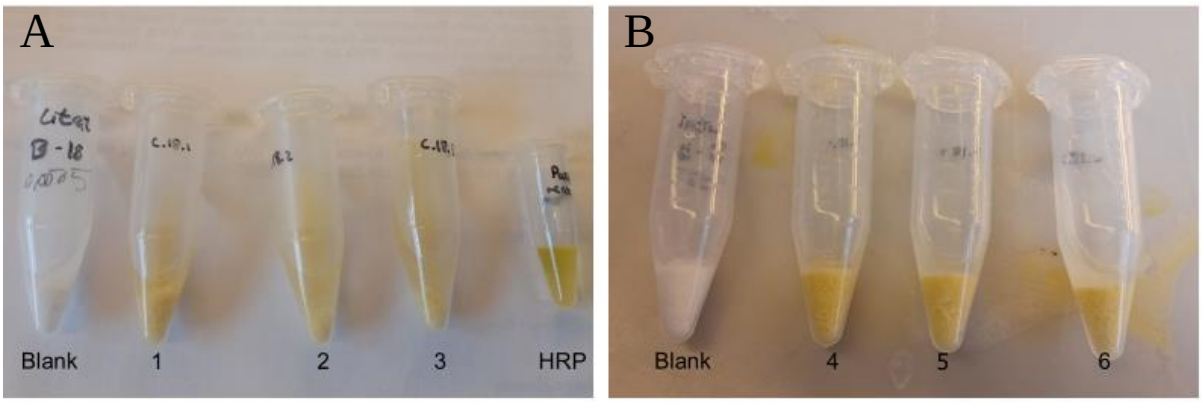
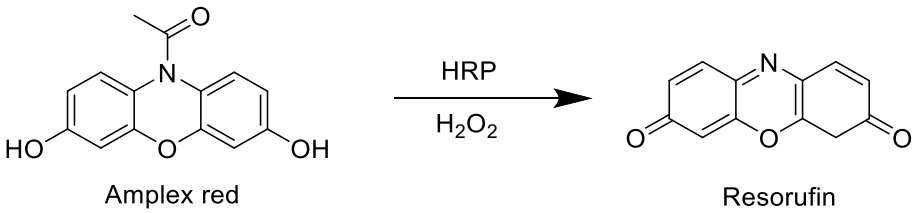


Figure 22: enzymatic assay using pyrogallol

A clear color change from the blank (white) to samples 1-6 (yellow) are presented in figure 20. On Figure 20, A, one sample with only HRP is presented, this solution was also clearly yellow, same as the samples.

Figure 20, B show that the yellow color, which was suspected to be the product (Purpurgallin) was attached to the particles and not in the solution. One possibility was that the product has an affinity for the PU-particles and therefore bind – both have several polar functional groups. Another possibility was that a different reaction has occurred. Because the product was attached to PU, identification was not possible.

2.4.2 Enzymatic assay using amplex red



As the data from the pyrogallol assay were ambiguous, we needed a different assay in order to test the enzymes. As we assumed that the observations with pyrogallol was due interactions between the reaction product and the PU, we went for a less polar assay.

A new assay using amplex red was attempted. Absorbance at 575 nm were detected for blank, only HRP, immobilized HRP, FT and W (Table 3), the color change are also shown in Figure 23.

Table 3: Results from enzymatic assay using amplex red.

SAMPLE	ABS (575 NM)
BLANK	0.00967
ONLY HRP	0.3709
IMMOBILIZED HRP (C, 0.1)	0.1148
IMMOBILIZED HRP (C, 0.025)	0.2691
FT	0.3755
W	0.5427

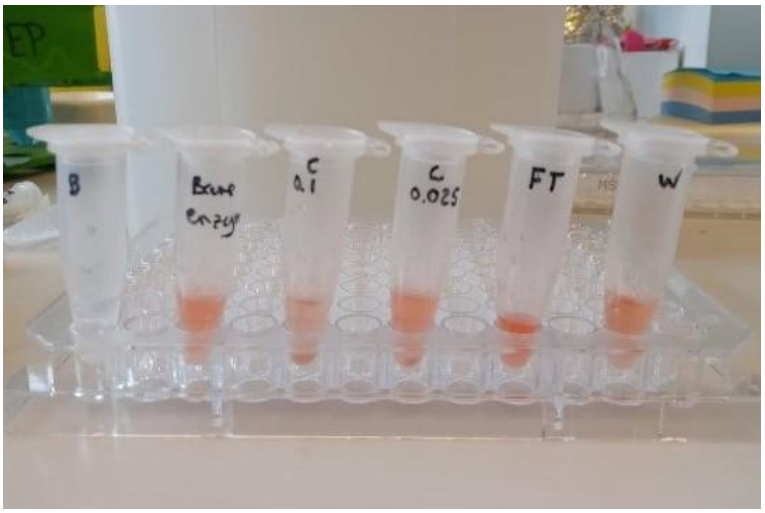
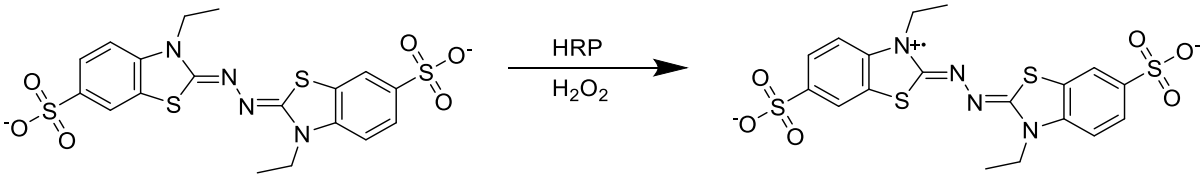


Figure 23: enzymatic assay using Amplex red.

Change in color occurs in all but the blank, which indicate that there was activity on the immobilized HRP, which was also confirmed by the absorption presented in table 3. After some time, it was observed that some of the pink/red color were attached to the PU-particles. PU might also have affinity to the product in this assay. Because of the results in table 3 we can be certain that the right product was formed. But a new assay still should be tested.

2.4.3 Enzymatic assay using ABTS



ABTS $ABTS^{\bullet+}$

The third attempt for testing the activity of immobilized HRP was by using ABTS[70]. In this assay oxidized ABTS was formed and a dark blue/black color was observed. Measuring the absorption at 405 nm were attempted, but due to particles a lot of noise were present, and the absorption were not detectable. However, color change was observed and pictured in Figure 24.

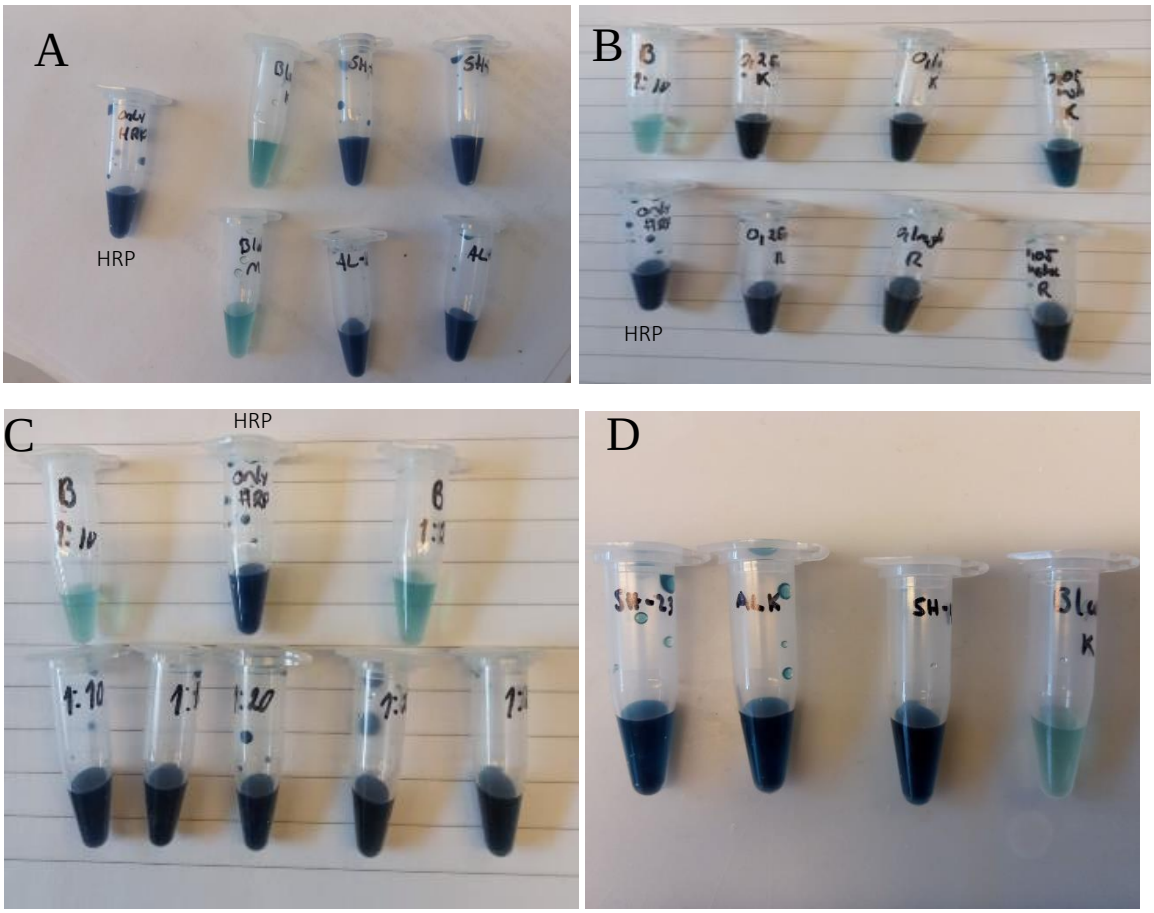


Figure 24: enzymatic assay using ABTS, A) samples from conventional heating and from the microwave. B) Samples from saturation of PU. C) PU synthesized with different mole ratios. D) stored PU.

Samples and only HRP show a clear change in color, there was therefore activity on all the immobilized HRP.

In Figure 24, D it was proven that stored PU (up to 90 days) can be used for immobilization of HRP, and the HRP was still active.

These reactions were performed rapidly, to ensure that the enzymes were still active, for further work they should be more controlled, and a method that are able to detect the changes should be found. A time course of the reaction should also be detected, and then the differences between free enzyme and immobilized enzyme could be decided.

2.4.4 Reuse of immobilized HRP

In order for it to be a good immobilization system, immobilized HRP needs to catalyze multiple reaction. Therefore, one sample of immobilized HRP was tested through five/six catalytic reactions, using ABTS.

The first five reactions were performed without cleaning steps between every reaction. The new reaction mixture (without HRP) was added to the same filter, and vacuum was used to

filter the samples. After five reactions both the well with immobilized HRP and free HRP show a dark color, indicating that oxidized ABTS was formed (see Figure 25).

One reason for a positive reaction to occur five times in the well with free HRP, might be caused by the dark color that was observed on the filter for all wells, excluding the blank. Product and HRP might be stuck on the filter, and a new reaction could occur. Therefore, one washing step was included between plate number five and six. For reaction six less color was observed in the filter with free HRP, but difficult to capture on a picture.

This method was not successful to show that the immobilized HRP can be reused and the free HRP was not. More washing steps might be the answer in confirming that immobilized HRP was more suitable for reuse than HRP in solution.

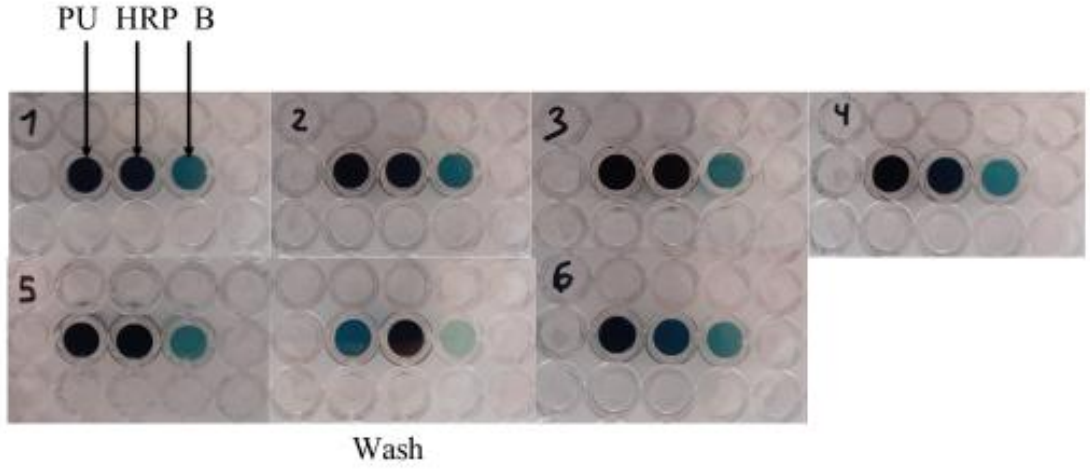


Figure 25: The attempt of reuse of immobilized HRP. The first well contain immobilized HRP (PU), well number two is free HRP (HRP) and the third well indicate the blank sample (B).

Another study on immobilized HRP used a Büchner funnel for testing the activity of immobilized HRP[76]. Therefore, a Büchner funnel was used in a new attempt on reuse of immobilized HRP, with addition of more washing steps. With HRP in solution dark color in the filter were observed after the first reaction, and after three washing steps (using buffer) some dark color was still observed. A new reaction mixture was added (not HRP) and washing steps were repeated. Eppendorf vials of each reaction is pictured in Figure 26.

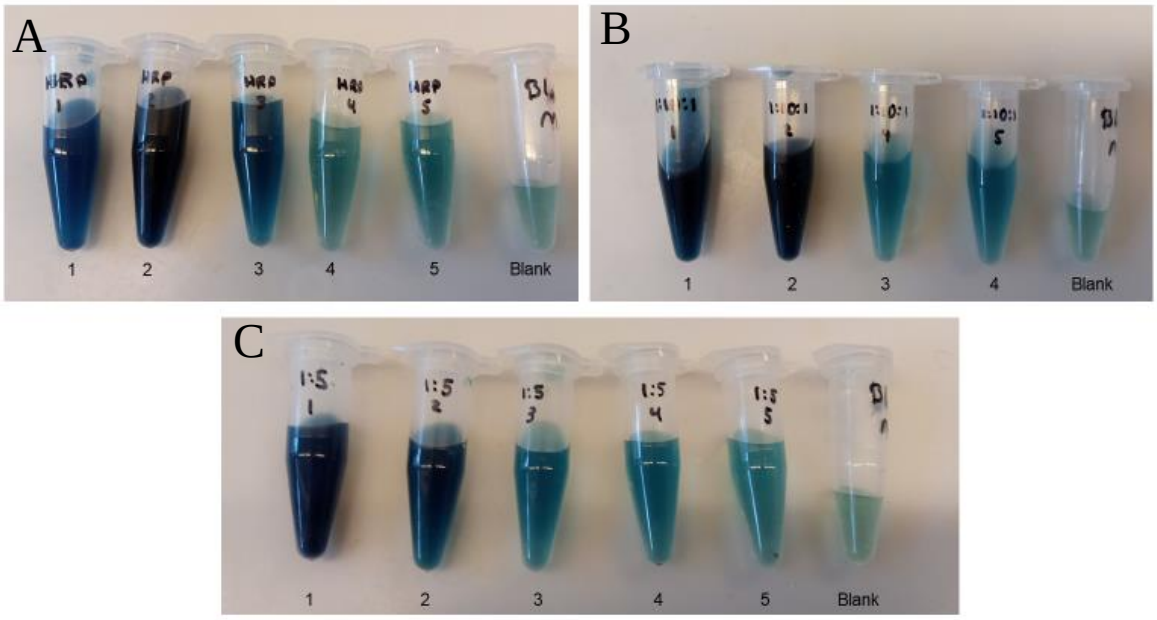


Figure 26: Reuse of HRP, A) show five reactions of free HRP, B) show four reactions of immobilized HRP (sample M.10.1, reaction three is not pictured because a clear color was observed.) C) show five reaction of immobilized HRP (sample M.15).

When only HRP was used, more of the product/dark color were observed in the filter after wash, compared to when immobilized HRP were used. This might be the reason why dark color was observed in reaction 2 and 3 in Figure 26 A, reaction 4 and 5 for free HRP was expected to be a negative reaction. For both samples pictured in Figure 26, B and C it was assumed that all reactions 1-5 were positive, but for reaction number 3-5, the reactions were slower, and less color was observed after 3 minutes. The change in color was only captured on pictures, therefore, it was difficult to confirm these assumptions.

The method using a Büchner funnel was not optimal, the filter size could be the reason for the observation in Figure 26. For further work, another method should be used for confirming that immobilized HRP can be reused.

2.5 Particle size

Particle size of synthesized PU (see table 4 sample information) was measured using Mastersizer 3000, which can measure particles from 0.01 – 3500 µm. The size-distribution for different PU samples are presented in Figure 27. For all samples there was a portion of particle that was large (over 500 µm), this was caused by oligomerization of particles.

Table 4: overview of samples

Sample	**Information**
C.11	1:5 mole ratio Conventional heating, 120 °C for 90 min.
M.8	1:5 mole ratio Microwave, 170 °C, 2 min
M.10	1:10 mole ratio Microwave, 170 °C, 2 min
M.11	1:15 mole ratio Microwave, 170 °C, 2 min
M.12	1:20 mole ratio Microwave, 170 °C, 2 min
M.13	1:25 mole ratio Microwave, 170 °C, 2 min
M.14	1:50 mole ratio Microwave, 170 °C, 2 min
M.6	1:5 mole ratio Microwave, 120 °C, 15 min

Figure 27: Size-distribution of PU-samples.

Dx-values are presented in Table 5, these values describe how much x vol% of the sample that was lower than this value. For example, the D (10) for sample C.11, describes that 10 % of the particles have a diameter of 38.3 µm or less.

Table 5: Dx-values and average D [4:3] and D [3:2] of all samples

Sample Name	Average of 'C.11'	Average of 'M.8'	Average 2 of 'M.8'	Average of 'M.10'	Average of 'M.11'	Average of 'M.12'	Average of 'M.13'	Average of 'M.14'	Average of 'M.6'
D [4:3]	222	469	581	564	501	596	826	780	1080
D [3:2]	69,9	53,7	57,5	79,4	60,6	68,8	134	129	374
D (10)	38,3	21	21,7	28,4	19,5	21,8	54,5	78,9	221
D (50)	77,6	111	137	300	247	314	622	545	890
D (90)	649	1530	1860	1630	1460	1710	1940	1910	2220

Studies have shown that PU particles under 100 µm give the best immobilization results[75]. In sample C.11 and M.8 there was almost 50 % of particles under 100 µm. While sample M.6 synthesized with the same mole ratio, but different settings, contain particles with larger diameter, only 10 % of these particles were smaller than 221 µm.

The size distribution for sample M.10 – M.14, show that a more significant portion of these particles were larger than 100 µm in diameter. These larger particles were caused by oligomerization and can be fixed by crushing the particles even more. At the same time there was also more particles that were very small (under 20 µm), compared to sample C.11.

D [4:3] describe the volume middle diameter and D [3:2] is the Sauter mean value (see table 5). The Sauter mean value describes the average particle size, sample C.11, M.8 and M.10 – M.12 all have an average particle size under 100 µm.

Since there was a large span of sizes of PU in each sample, where some of the larger particles were caused by oligomerization. The answer could be crushing the PU-particles more and therefore homogenize the particles. Another option is filtration of PU before immobilization to get a homogenous sample.

2.6 Cultivation of ChiA

To confirm that ChiA were purified using affinity chromatography, an SDS-PAGE gel of PPE, CPE, void, and eluate are shown in Figure 28.

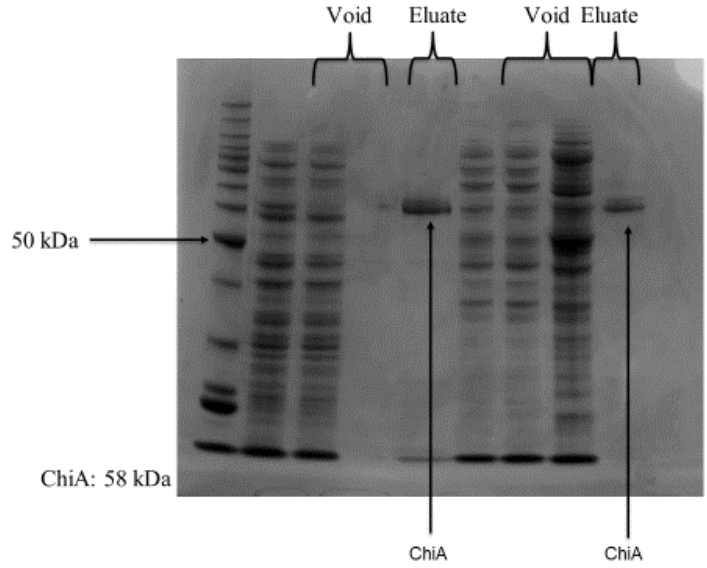


Figure 28: SDS-PAGE of PPE, CPE, void, and eluate after cultivation of ChiA.

In the well of the eluate only one bond is shown, and by using the benchmark ladder, it was expected to be the ChiA-enzyme (58 kDa). Therefore, ChiA was successfully purified using affinity chromatography.

Both eluates containing purified ChiA were mixed, the buffer was changed and concentrated to a final volume of 3-5 mL. The concentration of ChiA was tested using Bradford assay, this was not successful because the concentration was too low to be detected.

A new cultivation was started but because of the deadline and Covid-19 this was not completed.

3. Conclusion

The method for synthesizing polyurethane from Budriene[43] was optimized using PVA and HMDI. A method to successfully synthesize polyurethane using microwave assisted synthesis was also performed. Polyurethane was modified with nanoparticles, but due to limited time non-modified polyurethanes were tested most.

Immobilization of HRP onto polyurethane was successful, but the results were a bit varying when the same polyurethane was used. Particle distribution of polyurethane show that there was a broad range of particle size of polyurethanes. This might be the reason for a variation in immobilization when the same support material was used. Crushing the PU-particles even more, or a filtration step can be the answer to this problem.

Positive enzymatic assay of immobilized HRP, show that there was still activity after immobilization. Change in color occurs in the enzymatic assay, was clearly visible, but the product was difficult to detect.

The reuse of the immobilization system was difficult to perform, and a different method should be tested.

Cultivation of ChiA was successful, but the concentration was low and due to time limit and Covid-19 it was not possible to finish this process.

Future work

For further work PU samples should be better homogenized. One possibility is by crushing the PU samples even more. Another option is filtration of PU before immobilization. Immobilization of HRP using PU modified with nanoparticles of gold or silver should also be tested more in the future.

Another important aspect is that the enzymatic assay should be more controlled. A time course of the reaction should also be detected, and then the differences between free enzyme and immobilized enzyme could be decided.

Reusability of the system was difficult to identify; another method should be developed to test the reusability of the immobilization system.

Immobilization of ChiA and LMPOs onto PU particles should be tested.

4. Experimental

4.1 General information

The microwave that was used for synthesis of PU was a Biotage® Initiator+, Fourth Generation Microwave Synthesizer

Aglient Technologies – Cary 8454 UV-Vis was used for absorption measurements.

NanoDrop™ One Microvolume UV-Vis spectrophotometer was used for absorption measurements.

Mastersizer 3000 was used for particle size analyses

Eppendorf ThermoMixer™ C was used for stirring and incubation of samples.

Agilent 550 Series FT-IR instrument with an ATR diamond cell was used for IR spectra

Bruker Ascend 400 instrument at 25°C, at 400 MHZ for ^{1}H NMR, DMSO-d_6 was used as solvent. The reference peaks were calibrated to 2.50 ppm.

4.2 General synthesis of polyurethane

4.2.1 Synthesis using conventional heating

Poly (vinyl-alcohol) (PVA), 98-99% hydrolyzed, M_w 31.00-50.00, was used as polyol,

Method: Poly(vinyl-alcohol) (PVA) (0,0031 mol), Hexamethylene diisocyanate (0,0062-0,0155 mol), 25 mL DMSO and ca. 0,25 mL dH_2O were mixed in a three-necked flask fitted with magnetic stirrer, thermometer and a reflux condenser with nitrogen inlet. The mixture was carefully heated to a temperature of 120 °C and kept at 120 °C for 90 minutes (lumps were formed at approx. 90 °C). The mixture was then cooled to room temperature, and acetone: diethyl ether (200 mL, 1:1 volume ratio) were added. PU precipitated and stored at 4 °C overnight. The next day PU was filtered with a folded paper filter, transferred to a beaker, and dried in an exicator.

Details and comments on the different PU samples are described in Table 6.

Table 6: Details of PU samples

Sample (mole ratio)	Comment	Yield
C.1 (1:2)	PVA (0.00310 mol, 0.155g) and HMDI (0.0062 mol, 0.996 mL) were used.	-
C.2 (1:2)	PVA (0.00310 mol, 0.155g) and HMDI (0.0062 mol, 0.996 mL) were used. When lumps were formed at approx. 90 °C, more DMSO was added to dissolve the lumps.	21.8 g
C.3 (1:5)	PVA (0.00310 mol, 0.155g) and 4,4'-methylenebis (cyclohexyl isocyanate) (0.0155 mol, 3.80 mL) were used. When lumps were formed at approx. 90 °C, more DMSO was added to dissolve the lumps.	14.8 g
C.4 (1:5)	PVA (0.00310 mol, 0.155g) and HMDI (0.0155 mol, 2.5 mL) were used. When lumps were formed at approx. 90 °C, more DMSO was added to dissolve the lumps.	9.32 g
C.5 (1:5)	PVA (0.00310 mol, 0.155g) and HMDI (0.0155 mol, 2.5 mL) were used.	3.87 g
C.6 (1:5)	PVA (0.00310 mol, 0.155g) and HMDI (0.0155 mol, 2.5 mL) were used.	4.80 g
C.7 (1:5)	PVA (0.00310 mol, 0.155g) and HMDI (0.0155 mol, 2.5 mL) were used.	5.74 g
C.8 (1:5)	PVA (0.00310 mol, 0.155g) and HMDI (0.0155 mol, 2.5 mL) were used.	7.20 g
C.9 (1:5)	PVA (0.00310 mol, 0.155g) and HMDI (0.0155 mol, 2.5 mL) were used.	6.56 g
C.10 (1:5)	PVA (0.00310 mol, 0.155g) and HMDI (0.0155 mol, 2.5 mL) were used.	6.51 g
C.11 (1:5)	PVA (0.00310 mol, 0.155g) and HMDI (0.0155 mol, 2.5 mL) were used.	6.12 g

Data:

^{1}H NMR (400MHz, Dimethyl Sulfoxide-d_6): δ 5.75 – 5.78 (bs, 2H, NH), 2.95 – 3.00 (bs, 4H), 1.08 – 1.42 (bs, 8H)

IR: 3321, 2930, 2852, 1616, 1571 cm^{-1}

4.2.2 Synthesis using microwave

Method: PVA (0.000516 – 0.00155 mol), HMDI (0.00258 – 0.0258 mol), DMSO and dH_2O was mixed in a microwave glass with a magnet. Microwave settings and details are summarized in table 7. After synthesis in the microwave, the mixture was cooled to room temperature, and acetone: diethyl ether (50-100 mL, 1:1 volume ratio) were added. PU precipitated and stored at 4 °C overnight. The next day PU was filtered with a folded paper filter, transferred to a beaker, and dried in an exicator. An overview of each sample is described in Table 7.

Table 7: Details for the synthesis of PU in mocrowave.

Sample (Mole ratio)	Chemicals	Microwave settings	Yield
M.1 (1:5)	PVA: 0.00258 g ($5.16*10^{-4}$ mol) HMDI: 0.415 mL ($2.58*10^{-3}$ mol) DMSO: 4.16 mL dH_2O: 0.0416 mL	Vial: 2-5 mL Time: 15 min Temp: 120 °C	0.724 g
M.2 (1:5)	PVA: 0.00258 g ($5.16*10^{-4}$ mol) HMDI: 0.415 mL ($2.58*10^{-3}$ mol) DMSO: 4.16 mL dH_2O: 0.0416 mL	Vial: 2-5 mL Time: 15 min Temp: 120 °C	0.646 g
M.3 (1:5)	PVA: 0.00258 g ($5.16*10^{-4}$ mol) HMDI: 0.415 mL ($2.58*10^{-3}$ mol) DMSO: 4.16 mL dH_2O: 0.0416 mL	Vial: 2-5 mL Time: 15 min Temp: 120 °C	0.687 g
M.4 (1:5)	PVA: 0.00258 g ($5.16*10^{-4}$ mol) HMDI: 0.415 mL ($2.58*10^{-3}$ mol) DMSO: 4.16 mL dH_2O: 0.0416 mL	Vial: 2-5 mL Time: 15 min Temp: 120 °C	0.748 g

M.5 **(1:5)**	PVA: 0.00258 g ($5.16*10^{-4}$ mol) HMDI: 0.415 mL ($2.58*10^{-3}$ mol) DMSO: 4.16 mL dH_2O: 0.0416 mL	Vial: 2-5 mL Time: 15 min Temp: 120 °C	0.708 g
M.6 **(1:5)**	PVA: 0.00258 g ($5.16*10^{-4}$ mol) HMDI: 0.415 mL ($2.58*10^{-3}$ mol) DMSO: 4.16 mL dH_2O: 0.0416 mL	Vial: 2-5 mL Time: 15 min Temp: 120 °C	0.648 g
M.7 **(1:5)**	PVA: 0.00258 g ($5.16*10^{-4}$ mol) HMDI: 0.415 mL ($2.58*10^{-3}$ mol) DMSO: 4.16 mL dH_2O: 0.0416 mL	Vial: 2-5 mL Time: 3 min Temp: 170 °C	0.785 g
M.8 **(1:5)**	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 1.25 mL ($7.75*10^{-3}$ mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	3.13 g
M.9 **(1:5)**	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 1.25 mL ($7.75*10^{-3}$ mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	1.55 g
M.10 **(1:10)**	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 2.49 mL (0.0155 mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	1.53 g
M.11 **(1:15)**	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 3.74 mL (0.0233 mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	1.44 g
M.12 **(1:20)**	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 4.98 mL (0.031 mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	1.54 g

M.13 (1:25)	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 6.23 mL (0.03875 mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	1.99 g
M.14 (1:50)	PVA: 0.0516 g ($1.03*10^{-3}$ mol) HMDI: 8.30 mL ($5.16*10^{-3}$ mol) DMSO: 8.30 mL dH_2O: 0.0830 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	0.770 g
M.15 (1:5)	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 1.25 mL ($7.75*10^{-3}$ mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	2.19 g
M.16 (1:10)	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 2.49 mL (0.0155 mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	2.85 g
M.17 (1:15)	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 3.74 mL (0.0233 mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	2.81 g
M.18 (1:20)	PVA: 0.0775 g ($1.55*10^{-3}$ mol) HMDI: 4.98 mL (0.031 mol) DMSO: 12.5 mL dH_2O: 0.125 mL	Vial: 10-20 mL Time: 2 min Temp: 170 °C	1.83 g

Data:

^{1}H NMR (400MHz, Dimethyl Sulfoxide-d_6): δ 5.76 – 5.78 (bs, 2H, NH), 2.95 – 3.00 (bs, 4H), 1.05 – 1.35 (bs, 8H)

IR: 3333, 2930, 2852, 1622, 1577 cm^{-1}

4.3 Synthesis of silver nanoparticles

Synthesis of silver nanoparticles was performed by heating of 100 mL of aqueous silver nitrate ($3{,}0 * 10^{-4}$M) to 60 °C with magnetic stirring. 10 mL of aqueous sodium citrate

($3{,}88 * 10^{-2}$M) with the same temperature (60 °C) was added, the mix was heated to 98 °C for 2 minutes. Silver nanoparticles were synthesized when the solution turned yellow.

4.4 Synthesis of gold nanoparticles

Synthesis of gold nanoparticles was performed by heating 80 mL $HAuCl_4$ ($3{,}175 * 10^{-4}$ M) to 60 °C, a 20 mL solution of sodium citrate ($6{,}80*10^{-3}$ M) with the presence of tannic acid ($7{,}34*10^{-6}$ M) with the same temperature (60 °C) was added. These two solutions were mixed in a round-necked flask and heated until boiling (approximately 94 °C) for 2 minutes. The solution turned to a wine-red color when nanoparticles were formed.

4.5 Formation of PU-nanoparticles conjugates

PU-nanoparticles conjugates were synthesized from the method provided by Kochane *et al*.[57] 0,5 grams of PU was added to 100 mL of previous synthesized nanoparticles (Both Ag-Nps and Au-Nps) and heated until boiling for 300-600 seconds. PU/Ag-Nps conjugates turned yellow, and PU/Au-NPs conjugates turned pale purple when they were formed.

4.6 Immobilization method

Solutions used for immobilization

Solution	Content
1 M monobasic phosphate	13.98g HPO_4^- was dissolved in 100 mL dH_2O
1 M dibasic phosphate	2.84 g dibasic phosphate was dissolved in 20 mL of dH_2O
0.1 M Phosphate buffer (pH 6)	88 mL of 1 M monobasic phosphate solution was dissolved in 12 mL, 1 M dibasic phosphate, and diluted to 1 L.
0.1 M Citrate buffer (pH 5)	3.39 g sodium citrate dihydrate and 1.63 g citric acid was added to a container with 160 mL of dH_2O. pH was adjusted to pH 5 using HCl or NaOH, and then dH_2O was added until the final volume was 0.2 L.
50 µM HRP (stock solution)	3.20 mg of HRP was dissolved in 1.5 mL of buffer.
5 µM HRP (working solution)	Dilute the 50 µM HRP stock solution 1:10.

For samples in section 2.1 a solution of theoretically calculated 5 µM HRP was added to each vial with PU. 1 mL of 5 µM HRP was added unless something else is mention.

4.6.1 Immobilization of HRP without stirring

Horseradish peroxide (HRP) in buffer was mixed with PU, PU/Ag-Nps or PU/Au-Nps in Eppendorf vials. These vials were incubated (40 °C, 100 rpm) for 30 minutes, and then stored at 4 °C overnight.

4.6.2 Immobilization of HRP with stirring

HRP in buffer was mixed with PU in Eppendorf vials, the vials were incubated while shaking (40 °C, 1000 rpm) for 30 minutes. Then the vials were stored at 4 °C (1000 rpm) overnight.

Citrate buffer (0,1 M, pH 5) were used unless something else is mention, phosphate buffer (0,1 M, pH 6) were only used in section 2.2.2.

4.7 Testing Immobilization

4.7.1 Using UV

Beer-Lambert law was used to calculate the concentration of HRP using measured absorbance at 403 nm (Eq. 4.1).

Eq. 4.1 $$A = \varepsilon c l$$

A is absorbance

ε is the molar absorption coefficient ($M^{-1}cm^{-1}$)

c is molar concentration (M)

l is optical path length (cm)

The concentration was calculated using measured $A_{(403\ nm)}$, $\varepsilon_{(403\ nm)}$ = 100 $mM^{-1}cm^{-1}$, and l was 1 cm.

It was assumed that the amount of HRP that was no longer present in the solution has been immobilized onto PU-particles. Therefore, percentage of immobilized HRP was assumed to be the difference between concentration with only HRP and concentration when HRP was mixed with PU (Eq. 4.2).

Eq. 4.2 $$\%\ immobilized\ HRP = \left(1 - \frac{[PU/HRP_{sample}]}{[Only\ HRP]}\right) * 100$$

4.7.2 Using SDS-PAGE

Solutions used for SDS-page

SOLUTION	CONTENTS
SDS-BUFFER	4x - Sample buffer 10x - Reducing agent dH_2O For 200 µL (per sample) 50 µL sample buffer, 20 µL reducing agent and 130 µL dH_2O
CONCENTRATED SDS-BUFFER	4x - Sample buffer 10x - Reducing agent
MOPS BUFFER	20 X Mops SDS running buffer was diluted to a 1 X Mops SDS buffer.
STAINING-SOLUTION	0.1 % Coomassie Brilliant Blue 10 % ethanol 10 % acetic acid
DE-STAINING SOLUTION	10 % ethanol 7.5 % acetic acid

Preparation of samples:

200 µL of immobilized HRP was placed on a filter plate, vacuum was used to filter the samples. The flow through (FT) was collected. 200 µL of buffer was again added to PU-HRP conjugates on the filter plate, and vacuum was used to filter the samples, this wash (W) was collected. PU-HRP conjugates (C), FT and W were all tested for protein content on an SDS-PAGE.

Before being loaded onto an SDS-PAGE the samples need to be treated with SDS-buffer. Concentrated SDS buffer (9 µL) was added directly to 16 µL of both FT and W. The PU-HRP was still on the filter plate, 200 µL of SDS buffer were added to the filter, and the solution with the PU-HRP conjugates were transferred to Eppendorf vials. Because of the reducing agent and SDS the HRP immobilized on PU was released into the solution, and the amount of immobilized HRP was detectable on an SDS-PAGE.

SDS-PAGE

A precasted NuPAGE 10 % Bis-Tris Gel was placed in an electrophoresis chamber, and the chamber was filled with 1 x MOPS buffer. 7 µL of benchmark™ Protein ladder and 18 µL of the samples treated with SDS-buffer were added to the gel, and a power of 200 V was applied for 50 minutes.

Two different systems to visualize protein content on the gel was used.
For section 2.3.1 a system called BioRad GelDoc was used, which uses UV to make the protein visible, by using the fluorescence of certain amino acids.

The second system uses Coomassie-brilliant-blue, which makes the proteins visible by attaching to the basic amino acids. The gel was colored by staining solution in the microwave for 1 minute, and then shaking for 5 min. Then de-staining solution was added to the gel, with heating in the microwave for 1 minute and incubated with shaking for 5-10 minutes. De-staining step was repeated until the protein-bonds were visible.

4.7.3 Measuring concentration using Nanodrop

FT and W were tested for HRP content using Nanodrop one, 2 µL of each sample was added to the nanodrop and absorption at 403 nm was measured.

The same calculations as described in section 4.7.1 were used to estimate the percentage of immobilized HRP.

4.8 Enzymatic assay of HRP

4.8.1 Solutions used for enzymatic assay

SOLUTION	CONTENTS
0.5 % H_2O_2 SOLUTION	0,0857 mL H_2O_2 (35 %) was diluted to a final volume of 6 mL with dH_2O.
500 µM H_2O_2 SOLUTION	
0.35 % H_2O_2 SOLUTION	10 µL 35 % H_2O_2 990 µL dH_2O
0.5 MG/ML HORSERADISH PEROXIDASE	1,25 mg HRP was dissolved in 2,5 mL of buffer.
50 MG/ML PYROGALLOL SOLUTION	0,55 g pyrogallol was dissolved in 11 mL of dH_2O.
AMPLEX RED SOLUTION	A premade 10mM was used
5 MG/ML ABTS SOLUTION	1 tablet (10 mg) was dissolved in 1 mL 0.1 M citrate buffer (pH 5).

4.8.2 Enzymatic assay using Pyrogallol

For HRP in solution:

dH_2O, buffer, 0.5 % H_2O_2 and Pyrogallol (50 mg/mL) were mixed in an Eppendorf vial and transferred to a cuvette. Depending on whether it was a blank or a sample, buffer or HRP, respectively was added (amounts are shown in Table 8). The absorbance at 420 nm was measured every 20 seconds for three minutes.

A concentration of 0.05 mg/mL of HRP were observed to give the best result

For immobilized HRP

dH_2O, buffer, 0.5 % H_2O_2 and Pyrogallol (50 mg/mL) was mixed in an Eppendorf vial (amounts in table 5) and transferred to the vial with 0.05 mg/mL immobilized HRP. These vials were stirred and after one, two and three minutes 200 µL were taken out and centrifuged (1 minute, 1000 rpm). 100-150 µL of the supernatant were transferred to a cuvette and the absorbance at 420 nm was measured.

A blank sample was also tested were the mix of dH_2O, buffer, 0.5 % H_2O_2 and Pyrogallol (50 mg/mL) were added to a vial with PU and buffer.

Table 8: Amount of solutions used in the enzymatic assay of HRP.

	Blank	**Test**
1		
dH_2O	210 µL	210 µL
Buffer	32 µL	32 µL
H_2O_2	16 µL	16 µL
Pyrogallol	32 µL	32 µL
2		
Buffer	10 µL	-
Enzyme (HRP)	-	10 µL

4.8.3 Enzymatic assay using Amplex Red

Solutions described below was mixed in a vial, and a change in color was observed where HRP was active (turns pink). The change in color was detected by UV/Vis at 580 nm.

Blank:

2.5 µL of amplex red (10 mM), 25 µL of H_2O_2 (500 µM) and 222.5 µL of blank (PU mixed with buffer) were mixed in a vial.

For HRP in solution:

197.5 µL citrate buffer (0.1 M), 2.5 µL of amplex red (10 mM), 25 µL of H_2O_2 (500 µM) and 25 µL HRP (1 mg/mL) were mixed in a vial.

Immobilized HRP:

2.5 µL of amplex red (10 mM), 25 µL of H_2O_2 (500 µM) and 222.5 µL of immobilized HRP (0.1 mg/mL) were mixed in a vial.

4.8.4 Enzymatic assay using ABTS

The solutions in table 8 were mixed in Eppendorf vials, 5 µL of both HRP in solution and immobilized HRP were used. A change in color (turns dark blue/black) was observed where HRP was active.

Table 9 solutions used for enzymatic assay using ABTS

	Blank	**Test**
9.1 mM ABTS	290 µL	290 µL
0.3 % H_2O_2	10 µL	10 µL
Blank sample (PU and buffer)	5 µL	-
HRP	-	5 µL

4.8.5 Reuse of immobilized HRP

Using filter-plate

First a filter-plate was used, the reaction mixture was added to the filter and vacuum was used to filtrate the sample. A new reaction mixture was added to the filter-well, and a new collecting plate was used. Between reaction 1-5 the filter was not cleaned, but it was cleaned with buffer between reaction five and six.

Using a Büchner funnel

The reaction mixture was added to the funnel and after three minutes water-vacuum were used to filtrate the sample. Three washing steps (with buffer) were performed between each reaction. A new reaction mixture was added (without HRP), after every reaction the mixture was transferred to an Eppendorf vial.

4.9 Cultivation of His10-ChiA-D313N-F296W

4.9.1 Solutions used for cultivation of His10-ChiA-D313N-F296W

Solution	Content
For cultivating	
LB-medium	10 g Bacto Tryptone 5 g Bacto Yeast extract 10 g NaCl Fill it up to 1 L with dH_20
Ampicillin (100 mg/mL)	1,0 g ampicillin was dissolved in 10 ml dH_2O and filtrated through a 0,22 µm syringe filter.
IPTG (1M)	0,238 g IPTG was dissolved in 1 mL dH_2O
For periplasmatic and cytoplasmatic extract	
1 M Tris-HCl, pH 8.0	121,1 g Tris-Base in 800 mL of dH_2O Adjusted pH with HCl, added dH_2O until the total volume was 1 L.
1 M Tris-HCl, pH 7.5	Used 100 mL of 1 M Tris-HCl, pH 8.0 and adjusted pH using HCl.
0,5 M EDTA	46,5 g EDTA was dissolved in 200 mL dH_2O, adjusted the pH to 8.0 with NaOH pellets (a lot of pellets!). Then the final volume was adjusted to 250 mL (the pH was first adjusted to 8.3 and was then adjusted to 8.0 with acetic acid).
1 M $MgCl_2$	20,33 grams of MgCl was dissolved in 100 mL of dH_2O.
1 M NaCl	5,844 grams of NaCl was dissolved in 100 mL of dH_2O.
Spheroplast buffer	10 mL 1 M Tris-HCl, pH 8.0 17,1 g sucrose 1 tablet protease inhibitor 100 µL 0,5 M EDTA Adjust volume to 100 mL with dH_2O
Lysis buffer	7,5 mL 1 M Tris-HCl, pH 7.5 300 µL 0,5 M EDTA 600 µL 1 M $MgCl_2$ 150 mg lysozyme

	2 tablets protease inhibitor 7,5 mL 1 M NaCl Adjust volume to 150 mL with dH_2O
For purification	
Buffer A: Binding buffer (250 mL)	5 mL 1 M Tris-HCl, pH 8.0 (20 mM 1 M Tris-HCl, pH 8.0) 0,0851 g Imidazole (5 mM) 7,305 g NaCl (0,5 M) Adjust volume to 250 mL with dH_2O
Buffer B: Cleaning buffer (500 mL)	10 mL 1 M Tris-HCl, pH 8.0 (20 mM 1 M Tris-HCl, pH 8.0) 0,6808 g Imidazole (20 mM) 14,61 g NaCl (0,5 M) Adjust volume to 500 mL with dH_2O
Buffer C: Eluting buffer (250 mL)	5 mL 1 M Tris-HCl, pH 8.0 (20 mM 1 M Tris-HCl, pH 8.0) 4,255 g Imidazole (250 mM) 7,305 g NaCl (0,5 M) Adjust volume to 250 mL with dH_2O
0,5 M NaOH	2 g NaOH was dissolved in 100 mL dH_2O
20 % EtOH	21 mL 96 % EtOH diluted to a total volume of 100 mL.
For SDS-PAGE	
SDS-buffer	4x sample buffer 10x reducing agent dH_2O
MOPS buffer	20 X Mops SDS running buffer was diluted to a 1 X Mops SDS buffer.
Staining-solution	0.1 % Coomassie Brilliant Blue 10 % ethanol 10 % acetic acid
De-staining solution	10 % ethanol 7.5 % acetic acid
Concentration of enzyme	
50 mM NaAc buffer (pH 6,1)	48 mL, 0.2 M acetic acid 572 mL 0.2 M NaAc Dilute to a volume of 2 L pH was adjusted to 6.1

4.9.2 Method for cultivation of His10-ChiA-D313N-F396W

Overnight cultures were made in culture flasks by mixing 50 mL LB-medium, 25 µL ampicillin (100 mg/mL) and cells from a stock in the freezer. The cultures were incubated overnight (37 °C and 200 rpm).

The next day the cell density (OD) was measured in the cultures and was inoculated to 0.1 OD value in 1 L culture flask with 250 mL LB-medium and 250 µL ampicillin (100 mg/mL). These cultures were incubated (37 °C and 200 rpm), until the OD was between 0.8-1.0. In the next step the cell-cultures were induced with 250 µL ampicillin (100 mg/mL) and 25 µL IPTG (1 M) and was then incubated overnight (22 °C and 200 rpm).

The next day the cells were harvested by centrifugation in JA10 rotor for 15 minutes (4 °C, 8000 rpm). The cells were transferred to ziplock bags and placed in the freezer.

Periplasmatic extraction

Frozen cells were dissolved in spheroplast buffer, transferred to centrifuge-tubes, and incubated on ice for 5 minutes. After they were centrifuged for 10 minutes (8000 rpm and 4 °C), the pellets were incubated for 10 minutes in room temperature. Then the pellets were dissolved in 15 mL cold dH_2O and incubated on ice for 45 second, 1,5 mL $MgCl_2$ (1 M) was added, and centrifuged for 10 minutes (8000 rpm and 4 °C). The supernatant was filtrated and stored at 4 °C. The pellets were used in the next step, cytoplasmatic extraction.

Cytoplasmatic extraction

Each pellet from the previous step was dissolved in 30 mL lysis buffer, and was incubated for 30 minutes at 37 °C. After the suspension were centrifugated for 20 minutes (8000 rpm and 4 °C). The supernatant was filtrated and was stored at 4 °C.

Purification of periplasmatic and cytoplasmatic extract

A column with 7 mL Ni-NTA was prepared and equilibrated with buffer A for 10 minutes (2.5 mL/min flow). First the periplasmatic extract (PPE) was added to the column, then buffer B was added until baseline (the void was collected in a flask, PPE-void). Buffer C was then added, and the eluate was collected in a new flask (PPE-eluate).

To purify the cytoplasmatic extract (CPE), the buffer B was used until baseline in addition to 0.5 M NaOH for approx. 10 minutes to clean the column. The column was equilibrated with

buffer A (for 10 minutes), before the cytoplasmatic extract was added. Again, buffer B was added until baseline (the void was collected in a flask, CPE-void). Buffer C was used to elute the enzyme, and the eluate was collected in a new flask (CPE-eluate). The column was cleaned with buffer B until baseline, 10 minutes of NaOH (0.5 M) and 10 minutes of 20 % EtOH.

SDS-PAGE

SDS-PAGE were used to separate protein by molecular mass and are described in section 1.4.2.

PPE, PPE-void, PPE-eluate, CPE, CPE-void and CPE-eluate were tested for protein-content on an SDS-PAGE gel. 10 µL SDS-buffer and 10 µL of the protein solution were mixed and denatured at 70 °C for 10 minutes.

A precasted NuPAGE 10 % Bis-Tris Gel was placed in an electrophoresis chamber, and the chamber was filled with 1 x MOPS buffer. 7.5 µL of benchmark™ Protein ladder and 20 µL of the samples treated with SDS-buffer were added to the gel, and a power of 200 V was applied for 50 minutes. The gel was colored by staining solution in the microwave for 1 minute, and then shaking for 5 min. Then de-staining solution was added to the gel, with heating in the microwave for 1 minute and incubated with shaking for 5-10 minutes. De-staining step was repeated until the protein-bonds were visible.

Concentration of enzyme

Samples that indicated protein-content at 58 kDa (ChiA enzyme) on the SDS-PAGE gel, were concentrated using a Macrosep® Advance Centrifugal Device (from PALL Corporation). This to get a higher concentration of ChiA and change the buffer.
Method: Protein solution were transferred to a centrifugal filter with pore-size 30 kDa. The tube was centrifuged (4 °C, 4500 rof.). When the final volume of protein-solution was 2-5 mL, a new buffer (50 mM NaAc buffer, pH 6.1) was added, and again centrifuged (4°C, 4500 rof.). This buffer was added two more times, and the final volume was again 2-5 mL.

Bradford Assay

Bradford assay was used to determine the concentration of ChiA.
Method: 5 µL of concentrated enzyme was diluted with 795 µL of 50 mM NaAc buffer, pH 6.1 and mixed with 200 µL Coomassie Brilliant Blue, after 5 minutes the concentration was measured at a wavelength of 595 nm using spectrophotometer, Biophotometer.

5. References

1. Berg, J. M.; Tymoczko, J. L.; Gatto Jr., G. J.; Stryer, L., *Biochemistry*. **2015.** pp 215-285 ISBN 978-1-4641-2610-9.
2. Anthonsed, T., *Applied Biocatalysis*. **2005**. pp. 18-20. ISBN: 0-203-30563-9
3. Lesk, A. M., *Introduction to Protein Science*. **2016**. pp. 1-85. ISBN: 978-0-19-871684-6.
4. Elansary, M. Prediction of protein aggregation inducing mutations. **2012.**
5. Richter, M., *Natural Product Reports* **2013,** *30* (10), pp 1324-1345.
6. Blanco, A.; Blanco, G., *Medical Biochemistry*, **2017**. pp 153-175. ISBN: 978-0-12-803550-4.
7. Poulos, T. L., *Heme Enzymes*. **2005**; 6 (2), p 33-45.
8. May, S. W., *Current Opinion in Biotechnology* **1999,** *10* (4), pp 370-375.
9. Paul, P. E. V.; Sangeetha, V.; Deepika, R. G., *Recent Developments in Applied Microbiology and Biochemistry*, **2019**; pp 107-125. ISBN: 978-0-12-816328-3
10. Brurberg, M. B.; Synstad, B.; Klemsdal, S. S.; van Aalten, D. M.; Sundheim, L.; Eijsink, V. G., *Recent Res. Dev. Microbiol* **2001,** *5*, pp 187-204.
11. Banci, L., *Journal of Biotechnology* **1997,** *53* (2), pp 253-263.
12. Ayala, M.; Verdin, J.; Vazquez-Duhalt, R., *Biocatalysis and Biotransformation* **2007,** *25* (2-4), pp 114-129.
13. Sheldon, R. A., *Advanced Synthesis & Catalysis* **2007,** *349* (8-9), pp 1289-1307.
14. Regalado, C.; García-Almendárez, B. E.; Duarte-Vázquez, M. A., *Phytochemistry Reviews* **2004,** *3* (1), pp 243-256.
15. Veitch, N. C., *Phytochemistry* **2004,** *65* (3), pp 249-259.
16. Bank, R. p. D., Structure of horseradish peroxidase C1A compound 1. **2001.**
17. Sigmaaldric Enzymatic Assay of Peroxidase (EC 1.11.1.7). https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-peroxidase.html.
18. Ly, H. D.; Withers, S. G., *Annual Review of Biochemistry* **1999,** *68* (1), pp 487-522.
19. Rye, C. S.; Withers, S. G., *Current Opinion in Chemical Biology* **2000,** *4* (5), pp 573-580.
20. Zakariassen, H.; Hansen, M. C.; Jøranli, M.; Eijsink, V. G. H.; Sørlie, M., *Biochemistry* **2011,** *50* (25), pp 5693-5703.
21. Ravi Kumar, M. N. V., *Reactive and Functional Polymers* **2000,** *46* (1), pp 1-27.
22. Hines, D. A.; Saurugger, P. N.; Ihler, G. M.; Benedik, M. J., G. *Journal of Bacteriology* **1988,** *170* (9), pp 4141-4146.
23. Madhuprakash, J.; Dalhus, B.; Rani, T. S.; Podile, A. R.; Eijsink, V. G. H.; Sørlie, M., *Biochemistry* **2018,** *57* (29), pp 4325-4337.
24. Taira, T.; Fujiwara, M.; Dennhart, N.; Hayashi, H.; Onaga, S.; Ohnuma, T.; Letzel, T.; Sakuda, S.; Fukamizo, T., T. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **2010,** *1804* (4), pp 668-675.
25. Lü, Y.; Yang, H.; Hu, H.; Wang, Y.; Rao, Z.; Jin, C., *Glycoconjugate Journal* **2008,** *26* (5), pp 525-534.
26. Vaaje-Kolstad, G.; Horn, S. J.; Sørlie, M.; Eijsink, V. G. H., *The FEBS Journal* **2013,** *280* (13), pp 3028-3049.
27. Reese, E. T.; Siu, R. G. H.; Levinson, H. S., *Journal of bacteriology* **1950,** *59* (4), pp 485-497.
28. Vaaje-Kolstad, G.; Westereng, B.; Horn, S. J.; Liu, Z.; Zhai, H.; Sørlie, M.; Eijsink, V. G. H., *Science* **2010,** *330* (6001), pp 219-222.

29. Levasseur, A.; Drula, E.; Lombard, V.; Coutinho, P. M.; Henrissat, B., *Biotechnology for Biofuels* **2013,** *6* (1),
30. Forsberg, Z.; Vaaje-Kolstad, G.; Westereng, B.; Bunæs, A. C.; Stenstrøm, Y.; MacKenzie, A.; Sørlie, M.; Horn, S. J.; Eijsink, V. G. H., *Protein Science* **2011,** *20* (9), pp 1479-1483.
31. Bissaro, B.; Várnai, A.; Røhr, Å. K.; Eijsink, V. G. H., *Microbiology and Molecular Biology Reviews* **2018,** *82* (4), pp e00029-18.
32. Quinlan, R. J.; Sweeney, M. D.; Lo Leggio, L.; Otten, H.; Poulsen, J.-C. N.; Johansen, K. S.; Krogh, K. B. R. M.; Jørgensen, C. I.; Tovborg, M.; Anthonsen, A.; Tryfona, T.; Walter, C. P.; Dupree, P.; Xu, F.; Davies, G. J.; Walton, P. H., *Proceedings of the National Academy of Sciences* **2011,** *108* (37), pp 15079-15084.
33. Walton, P. H.; Davies, G. J., *Current Opinion in Chemical Biology* **2016,** *31*, pp 195-207.
34. Bissaro, B.; Røhr, Å. K.; Müller, G.; Chylenski, P.; Skaugen, M.; Forsberg, Z.; Horn, S. J.; Vaaje-Kolstad, G.; Eijsink, V. G. H., *Nature Chemical Biology* **2017,** *13* (10), pp 1123-1128.
35. Bissaro, B.; Kommedal, E.; Røhr, Å. K.; Eijsink, V. G. H., *Nature Communications* **2020,** *11* (1).
36. Cannella, D.; Möllers, K. B.; Frigaard, N. U.; Jensen, P. E.; Bjerrum, M. J.; Johansen, K. S.; Felby, C., *Nature Communications* **2016,** *7* (1).
37. Bissaro, B.; Forsberg, Z.; Ni, Y.; Hollmann, F.; Vaaje-Kolstad, G.; Eijsink, V. G. H., *Green Chemistry* **2016,** *18* (19), pp 5357-5366.
38. Fisher, C. H., *Pioneers in Polymer Science*, **1989**; pp 47-61. ISBN: 978-94-009-2407-9
39. Gomaa, A. M., *Journal of Nutrition and Foot Science Forecast* **2018,** *1* (1), pp 1-5.
40. Bilal, M.; Asgher, M.; Cheng, H.; Yan, Y.; Iqbal, H. M. N., *Critical Reviews in Biotechnology* **2019,** *39* (2), pp 202-219.
41. Tran, D. N.; Balkus, K. J., *ACS Catalysis* **2011,** *1* (8), pp 956-968.
42. Nelson, J. M.; Griffin, E. G., *Journal of the American Chemical Society* **1916,** *38* (5), pp 1109-1115.
43. Budriene, S.; Romaskevic, T.; Pielichowski, K.; Pielichowski, J., *Polymers for Advanced Technologies* **2007,** *18* (1), pp 67-71.
44. Hanefeld, U.; Gardossi, L.; Magner, E., *Chem Soc Rev* **2009,** *38* (2), pp 453-468.
45. easybiologyclass Enzyme Immobilization

Techniques of Enzyme & Whole Cell Immobilization.
https://www.easybiologyclass.com/enzyme-cell-immobilization-techniques/.
46. Lotha, G.; et.al Polymer. https://www.britannica.com/science/polymer (accessed 21.02.2020).
47. Carey, F. A., *Organic chemistry*. **2006.** pp 1242-1271. ISBN: 0-07-111562-5
48. Lithner, D.; Larsson, Å.; Dave, G., E., *Science of The Total Environment* **2011,** *409* (18), pp 3309-3324.
49. Szycher, M., *Szycher's Handbook of Polyurethanes*. **1999**. pp 1.1-1.9. ISBN: 0-8493-0602-7
50. Heath, D.; Cooper, S., A. Polyurethanes. **2013**; p 79-82. DOI:10.1016/B978-0-08-087780-8.00009-7
51. Shelke, N. B.; Nagarale, R. K.; Kumbar, S. G., *Natural and Synthetic Biomedical Polymers*, **2014;** pp 123-144. ISBN: 978-0-12-396983-5
52. AmericanChemistryCouncil Introduction to Polyurethanes: History. https://polyurethane.americanchemistry.com/History/ (accessed 21.02.2020).

53. Kauffman, G. B.; Gent, A. N.; Bierwagen, G. P.; Stevens, M. P.; Preston, J.; Rodrigues, F. Major Industrial Polymer. https://www.britannica.com/topic/industrial-polymers-468698 (accessed 21.02.2020).
54. AmericanChemistryCouncil Introduction to Polyurethanes: Polyurethane Applications. https://polyurethane.americanchemistry.com/Applications/ (accessed 05.06).
55. Romaškevič, T.; Budrienė, S.; Pielichowski, K.; Pielichowski, J., *Chemija* **2006,** *17* (4), pp 74-89.
56. Janik, H.; Sienkiewicz, M.; Kucinska-Lipka, J., *Handbook of Thermoset Plastics*. **2013**. pp 253-290. ISBN: 978-1-4557-3107-7
57. Kochane, T.; Budriene, S.; Miasojedovas, S.; Ryskevic, N.; Straksys, A.; Maciulyte, S.; Ramanaviciene, A., *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2017,** *532*, pp 436-443.
58. Phadtare, S.; Kumar, A.; Vinod, V. P.; Dash, C.; Palaskar, D. V.; Rao, M.; Shukla, P. G.; Sivaram, S.; Sastry, M., *Chemistry of Materials* **2003,** *15* (10), pp 1944-1949.
59. Zdarta, J.; Meyer, S. A.; Jesionowski, T.; Pinelo, M., *Catalysts* **2018,** *8* (2).
60. Kappe, C. O.; Dallinger, D.; Murphree, S. S., *Practical Microwave Synthesis for Organic Chemist*. **2009**. pp 11-200. ISBN: 978-3537-32097
61. Gaba, M.; Passi, N., *Indian Journal of Pharmaceutical Education and Research* **2010**.
62. Giguere, R. J.; Bray, T. L.; Duncan, S. M.; Majetich, G., *Tetrahedron Letters* **1986,** *27* (41), pp 4945-4948.
63. Gedye, R. N.; Smith, F. E.; Westaway, K. C., *Canadian Journal of Chemistry* **1988,** *66* (1), pp 17-26.
64. de la Hoz, A.; Díaz-Ortiz, Á.; Moreno, A., *Chem Soc Rev* **2005,** *34* (2), pp 164-178.
65. Zong, L.; Zhou, S.; Sgriccia, N.; Hawley, M. C.; Kempel, L. C., *Journal of Microwave Power and Electromagnetic Energy* **2003,** *38* (1), pp 49-74.
66. Laemmli, U. K., *Nature* **1970,** *227* (5259), pp 680-685.
67. AppliedproteinTechnology., Purity analysis SDS-PAGE. NA. http://www.aptbiotech-en.com/Cn/Index/pageView/catid/80.html (accessed 02.07).
68. Merck Enzymatic Assay of Peroxidase (EC 1.11.1.7). https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-peroxidase.html (accessed 08.06).
69. Scientific, T. Amplex Red Reagent. https://www.thermofisher.com/order/catalog/product/A12222?CID=AFLBC-A12222#/A12222?CID=AFLBC-A12222 (accessed 22.06).
70. Merck Enzymatic Assay of Peroxidase (EC.1.11.1.7) 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid) as a Substrate. https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-peroxidase-abts-as-substrate.html (accessed 12.06).
71. Bradford, M. M., *Analytical Biochemistry* **1976,** *72* (1), pp 248-254.
72. Ninfa, A. J.; Ballou, D. P.; Benore, M., *Fundamental Laboratory Approaches for Biochemistry and Biotechnology*. **2010.** pp 113 ISBN: 978-0-470-08766-4
73. Bornhorst, J. A.; Falke, J. J., *Methods in Enzymology*, **2000**; 326(1), pp 245-254.
74. CaframoLabSolutions., Protein Affinity Chromatography. **2016**. https://www.caframolabsolutions.com/application/homogenizing/protein-affinity-chromatography/ (accessed 02.07)
75. Romaskevic, T.; Viskantiene, E.; Budriene, S.; Ramanaviciene, A.; Dienys, G., *Journal of Molecular Catalysis B: Enzymatic* **2010,** *64* (3), pp172-176.
76. Fernandes, K. F.; Lima, C. S.; Pinho, H.; Collins, C. H., *Process Biochemistry* **2003,** *38* (9), pp 1379-1384.

Appendix A – Immobilization results using PU with 1:2 mole ratio

Immobilization of HRP using PU synthesized with 1:2 mole ratio between PVA and HMDI

Non-modified PU

Sample	PU (g)	Abs 403	C (µM)	% immobilized
Only HRP		0.2503	2.503	
2.1.1.1	0.0236	0.1876	1.876	25.05
2.1.1.2	0.0484	0.2019	2.019	19.33
2.1.1.3	0.0675	0.197	1.970	21.28
Only HRP		0.2451	2.451	
2.1.1.4	0.034	0.1914	1.914	21.9
2.1.1.5	0.0588	0.2286	2.286	6.736
2.1.1.6	0.0895	0.2233	2.233	8.915
Only HRP		0.3314	3.314	
2.1.1.7	0.0316	0.3056	3.056	7.774
2.1.1.8	0.065	0.2882	2.882	13.03
2.1.1.9	0.0709	0.2825	2.825	14.74
Only HRP		0.3297	3.297	
2.1.1.10	0.0239	0.3182	3.182	3.494
2.1.1.11	0.0651	0.3232	3.232	1.984
2.1.1.12	0.0914	0.3132	3.132	5.013

PU/Ag-Nps and PU/Au-Nps

Sample	PU/Ag-Nps (g)	Abs 403	C (µM)	% immobilized
Only HRP		0.2793	2.703	
2.1.1.13	0.0208	0.3023	3.023	-8.227
2.1.1.14	0.0467	0.2964	2.964	-6.119
2.1.1.15	0.0795	0.3128	3.128	-11.97
Only HRP		0.2524	2.524	
2.1.1.16	0.0246	0.2199	2.199	12.85
2.1.1.17	0.0440	0.2101	2.101	16.75
2.1.1.18	0.0697	0.1743	1.743	30.92
Only HRP		0.2989	2.989	
2.1.1.19	0.0260	0.2608	2.608	12.75
2.1.1.20	0.0563	0.2539	2.539	15.07
2.1.1.21	0.0788	0.2349	2.349	21.41
Only HRP		0.3297	3.297	
2.1.1.22	0.0229	0.3240	3.240	1.741
2.1.1.23	0.0449	0.3084	3.084	6.463
2.1.1.24	0.0858	0.3028	3.028	8.177
Sample	**PU/Au-Nps (g)**	**Abs 403**	**C (µM)**	**% immobilized**
only HRP		0.2995	2.995	
2.1.1.25	0.0404	0.2708	2.708	9.584
2.1.1.26	0.0511	0.2585	2.585	13.68
2.1.1.27	0.0640	0.2530	2.530	15.50
only HRP		0.3297	3.297	
2.1.1.28	0.0175	0.3027	3.027	8.192
2.1.1.29	0.0503	0.3172	3.172	3.791
2.1.1.30	0.1092	0.2916	2.916	11.55

Test on different amounts of non-modified PU and PU/Au-Nps and different amount of HRP.

Sample	HRP (mL)	PU (g)	Abs 403	C (µM)	% immobilized
only HRP			0.3051	3.051	
2.1.2.1	0.5	0.0302	0.2926	2.926	4.087
2.1.2.2	1	0.0248	0.2769	2.769	9.253
2.1.2.3	2	0.024	0.2586	2.586	15.23
2.1.2.4	0.5	0.0500	0.2693	2.693	11.72
2.1.2.5	1	0.0518	0.2494	2.494	18.26
2.1.2.6	2	0.0511	0.2540	2.540	16.74
2.1.2.7	0.5	0.0926	0.2871	2.871	5.906
2.1.2.8	1	0.0907	0.2665	2.665	12.64
2.1.2.9	2	0.0898	0.2889	2.889	5.294
Sample	**HRP (mL)**	**PU/Au-Nps (g)**	**Abs 403**	**C (µM)**	**% immobilized**
only HRP			0.3046	3.046	
2.1.2.10	0.5	0.025	0.2967	2.967	2.613
2.1.2.11	1	0.0307	0.2785	2.785	8.591
2.1.2.12	2	0.0195	0.2771	2.771	9.028
2.1.2.13	0.5	0.0559	0.3024	3.024	0.745
2.1.2.14	1	0.0489	0.2875	2.875	5.637
2.1.2.15	2	0.058	0.2618	2.618	14.05
2.1.2.16	0.5	0.0925	0.3062	3.062	-0.519
2.1.2.17	1	0.0919	0.2751	2.751	9.704
2.1.2.18	2	0.0893	0.2885	2.885	5.292

Appendix B – Immobilization results using PU synthesized from new method

Immobilization results when 1:2 mole ratio between PVA and HMDI were used.

Sample	PU (g)	Abs 403	C (µM)	% immobilized
only HRP		**0.3268**	**3.268**	
C.1.1	0.0521	0.2083	2.083	36.27
C.1.2	0.0532	0.2404	2.404	26.43
C.1.3	0.0542	0.2617	2.617	19.92
Only HRP		**0.6983**	**6.983**	
C.1.4	0.0550	0.5364	5.364	23.18
C.1.5	0.0535	0.5571	5.571	20.23
C.1.6	0.0545	0.5805	5.805	16.88
Sample	**PU/Ag-Nps (g)**	**Abs 403**	**C (µM)**	**% immobilized**
Only HRP		0.3268	3.268	
C.1.7	0.0540	0.4823	4.823	-47.60
C.1.8	0.0531	0.3292	3.292	-0.7253
Sample	**PU/Au-Nps (g)**	**Abs 403**	**C (µM)**	**% immobilized**
Only HRP		**0.3268**	**3.268**	
C.1.9	0.0532	0.3133	3.133	4.122
C.1.10	0.0543	0.3260	3.260	0.2356
Only HRP		**0.6983**	**6.983**	
C.1.11	0.0510	0.6441	6.441	7.763
C.1.12	0.0545	0.6060	6.060	13.23

Immobilization when 1:5 of PVA and HMDI were used

Sample	PU (g)	Abs 403	C (µM)	% immobilized
only HRP		0.2759	2.759	
C.2.1	0.0264	0.2634	2.634	4.548
C.2.2	0.0252	0.2403	2.403	12.93
C.2.3	0.0262	0.2044	2.044	25.93
C.2.4	0.059	0.2538	2.538	8.042
C.2.5	0.0607	0.2511	2.511	9.013
C.2.6	0.055	0.2555	2.555	7.411

Sample	PU/Ag-Nps (g)	Abs 403	C (µM)	% immobilized
only HRP		0.2759	2.759	
C.2.7	0.0264	0.2790	2.790	-1.113
C.2.8	0.0295	0.2790	2.790	-1.120
C.2.9	0.028	0.2745	2.745	0.522
C.2.10	0.0541	0.2611	2.611	5.364
C.2.11	0.0488	0.2660	2.660	3.595
C.2.12	0.0537	0.2633	2.633	4.590
Sample	**PU/Au-Nps (g)**	**Abs 403**	**C (µM)**	**% immobilized**
only HRP		0.2759	2.759	
C.2.13	0.0255	0.2614	2.614	5.277
C.2.14	0.0298	0.1895	1.895	31.33
C.2.15	0.0292	0.2379	2.379	13.78
C.2.16	0.0577	0.1968	1.968	28.69
C.2.17	0.0485	0.2199	2.199	20.32
C.2.18	0.0493	0.2245	2.245	18.65

Immobilization using PVA and 4,4’-methylenebis (cyclohexyl isocyanate) (1:5 mole ratio)

Sample	PU (g)	Abs 403	C (µM)	% immobilized
only HRP		0.3105	3.105	
C.3.1	0.0499	0.1859	1.859	40.13
C.3.2	0.0535	0.1655	1.655	46.69
C.3.3	0.0501	0.1717	1.717	44.71
Sample	**PU/Ag-Nps (g)**	**Abs 403**	**C (µM)**	**% immobilized**
Only HRP		0.3105	3.105	
C.3.4	0.0531	0.2716	2.716	12.55
C.3.5	0.0547	0.2247	2.247	27.65
C.3.6	0.0560	0.2549	2.549	17.92
Sample	**PU/Au-Nps (g)**	**Abs 403**	**C (µM)**	**% immobilized**
only HRP		0.3105	3.105	
C.3.7	0.0512	0.1922	1.922	38.11
C.3.8	0.0520	0.2330	2.330	24.96
C.3.9	0.0537	0.1779	1.779	42.72

Appendix C – Immobilization results using homogenized PU (1:5 mole ratio)

Immobilization when 1:5 mole ratio between PVA and HMDI, addition of more DMSO when lumps were formed.

Sample	PU (g)	Abs 403	C (µM)	% immobilized
only HRP		0.3014	3.014	
C.4.1	0.0284	0.2327	2.327	22.81
C.4.2	0.0261	0.2432	2.432	19.30
C.4.3	0.0202	0.2308	2.308	23.43
C.4.4	0.0514	0.1996	1.996	33.76
C.4.5	0.0517	0.2007	2.007	33.42
C.4.6	0.0512	0.2062	2.062	31.59
C.4.7	0.1064	0.1784	1.784	40.81
C.4.8	0.1086	0.1772	1.772	41.21
C.4.9	0.1004	0.1702	1.702	43.54

Immobilization using 1:5 mole ratio between PVA and HMDI, no addition of DMSO when lumps were formed.

Sample	PU (g)	Abs 403	C (µM)	% immobilized
only HRP		0.3014	3.014	
C.5.1	0.0254	0.2277	2.277	24.44
C.5.2	0.0262	0.2071	2.071	31.30
C.5.3	0.025	0.2089	2.089	30.70
C.5.4	0.0512	0.1747	1.747	42.02
C.5.5	0.0517	0.1846	1.846	38.74
C.5.6	0.0504	0.1853	1.853	38.50
C.5.7	0.108	0.1418	1.418	52.94
C.5.8	0.1012	0.1407	1.407	53.33
C.5.9	0.1035	0.1296	1.296	56.99

Appendix D – Immobilization results using different buffers

Immobilization with citrate buffer and using PVA and HMDI (1:5 mole ratio). Sample C.6.1 – C.6.3 and C.7.1 – C.7.3 were synthesized using conventional heating, while sample M.1.1 – M.1.3, M.2.1 – M.2.3, and M.3.1 – M.3.3 were synthesized in the microwave.

Sample	PU(g)	Abs 403 nm	C (µM)	% immobilized
only HRP		0.2743	2.743	
C.6.1	0.0502	0.1271	1.271	53.65
C.6.2	0.0507	0.1514	1.514	44.81
C.6.3	0.0506	0.1557	1.557	43.23
C.7.1	0.0502	0.1444	1.444	47.36
C.7.2	0.0503	0.1670	1.670	39.12
C.7.3	0.0504	0.1572	1.572	42.68
M.1.1	0.0512	0.0965	0.965	64.82
M.1.2	0.0509	0.1188	1.188	56.70
M.1.3	0.0512	0.0802	0.802	70.77
M.2.1	0.0501	0.0718	0.718	73.83
M.2.2	0.0503	0.0818	0.818	70.16
M.2.3	0.0506	0.0903	0.903	67.08
M.3.1	0.0502	0.0987	0.987	64.02
M.3.2	0.0509	0.0998	0.998	63.61
M.3.3	0.0501	0.0958	0.958	65.06

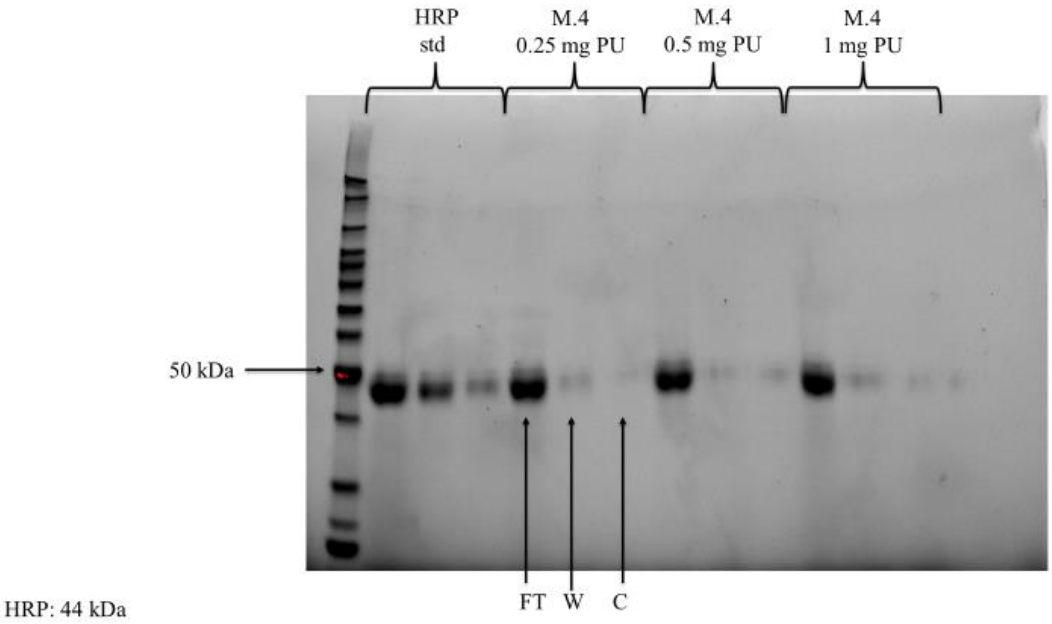
Immobilization with phosphate and using PVA and HMDI (1:5 mole ratio). Sample C.6.1 – C.6.3 and C.7.1 – C.7.3 were synthesized using conventional heating, while sample M.1.1 – M.1.3, M.2.1 – M.2.3, and M.3.1 – M.3.3 were synthesized in the microwave.

Sample	PU(g)	Abs 403 nm	C (µM)	% immobilized
Only HRP		0.2996	2.996	
C.6.1	0.0509	0.2382	2.382	20.50
C.6.2	0.0512	0.2294	2.294	23.42
C.6.3	0.0512	0.2122	2.122	29.17
C.7.1	0.0504	0.2352	2.352	21.49
C.7.2	0.0507	0.2247	2.247	24.99
C.7.3	0.0509	0.2226	2.226	25.71

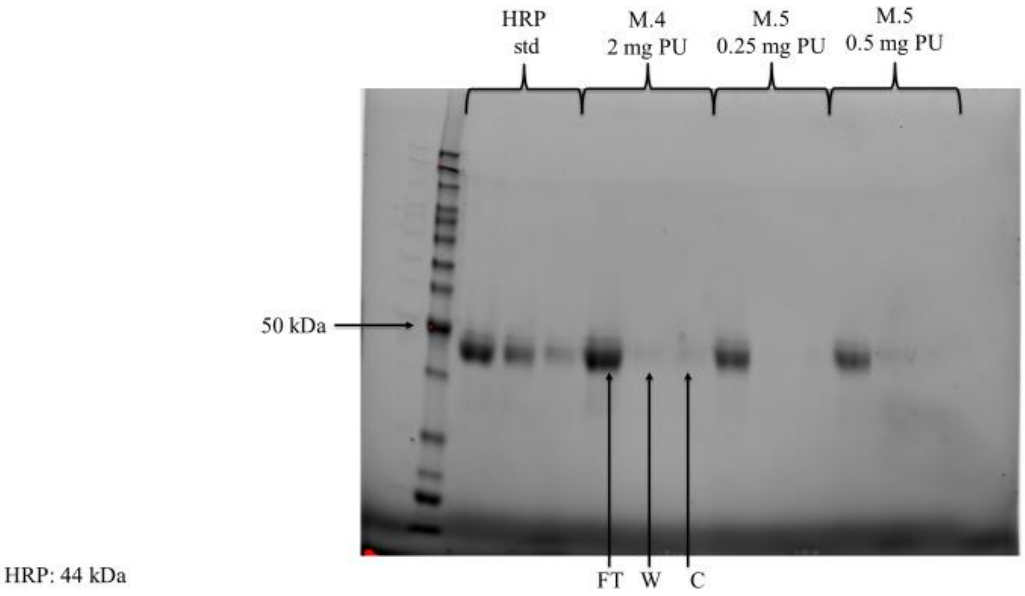
M.1.1	0.0509	0.1127	1.127	62.38
M.1.2	0.0507	0.1062	1.062	64.56
M.1.3	0.0502	0.0785	0.785	73.80
M.2.1	0.0512	0.1211	1.211	59.58
M.2.2	0.0503	0.1191	1.191	60.24
M.2.3	0.0506	0.1344	1.344	55.13
M.3.1	0.0511	0.1209	1.209	59.65
M.3.2	0.0501	0.0966	0.966	67.76
M.3.3	0.0508	0.1074	1.074	64.16

Appendix E – Detecting immobilization using SDS-PAGE

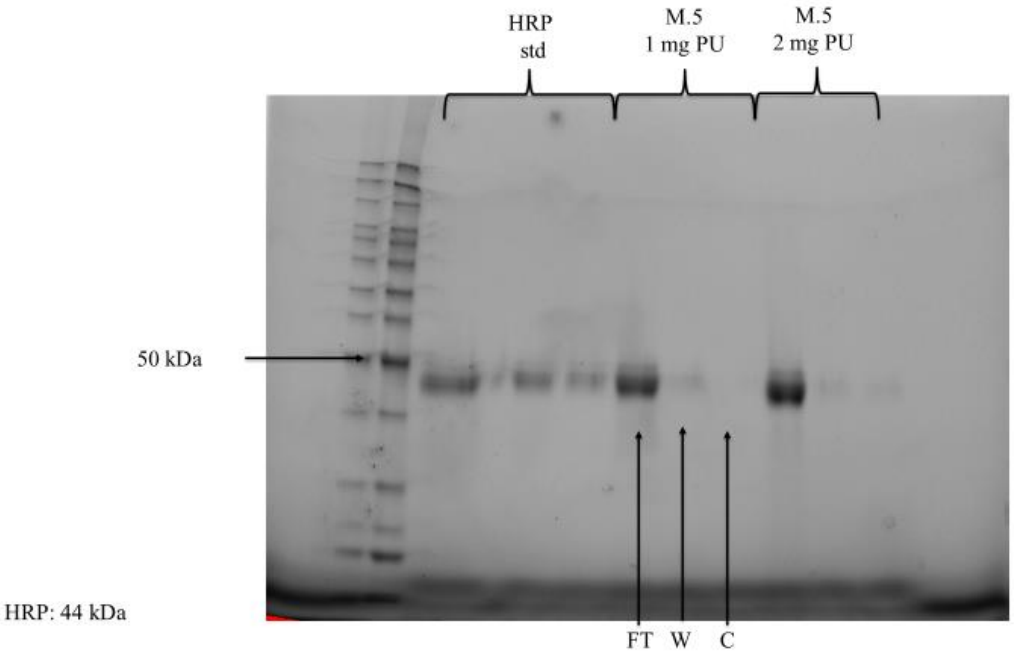
Immobilization results from sample M.4 – M.6 and C.8 and C.9. Sample number, amount PU and arrows indicating the pattern of FT, W and C are presented on each gel.

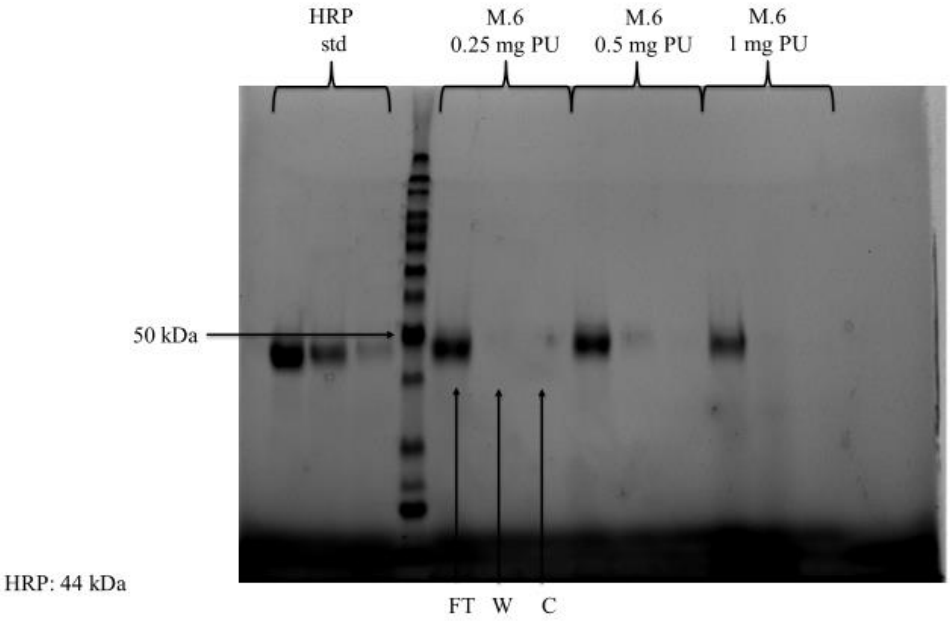


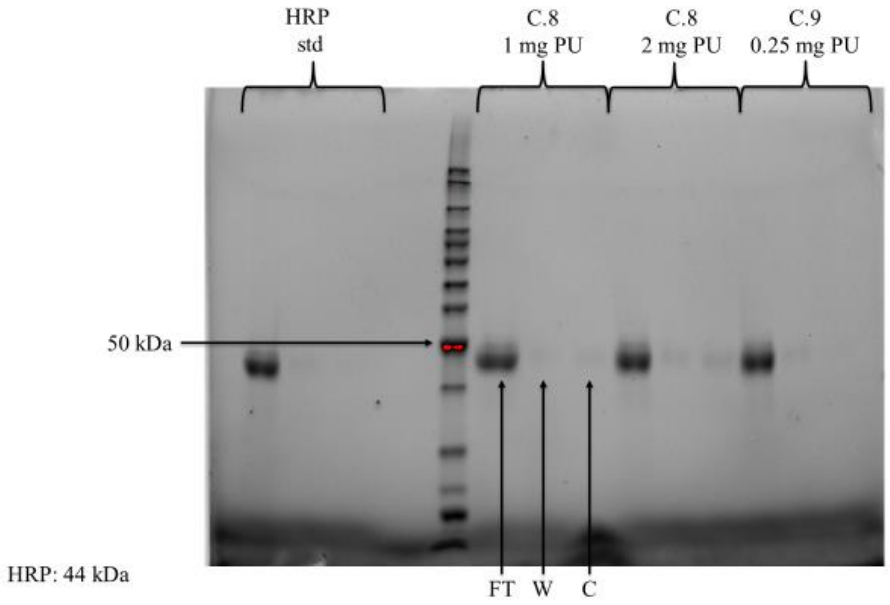
HRP: 44 kDa



HRP: 44 kDa

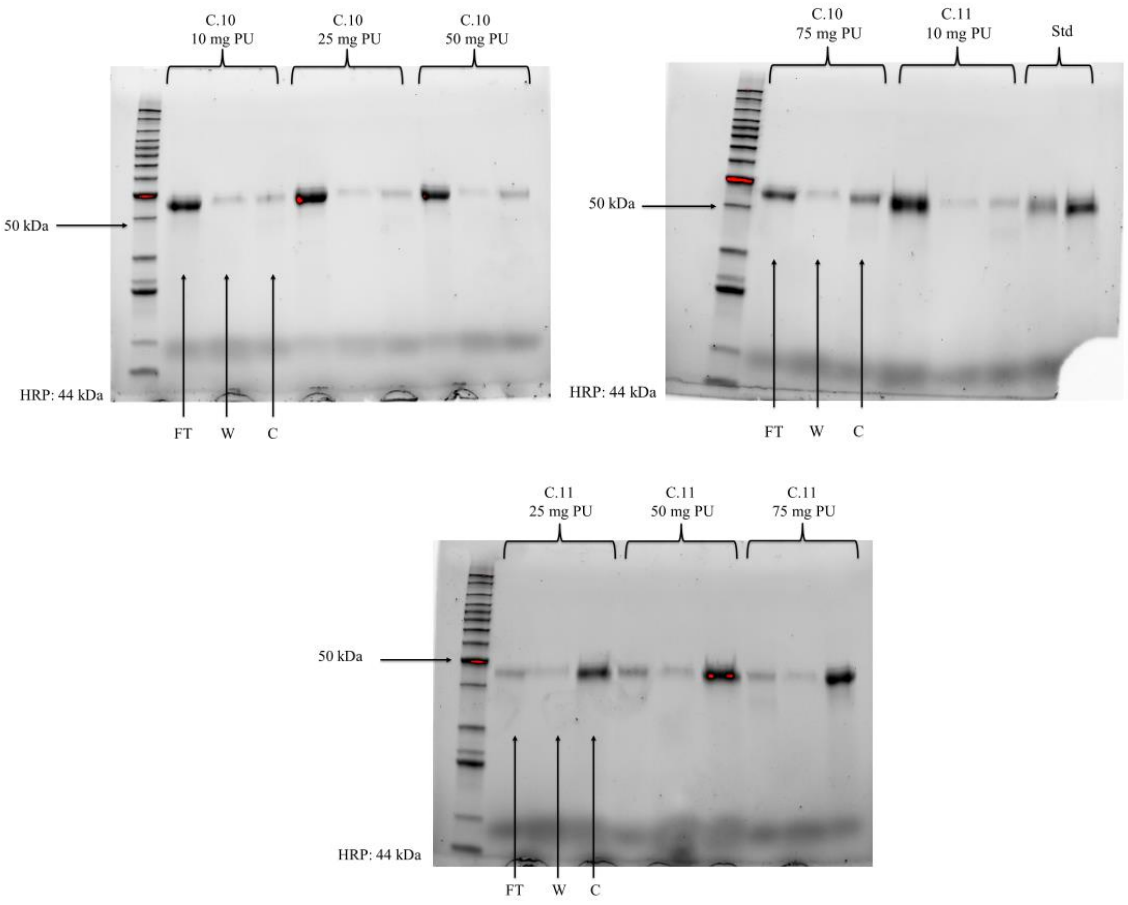

HRP
std
M.5
1 mg PU
M.5
2 mg PU
50 kDa
HRP: 44 kDa
FT
W
C


HRP
std
M.6
0.25 mg PU
M.6
0.5 mg PU
M.6
1 mg PU
50 kDa
HRP: 44 kDa
FT
W
C



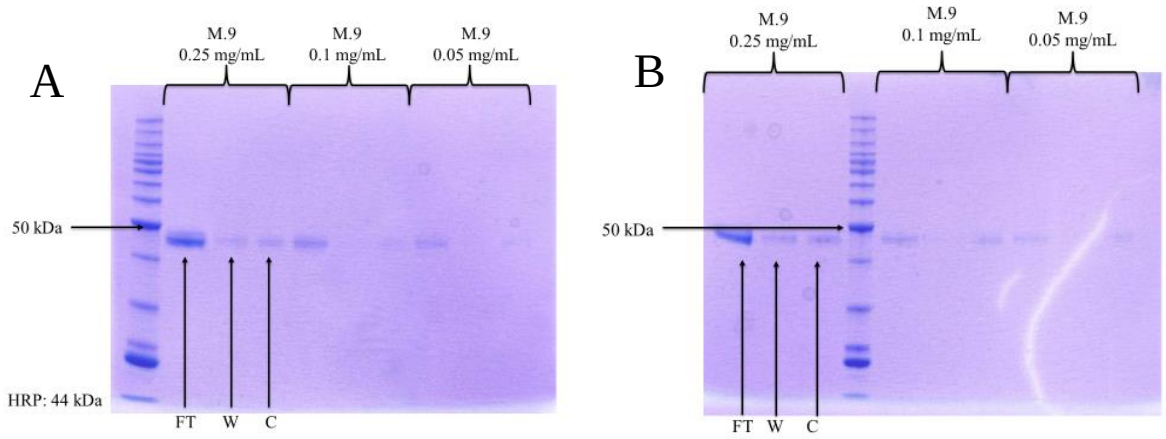
Appendix F – Saturation of PU from conventional heating

Saturation of PU, using different amount of PU with the same amount of HRP. Sample number, amount of PU, and arrows indicating the pattern of FT, W and C.



Appendix G – Saturation of PU from microwave heating

SDS-PAGE of attempt of saturation between PU and HRP. A is without stirring of sample, while B is with stirring overnight.



Nanodrop results and estimation of immobilized HRP

Sample	**Abs (403 nm)**	**Concentration (mg/mL) (in FT and W)**	**mg_{HRP} /mg_{PU}**	**% immobilized**
Blank (0.5 mg/mL) (0.25 mg/mL) (0.1 mg/mL) (0.05 mg/mL)	 0.830 0.415 0.120 0.0725	 0.3650 0.1826 0.0528 0.0319		
M.9 (0.25 mg/mL) No stirring	0.350	0.151	$0.84 * 10^{-3}$	17.27
M.9 (0.10 mg/mL) No stirring	0.130	0.056	-	-
M.9 (0.05 mg/mL) No stirring	0.060	0.025	$0.19 * 10^{-3}$	21.84
M.9 (0.25 mg/mL) Stirring	0.290	0.129	$1.42 * 10^{-3}$	29.32
M.9 (0.10 mg/mL) Stirring	0.040	0.018	$0.94 * 10^{-3}$	66.67
M.9 (0.05 mg/mL) Stirring	0.010	0.006	$0.69 * 10^{-3}$	81.61

Appendix H – Immobilization results using PU with different mole ratios

Results presented in figure 23

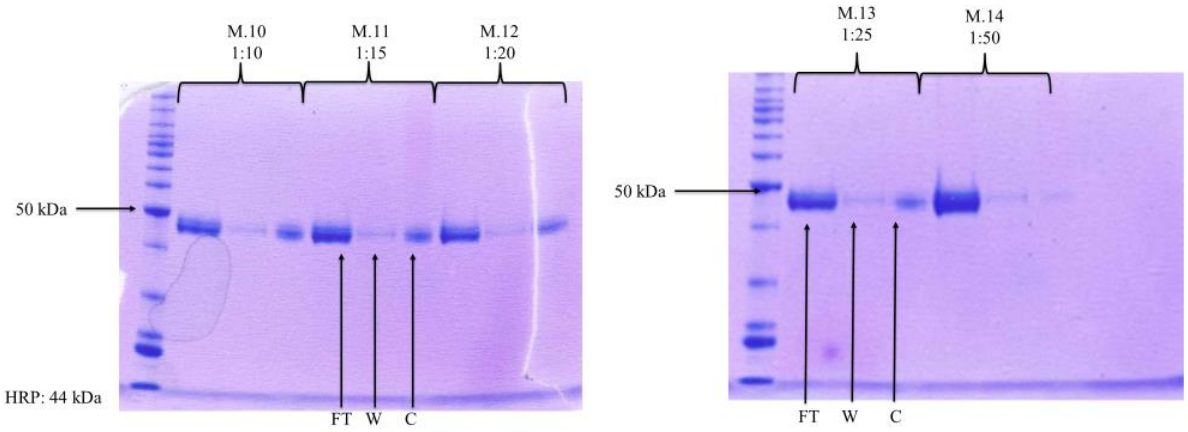
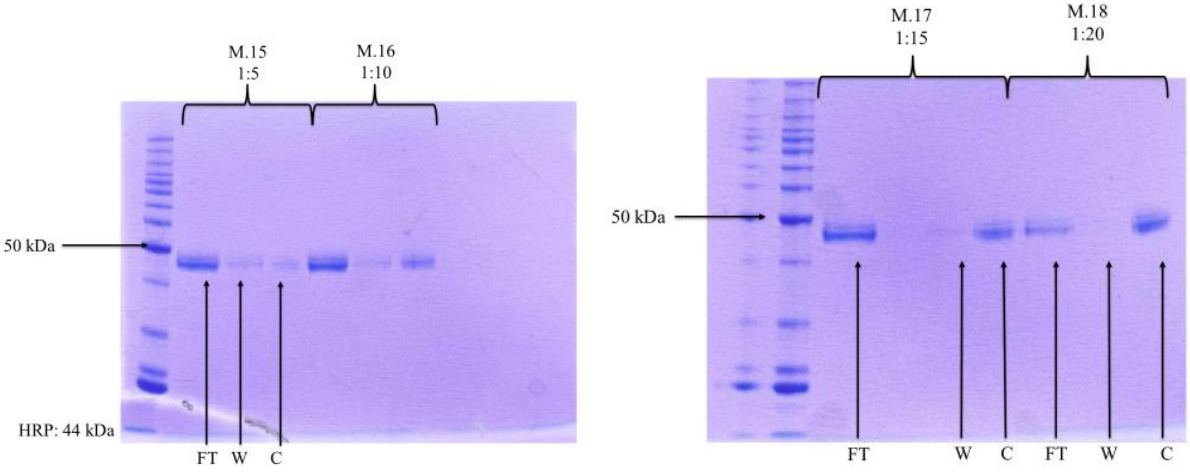


Figure 29: SDS-PAGE of FT, W, and C when sample M.10 – M.14 were used.

Sample	**Abs (403 nm)**	**Concentration (mg/mL) (in FT and W)**	**mg_{HRP} /mg_{PU}**	**% immobilized**
Blank (0.5 mg/mL)	0.830	0.3650		
M.10 (1:10)	0.223	0.098	$7.12 * 10^{-3}$	73.09
M.11 (1:15)	0.383	0169	$5.24 * 10^{-3}$	53.82
M.12 (1:20)	0.260	0.114	$6.69 * 10^{-3}$	68.67
M.13 (1:25)	0.387	0.170	$5.20 * 10^{-3}$	53.41
M.14 (1:50)	0.727	0.320	$1.21 * 10^{-3}$	12.45

SDS-PAGE indicating immobilization of sample M.15 – M.18.

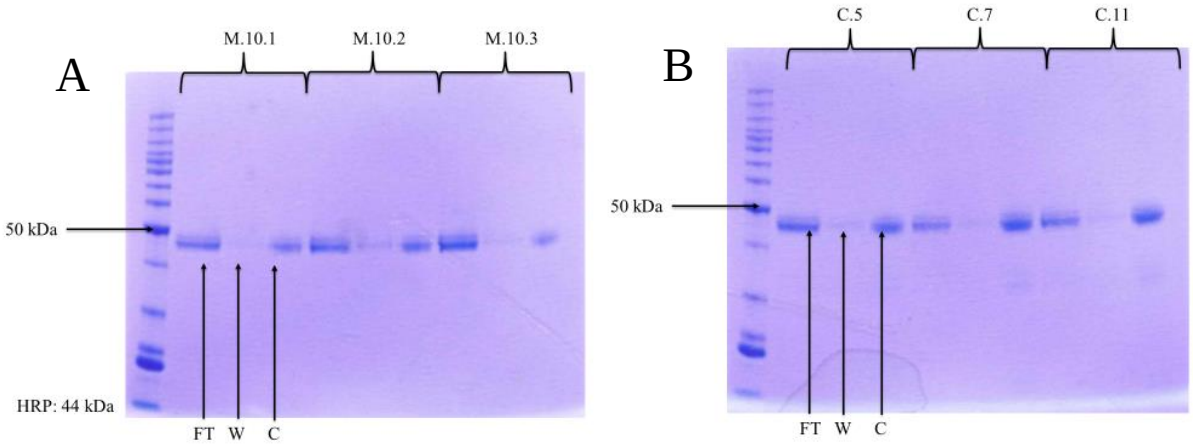


Nanodrop results and estimation of immobilized HRP

Sample	**Abs (403 nm)**	**Concentration (mg/mL) (in FT and W)**	**mg_{HRP} /mg_{PU}**	**% immobilized**
Blank (0.5 mg/mL)	0.830	0.3650		
M.15 (1:5)	0.263	0.150	$5.75 * 10^{-3}$	59.04
M.16 (1:10)	0.313	0173	$5.12 * 10^{-3}$	52.61
M.17 (1:15)	0.300	0.160	$5.48 * 10^{-3}$	56.22
M.18 (1:20)	0.137	0.075	$7.74 * 10^{-3}$	79.52

Appendix I – Immobilization results using stored PU

SDS-PAGE of stored PU



Nanodrop results and estimation of immobilized HRP

Sample	**Abs (403 nm)**	**Concentration (mg/mL) (in FT and W)**	**mg_{HRP} /mg_{PU}**	**% immobilized**
Blank (0.5 mg/mL)	0.830	0.3650		
M.10.1 (one week)	0.270	0.153	$5.67 * 10^{-3}$	58.23
M.10.2 (one week)	0.267	0.144	$5.91 * 10^{-3}$	60.64
M.10.3 (one week)	0.423	0.208	$4.19 * 10^{-3}$	42.97
C.5 (approx. 90 days)	0.390	0.197	$4.50 * 10^{-3}$	46.18
C.7 (4 weeks)	0.210	0.110	$6.81 * 10^{-3}$	69.88
C.11 (2 weeks)	0.300	0.155	$5.59 * 10^{-3}$	57.43

Appendix J – Particle size

Data used for particle distribution, figure XX

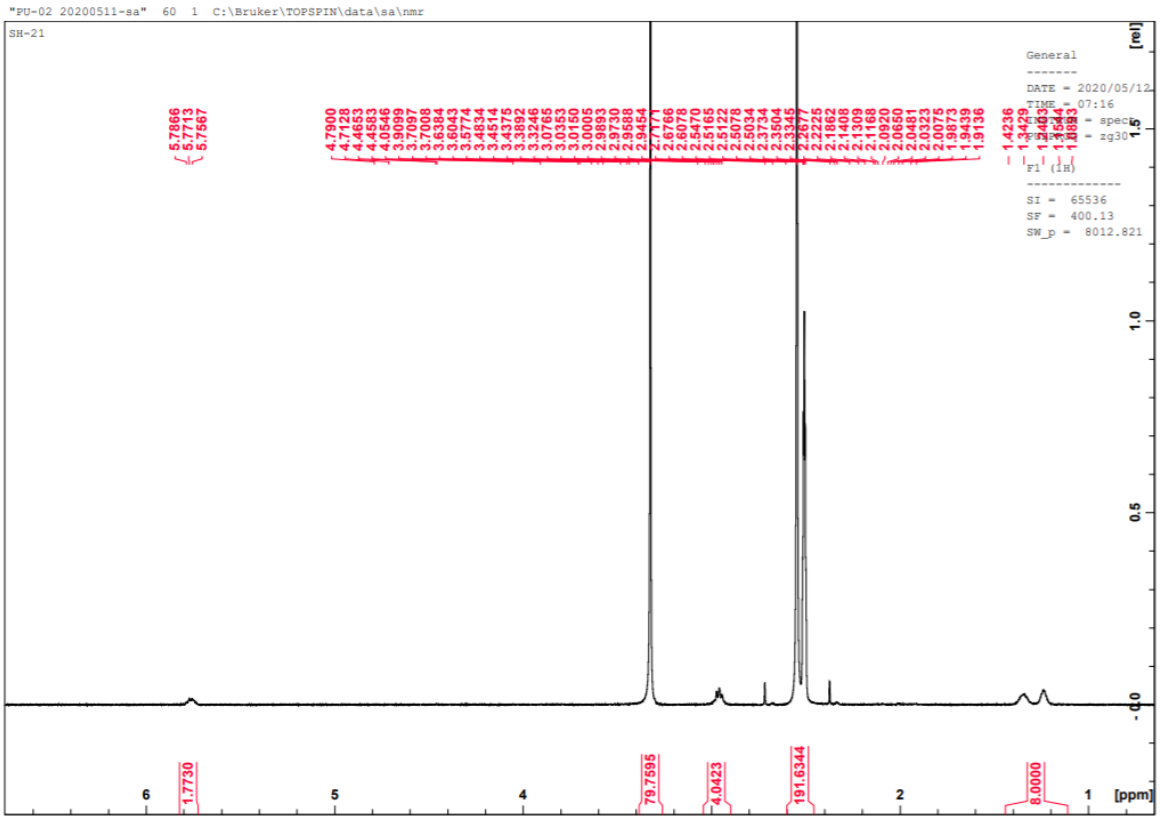
Sample Name	Average of 'C.11'	Average of 'M.8'	Average 2 of 'M.8'	Average of 'M.10'	Average of 'M.11'	Average of 'M.12'	Average of 'M.13'	Average of 'M.14'	Average of 'M.6'
2,75	0,01	0	0	0	0	0	0	0,03	0
3,12	0,01	0	0,01	0	0	0	0	0,08	0
3,55	0,01	0,08	0,07	0,08	0	0	0	0,1	0
4,03	0,01	0,1	0,1	0,11	0,08	0,08	0	0,12	0
4,58	0,01	0,14	0,13	0,14	0,12	0,12	0	0,14	0
5,21	0,01	0,18	0,17	0,18	0,19	0,18	0,08	0,16	0
5,92	0,08	0,23	0,22	0,24	0,28	0,26	0,11	0,18	0
6,72	0,11	0,3	0,28	0,3	0,4	0,36	0,15	0,2	0,03
7,64	0,14	0,39	0,37	0,37	0,56	0,5	0,21	0,24	0,04
8,68	0,15	0,52	0,49	0,45	0,75	0,66	0,28	0,27	0,07
9,86	0,14	0,7	0,66	0,55	0,96	0,83	0,35	0,31	0,07
11,2	0,11	0,91	0,86	0,67	1,19	1,02	0,43	0,35	0,07
12,7	0,03	1,18	1,11	0,79	1,41	1,2	0,51	0,39	0,07
14,5	0,02	1,48	1,39	0,92	1,61	1,36	0,59	0,42	0,08
16,4	0,02	1,81	1,7	1,05	1,78	1,49	0,65	0,44	0,1
18,7	0,12	2,15	2	1,16	1,89	1,58	0,7	0,45	0,11
21,2	0,42	2,46	2,29	1,26	1,95	1,63	0,73	0,45	0,12
24,1	0,98	2,74	2,54	1,34	1,96	1,63	0,75	0,45	0,13
27,4	1,85	2,98	2,75	1,39	1,92	1,61	0,77	0,46	0,14
31,1	3	3,16	2,9	1,43	1,87	1,57	0,79	0,48	0,14
35,3	4,36	3,29	3,02	1,46	1,8	1,53	0,81	0,51	0,16
40,1	5,75	3,37	3,08	1,48	1,74	1,51	0,84	0,56	0,18
45,6	7,01	3,41	3,11	1,5	1,71	1,5	0,88	0,62	0,21
51,8	7,92	3,39	3,09	1,52	1,69	1,51	0,93	0,7	0,27

58,9	8,35	3,33	3,04	1,55	1,7	1,55	0,99	0,77	0,34
66,9	8,21	3,22	2,94	1,6	1,73	1,59	1,06	0,85	0,42
76	7,53	3,07	2,81	1,66	1,76	1,64	1,13	0,91	0,51
86,4	6,45	2,89	2,65	1,74	1,82	1,7	1,2	0,96	0,61
98,1	5,14	2,69	2,48	1,86	1,89	1,77	1,27	1	0,7
111	3,83	2,5	2,31	2,03	1,99	1,86	1,34	1,04	0,79
127	2,69	2,33	2,16	2,26	2,12	1,98	1,42	1,1	0,88
144	1,83	2,17	2,02	2,54	2,29	2,12	1,5	1,22	0,98
163	1,27	2,02	1,89	2,86	2,49	2,29	1,6	1,42	1,09
186	0,98	1,85	1,75	3,19	2,7	2,47	1,72	1,73	1,22
211	0,87	1,68	1,6	3,51	2,92	2,67	1,87	2,15	1,38
240	0,9	1,51	1,46	3,78	3,12	2,86	2,08	2,71	1,6
272	1	1,38	1,35	4	3,31	3,05	2,35	3,37	1,91
310	1,14	1,3	1,28	4,12	3,45	3,22	2,69	4,1	2,32
352	1,31	1,3	1,27	4,14	3,53	3,36	3,1	4,81	2,85
400	1,48	1,37	1,32	4,04	3,54	3,45	3,55	5,42	3,47
454	1,63	1,51	1,42	3,82	3,45	3,46	3,99	5,81	4,11
516	1,74	1,7	1,54	3,5	3,27	3,39	4,38	5,91	4,72
586	1,78	1,9	1,68	3,13	3,03	3,25	4,67	5,7	5,21
666	1,73	2,09	1,84	2,75	2,75	3,06	4,84	5,23	5,55
756	1,59	2,25	2,01	2,42	2,48	2,87	4,88	4,61	5,74
859	1,38	2,37	2,22	2,2	2,26	2,71	4,82	3,99	5,8
976	1,14	2,46	2,47	2,11	2,12	2,63	4,69	3,49	5,79
1110	0,91	2,49	2,73	2,14	2,06	2,61	4,53	3,21	5,77
1260	0,73	2,48	2,97	2,24	2,06	2,65	4,35	3,14	5,76
1430	0,58	2,41	3,15	2,35	2,06	2,69	4,15	3,21	5,73
1630	0,48	2,27	3,18	2,39	2,03	2,66	3,89	3,27	5,58
1850	0,38	2,04	3,03	2,29	1,9	2,51	3,52	3,18	5,21
2100	0,3	1,73	2,67	2,03	1,66	2,2	3,02	2,86	4,55

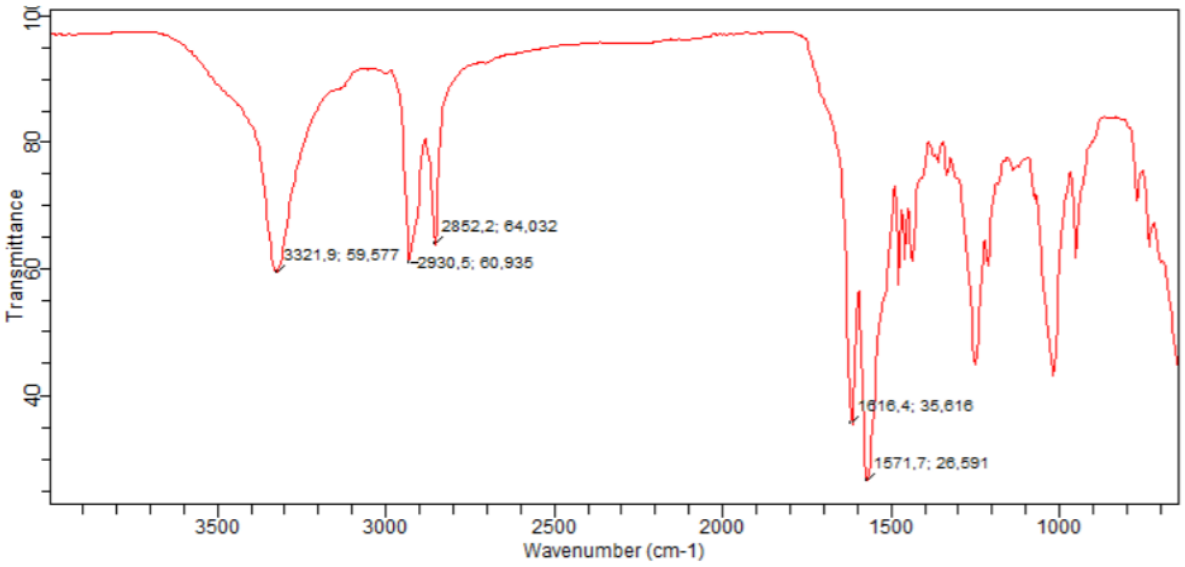
2390	0,22	1,35	2,14	1,63	1,32	1,76	2,38	2,31	3,63
2710	0,14	0,92	1,49	1,13	0,91	1,22	1,63	1,6	2,51
3080	0,07	0,47	0,76	0,58	0,47	0,62	0,83	0,82	1,28
3500	0	0	0	0	0	0	0	0	0

Appendix K – NMR and IR spectra

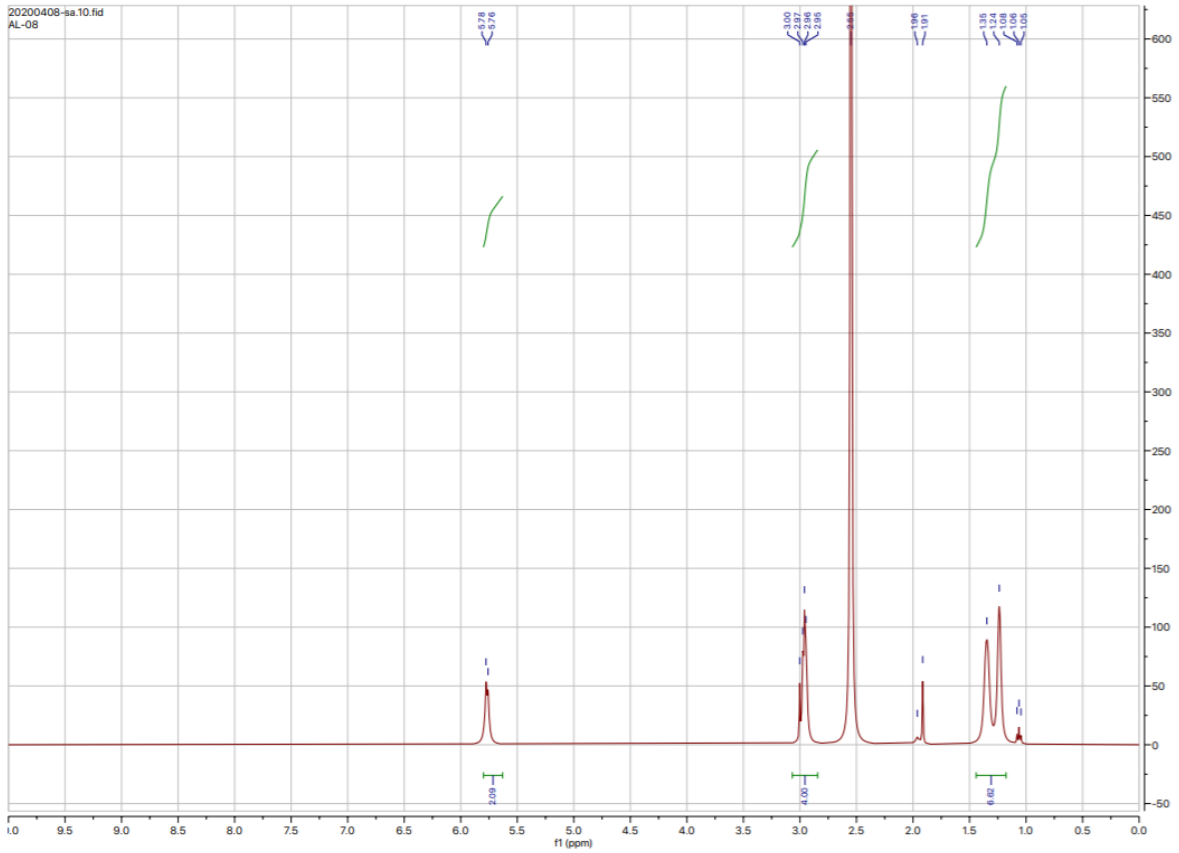
NMR spectrum for PU synthesized from conventional heating.



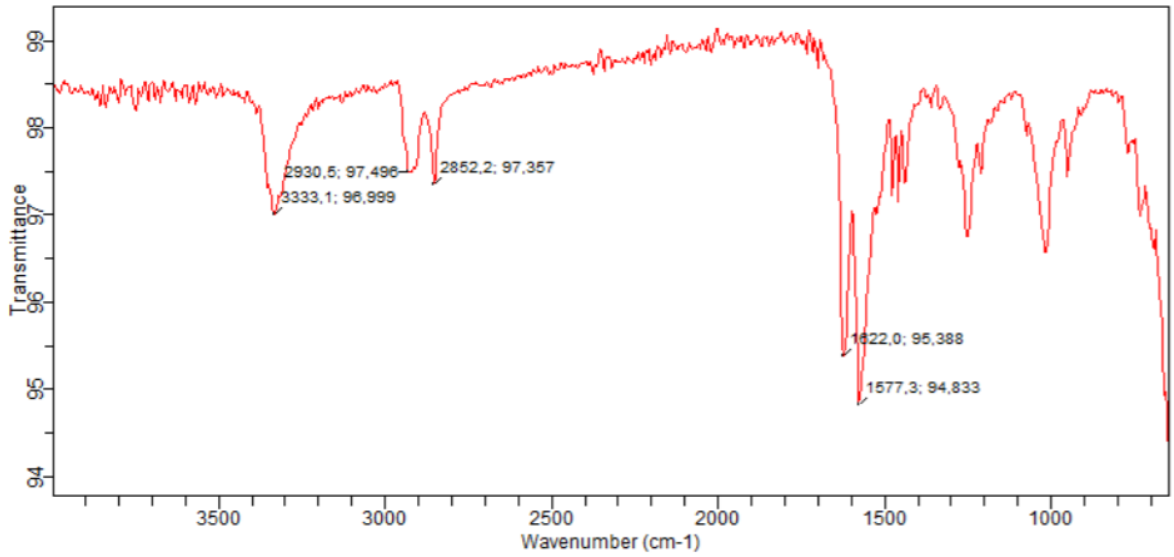
IR-spectrum for PU synthesized from conventional heating.



NMR spectrum for PU synthesized in microwave



IR-spectrum for PU synthesized from conventional heating.



Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway