Enzyme-polymer hybrid layers for self-cleaning surfaces

Urmil Shah

Thesis submitted in partial fulfilment of the requirements for the degree of

Master of Sciences in Biomedical Science (IMBS)

Supervisor I: Prof. Dr. Jürgen Rühe

Supervisor II: Prof. Dr. Monica Galleano

Date: 4th May 2015

The thesis was accomplished at the Laboratory of Chemistry and Physics of Interfaces
Prof. Dr. Jürgen Rühe
Department of Microsystems Engineering IMTEK
University of Freiburg
Freiburg im Breisgau, Germany

Author: Urmil Ajay Shah

Supervisor I: Prof.Dr.Jürgen Rühe, Department of Microsystems Engineering, Lab of Chemistry and Physics of Interface, University of Freiburg IMTEK,Freiburg, Gremany

Supervisor II: Prof.Dr.Monica Galleano, Department of Analytical Chemistry and Physical Chemistry, School of Pharmacy and Biochemistry University of Buenos Aires, Buenos Aires, Argentina


UNIVERSIDAD · DE · BUENOS · AIRES
ARCTA VIRTVS ROBVR ET STVDIVM

Satisfaction lies in the effort, not in the attainment, Full effort is full victory.

-Mahatma Gandhi.

Abstract

This work focused on the generation and characterization of ultrathin coatings with a self-cleaning ability. The general idea was to immobilize a typical detergent enzyme on model surfaces in a way that retains the activity of the enzyme. The specific system under investigation was a hydrogel coating which was chemically anchored on the surface and which also served as a carrier for the enzyme. The hydrogel coating was prepared from water-soluble polymers that carry a small percentage of photo-reactive groups suitable for reaction with neighbouring chains in a way that leads to crosslinking. Similar groups on the surface provide a surface anchoring of the layers by the establishment of covalent bonds between the two components. Amylase was chosen as the enzyme because it is readily available and it cleaves carbohydrates which are typically present in many surface stains. The resulting molecules are smaller and typically more soluble in water such that a stain containing such components is more easily rinsed off the surface.

This enzyme was to be incorporated into the hydrogel carrier coating by means of co-deposition via dip coating. The subsequent UV crosslinking which lead to a surface anchoring not only of the film but also of the enzyme by means of either physical entrapment or chemical anchoring through the surface reaction.

Previous finding suggested an insufficient anchoring of enzymes via this route as the enzyme needs to be deposited from buffer solutions which contain high amounts of salt. This is also deposited and keeps much of the enzyme from being incorporated into the hydrogel coating. For this reason the enzyme was modified with another hydrophilic polymer, Polyethylene glycol chains were chemically attached to the amylase via an active ester. Using this procedure the amylase was rendered soluble in ethanol without losing any activity and overall increased in enzymatic activity as compared to the native enzyme.

An investigation of the coating procedure by various techniques revealed indeed that the route via PEGylated amylase and deposition from ethanol in the absence of any salt yielded much smoother layers in which the enzyme was rather homogenously distributed. Control coatings generated from native amylase and deposited from PBS buffer gave very rough coatings that were covered with salt crystals.

A comparison of the enzymatic activity via colorimetric measurements first demonstrated that enzymatically coatings could be generated via both routes. The coatings prepared from PEG-conjugated amylase however were always more active than those prepared from native amylase and they also retained this active character better if exposed to UV light or heat.

A simple self-cleaning test also showed that coatings prepared from native amylase lost their initially good self-cleaning character after a first rinse. Repeated use of such samples was not possible. The coatings carrying PEGylated enzyme also lost much of their initial activity after the first test but repeated use was possible and clear degradation of starch in a test involving mayonnaise was clearly visible.

The general concept of enzyme carrying coatings based on surface-attached hydrogels was successfully demonstrated. Further investigations should concentrate on the analysis of the influence of the deposition conditions on the enzyme activity. Such investigations should include a more thorough characterization of the resulting layers. Such research may eventually lead to self-cleaning surfaces that are stable on a time scale which is suitable at least for delicate applications e.g. in a biomedical environment.

Resumen

Este trabajo se centró en la generación y caracterización de recubrimientos ultrafinos con capacidad autolimpiante. El objetivo general fue inmovilizar una enzima detergente típica en superficies modelo a través de un procedimiento que permitiera conservar la actividad enzimática. El sistema específico bajo investigación fue un recubrimiento de hidrogel capaz de unirse químicamente a la superficie y simultáneamente servir como vehículo para la enzima. El recubrimiento de hidrogel se preparó a partir de polímeros solubles en agua con un pequeño porcentaje de grupos fotorreactivos adecuados para reacciones de entrecruzamiento entre cadenas vecinas. Grupos similares en el recubrimiento proporcionan un anclaje para el establecimiento de enlaces covalentes con la enzima. La enzima amilasa fue elegida por su amplia disponibilidad y su capacidad de degradar hidratos de carbono. Los hidratos de carbono son moléculas típicamente presentes en muchas manchas de superficie y como resultado de la acción de la amilasa se producen moléculas más pequeñas y generalmente más solubles, de manera que una mancha que contiene estos componentes se elimina más fácilmente.

La enzima fue incorporada en el recubrimiento de hidrogel por co-deposición mediante inmersión. Posteriormente, a través de la exposición a radiación UV se generó un entrecruzamiento que condujo al anclaje, no solo de la película a la superficie, sino también de la enzima a la película, a través de atrapamiento físico o anclaje químico.

Estudios previos indicaron un anclaje insuficiente de las enzimas cuando el proceso se realizaba a través de esta técnica de co-deposición con inmersión, dado que la enzima era depositada a partir de soluciones buffer con altas cantidades de sal, que también se depositaba sobre la superficie. Este fenómeno impedía la incorporación de gran parte de la enzima al recubrimiento de hidrogel. Por esta razón la enzima se modificó con otro polímero hidrófilo, el polietilenglicol (PEG). Se unieron químicamente cadenas de PEG a la amilasa a través de un éster activo. Utilizando este procedimiento, la amilasa se solubilizó en etanol sin perder actividad.

Se investigaron diversas técnicas de recubrimiento y se demostró que la utilización de la amilasa conjugada con PEG y la deposición a partir de etanol en ausencia de cualquier sal produjo recubrimientos con una distribución homogénea de la enzima y de características muy lisas, mientras que los recubrimientos generados con amilasa nativa depositada a partir de solución buffer salina dieron origen a recubrimientos muy ásperos y cubiertos con cristales de sal.

Un análisis comparativo de la actividad enzimática, a través de mediciones colorimétricas, demostró que los recubrimientos tenían actividad enzimática, cualquiera fuera el procedimiento utilizado para su generación. Los recubrimientos preparados a partir de amilasa conjugada con PEG mostraron siempre mayor actividad que los preparados a partir de amilasa nativa y retuvieron mejor la actividad enzimática después de la exposición a radiación UV o calor.

A través de una prueba de autolimpieza sencilla se demostró que los recubrimientos preparados a partir de amilasa nativa perdieron su propiedad autolimpiante, inicialmente buena, después de un primer enjuague. El uso repetido

de dichos recubrimientos no fue posible. Los recubrimientos producidos con la amilasa conjugada con PEG también perdieron gran parte de su actividad autolimpiante inicial después del primer enjuague, pero fueron reutilizables. Se utilizó un ensayo de degradación del almidón en una muestra de mayonesa para estos estudios.

El objetivo general del recubrimiento de hidrogel con enzimas unidas a superficie se cumplió exitosamente en este trabajo. Investigaciones futuras se deberán enfocar en el análisis de la influencia de las condiciones de deposición sobre la actividad de la enzima. Esas investigaciones deberían incluir una caracterización más profunda de la estructura del recubrimiento resultante. Este tipo de investigación podría conducir eventualmente a desarrollar superficies autolimpiantes estables en escalas de tiempo adecuadas para aplicaciones específicas, por ejemplo, en el entorno biomédico.

Abbreviations

λ	wavelength
α-Amylase(Aspargillus oryzae)	α-Amylase
AFM	Atomic Force Microscopy
AIBN	azo-bis-isobutyronitrile
BP	benzophenone
BSA	bovine serum albumin
CPI	Laboratory for Chemistry & Physics of Interfaces
DMAA	dimethyl acrylamide
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EtOH	ethanol
kD	Kilo Dalton
MW	weight average molecular weight
MABP	methacryloyloxybenzophenone
PU	polyurethane
PMMA	poly (methyl methacrylate)
P (DMAA)	poly (dimethylacrylamide)
PEG	polyethylene glycol
PBS	phosphate buffer saline
PAGE	polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SDS	sodium dodecyl sulphate
t	time
T	temperature

Table of Content

1. Introduction

1.1 Self-cleaning surfaces

Many technologies existing in today's world have been derived or at least inspired from nature. An interesting question is how nature handles the contamination of surfaces with particles such as dust or with compounds such as proteins, polymeric sugars or lipids. Many surfaces in nature exhibit self-cleaning properties [1]. The wings of butterflies and the leaves of plants, such as cabbage and lotus, are typical examples for surfaces which require self-cleaning [2]. Also in many technical applications, from window glass cleaning, solar panel cleaning and cements to textiles, this technology received a great deal of attention during the late 20th century[1]. In most cases cleaning is achieved by washing with detergents, however also several approaches have been developed, which are inspired by nature and aim at the generation of self-cleaning surface. Today numerous research efforts around the world are directed to develop highly efficient and durable self-cleaning coating surfaces with good optical qualities. Apart from the wide range of applications, this technology also offers various benefits, which including the reduction of the use of chemicals, energy and in maintenance cost, through elimination of tedious manual effort spent in cleaning work [2].

One way to classify self-cleaning coatings for the protection of surfaces against contamination could be to divide them into surface protection by physical means or the removal of impurities by chemical reactions. The prior principle, ie. Surface protection by physical means can be subdivided into strategies based on hydrophilic or hydrophobic coatings [3]. In a hydrophilic coating, the water spreads on the surface, which leads to formation of a continuous water film, so that the impurities cannot adsorb and are just floating on the water film. When the water is removed, this carries away the dirt and other impurities. In contrast to this, when super hydrophobic coatings are applied, the water droplets slide and roll over the surfaces. While rolling they pick up dust particles in order to reduce the surface energy of the system. Thus the roll-off of the water drops leads to a dust removal from the surfaces.

In other systems, which are based on the chemical degradation of impurities on the surfaces suitable metal oxides such as titanium dioxide photo-chemically break down the complex dirt deposits by sunlight assisted cleaning mechanism [1,2,3]. Details of this approach will be discussed further below.

1.1.1 Strategies for the generation of self-cleaning surfaces

- **Self-cleaning through water-roll off**

The wettability is an important property of a solid surface, and contact angle (θ) measurement has been commonly used to characterize the behaviour of the surface against water and other liquids surface.

For a perfectly smooth and chemically homogeneous solid surface, the contact angle of a liquid is given by Young's equation [4].

$$\text{Cos } \theta = (\gamma_{SV} - \gamma_{SL})/\gamma_{LV} \qquad \text{Eq.1}$$

Where, γSV, γSL, and γLV are the interfacial tensions of the solid–vapour, solid–liquid and liquid–vapour interfaces, [4] which is depicted in the figure 1.

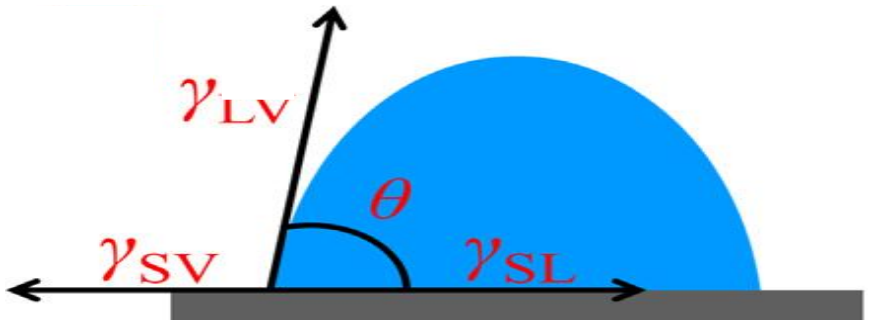


Figure 1. A liquid droplet on a smooth surface.

There are two kinds of surface with special wettability, super hydrophobic surface (water contact angle WCA > 150˚ and low contact angle hysteresis) and super hydrophilic surface (water contact angle <5˚ within 0.5 or less) [1]. A self-cleaning super hydrophobic surface should always have a low contact angle hysteresis [4]. The leaf of the lotus plant is a representative model for super hydrophobic and self-

cleaning surface. Due to the papillose epidermal cells covered with wax crystals, the leaf surface is very rough and water-repelling [5]. Water drops on such surfaces exhibit a high contact angle and a low contact angle hysteresis and are able to roll off to leaf easily. When a water droplet rolls over particular contaminants, such as dirt particles, they become adsorbed to the water droplet, and upon roll off of the drop are removed from the surface. On such intrinsic hierarchically structured surfaces, both the contact area and adhesion to the surface are dramatically reduced [5, 6].

Besides, special wettability also shows the surfaces with antifogging function [6]. Fogging occurs when water vapour condenses on the surface owing to temporary changes in humidity, temperature, or convection [7]. If the surface has a rather low contact angle, it could form a continuous or near continuous film, scattering is suppressed and optical transmission is enhanced. On the other hand, if the surface has a quite high contact angle and low contact angle hysteresis, the droplets may slide from the surface rapidly [6, 7]. Fog may thus be difficult to cover the surface. Super hydrophobic or super hydrophilic coatings are in line with the above requirements and have shown good prospects for practical applications [6, 7].

- ## Photo catalysis induced self-cleaning

Since Frank and Bard first discovered that TiO_2 can decompose cyanide in water in 1977 TiO_2 has been used as photo catalyst to decompose organic contamination for environmental applications [8]. TiO_2 which is widely used as a white pigment in coating application [8] has a strong oxidation power under UV illumination. Various organics accumulating on TiO_2 thin films could be oxidized into CO_2, H_2O, NO_3 or other simple products [9]. Moreover, as it can be evaporate under UV illumination, the surface of TiO_2 becomes super hydrophilic with a water contact angle close to 0˚. Due to these characteristics, TiO_2-coated substrates can achieve outstanding self-cleaning property when they are exposed to sun light [8, 9]. The self-cleaning effect is, however, limited if the surfaces in question are not exposed to direct sunlight.

1.2 Self-cleaning surfaces through immobilized enzymes

1.2.1 Enzymes

Enzymes are nature's catalysts. They facilitate chemical reactions in all biological systems on Earth. Enzymes are also used in many technical applications. A wide range of sources are used for commercial enzyme production and over 90% of these are from fungi, yeast and bacteria with animal and plant enzymes accounting for the rest [10]. At present, microbial enzymes are the preferred candidates in many applications due to their higher activities and neutral pH optimization. Most enzymes today are produced by the fermentation of bio based materials. They are increasingly replacing conventional chemical catalysts in many industrial processes.

1.2.2 Industrial applications of enzyme

Enzymes offers major advantages over conventional chemical catalysts in many industrial applications, due to the fact that they require mild reaction conditions, have high substrate specificity and cause reduced side products in the waste stream [10]. Enzymes have the ability to function under relatively mild conditions concerning temperature, pH and pressure, which results in the consumption of less energy. Ordinary chemical reactions often produce a variety of products due to a several reaction pathways [11]. Enzymes on the other hand are specific, often responsible for the catalysis of a very narrow range of substrates and hence do not produce unwanted by-products. Consequently, enzyme based reactions have and thus lower waste treatment costs. Finally, in comparison to chemical processes, enzyme-based processes are environmentally friendly, as their products are biodegradable [10, 11]. All of these factors account for the increase in process productivity with reduced manufacturing costs from enzyme-catalysed reactions.

1.2.3 Detergent Enzyme

Certain enzymes like proteases, amylases, lipases, cellulases, mannanases, and pectinases are very effective in small amounts, for removing certain target substances from the surfaces of textiles [12]. The enzymes used in laundry detergents act on materials that make up a variety of stains and soils so that these

materials can be washed away more easily. These enzymes are named after the materials they can act upon, for example [12],

- Proteases break down protein based stains,
- Lipases break down lipid (fat) based stains,
- Amylases break down starches and other carbohydrate based stains,
- Pectinase remove fruit and pectin-based stains that traditional detergent ingredients have trouble removing, doing it efficiently at low wash temperatures.

Since one enzyme molecule can act on many substrate molecules (i.e. those present in a stain), a small amount of enzyme added to a laundry detergent can provide a big cleaning benefit to the consumer [12].

1.2.4 Enzyme as a catalyst

The rate of a chemical reaction is determined by the activation energy of the process. A high activation energy might cause a very slow reaction despite that the driving force for a chemical reaction is very strong. Catalysts, including enzymes as biocatalysts, lower this activation energy and thus increases the reaction rate strongly [13]. The energy variation as a function of reaction coordinate shows the stabilisation of the transition state by an enzyme which is depicted into figure 2a.

Like other types of catalysts, an enzyme can complete its chemical reaction without being used up or destroyed, leaving the enzyme protein available for another reaction [10, 13]. This means that one enzyme protein molecule can act on many substrate molecules. Eventually, all the substrate is gone and the enzyme stops working and will eventually break down on its own [13].

The active site of the enzyme is open only to specific target substances with a matching chemical and 3-dimensional shape. If the substrate does not fit, it cannot enter and no reaction occurs. This makes the action of enzymes highly specific for their substrates. The simplest model to explain the action of enzymes is consider that enzymes fits their targets like a key fit to a lock see figure 2b.

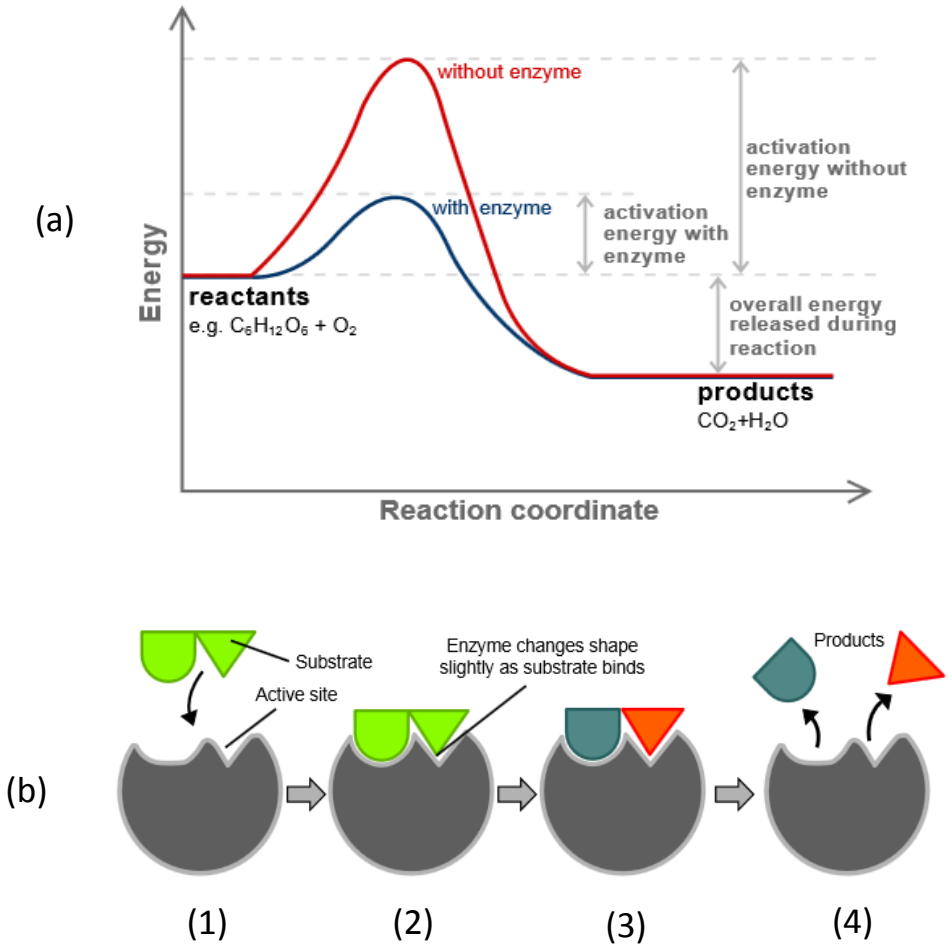
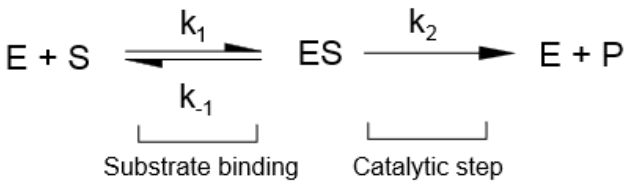


Figure 2. (a). Progress of a chemical reaction along the reaction coordinate without and with the presence of an enzyme. (b). Schematic depiction of the the key-lock principle (1) Substrate enter at the active site of enzyme; (2) complex of enzyme/substrate formed;(3) enzymatic reaction of the enzyme/substrate occur;(4) product leaves the active site of the enzyme [14].

The Michaelis–Menten theory describes how the (initial) reaction rate v_0 depends on the position of the substrate-binding equilibrium and the rate constant k_2. It also involves an enzyme E binding to a substrate S to form a complex ES, which in turn is converted into a product P and the free enzyme see equation 2.



Eq.2

After formation of ES, this complex either decomposes again to its initial components E+S or the reaction proceeds to yield the product P, while E is recovered. The Michaelis-Menten constant Km is composed of the individual rate constants of this three processes, as given into above equation.

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

Eq.3

Using this constant we can derived the reaction rate vo for any substrate concentration [s] as a fraction of maximum reaction rate v_{max}.

$$Vo = \frac{Vmax[s]}{Km+[s]}$$

Eq.4

This equation is frequently called the Michaelis-Menten equation. So with Michaelis-menten constant K_m we can also summarised the rate constant k_2. The maximum speed v_{max} describes the speed at which all enzymes are saturated with substrate, therefore [ES] = [Etotal] [13].

Enzymes can be incorporated in industrial or commercial coatings to create novel biologically and chemically active surfaces [10, 13]. This work is aimed to present some fundamental characteristics of enzymes with respect to their stability and activity in polymer solution and associated coating formulations. The development of an effective enzyme-based biocatalytic coating demands an understanding of the means of enzymatic deactivation. When designing a biocatalytic system, particular attention needs to be given to both the catalytic and the non-catalytic components of the system. In order to have a system with long term stability and

activity in various conditions it needs to be optimised and this issue is addressed by a series of stabilising techniques [10, 13].

1.2.5 Enzyme Stability

- **Enzyme stability in aqueous and non-aqueous systems**

Enzymes can undergo a variety of denaturation reactions during their application. Denaturation is the unfolding of the enzyme tertiary structure to a disordered polypeptide in which key residues are no longer aligned closely enough for continued participation in functional or structure stabilizing interactions [15]. This leads in most cases to an irreversible loss of activity or inactivation particularly following unfolding [15]. The extent of denaturation depends on the reaction conditions. Under some conditions the process can be still reversible and the native form of the enzyme is restored. Under other conditions the process is irreversible and no return to the native form is possible and the enzyme remains permanently inactive.

These molecular phenomena give rise to two distinct definitions of in vitro protein stability via thermodynamic (or conformational) stability and long term (or kinetic) stability. Thermodynamic stability concerns the resistance of the folded protein conformation to denature while long term stability measures the resistance to irreversible inactivation (i.e., persistence of biological activity).

Enzyme may be stabilised by attaching them to a surface. Multipoint covalent attachment of enzymes on highly activated pre-existing supports via short spacer arms and involving many residues placed on the enzyme surface promotes a rigidification of the enzyme structure of the immobilized enzyme [13]. This should reduce any conformational change involved in enzyme inactivation and greatly increase the enzyme stability which depicted in figure 3.

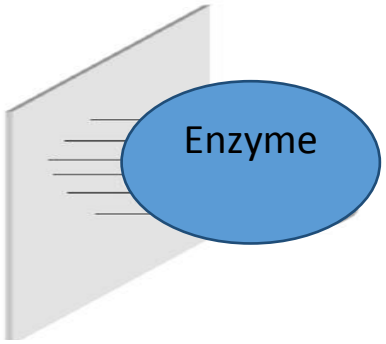


Figure 3. Immobilization of an enzyme to a surface through multipoint attachment.

- Stability of enzymes in non-aqueous environments

There are many enzymes which are required to function in organic/non aqueous media. In our research work we focus on enzyme technology involves enhancement of enzyme activity and stability under non-conventional conditions of organic solvents. The non-aqueous media permits the advantages of using organic solvents as reaction media such as increased solubility of a polar substrates, shift of thermodynamic equilibrium to favour synthesis over hydrolysis, suppression of many water dependent side reactions and decreased potential for microbial contamination [16].

1.2.6 Enzyme Immobilization

The immobilization of enzymes is a standard approach in applied biotechnology [10]. The primary objective for enzyme immobilization in biotechnology is to isolate the biocatalyst from the reactive product thereby increasing productivity and reusability. Enzymes can be incorporated in industrial or commercial coatings to create novel biologically and chemically active surfaces [10].

The immobilization of enzyme on a solid surface can be effected by different either by covalent attachment or through non-covalent attachment. A non-covalent attachment the enzyme can be achieved by adsorption or by incorporation into a hydrogel. Covalent immobilization of the enzyme at the surface of the substrate

can be achieved by use of reactive functional groups on the side chain of the amino acids.

Due to the high content of lysine in the proteins and the high reactivity of amines, the covalent immobilization often occurs via the amino group of lysine. Therefore a pathway, which is frequently followed for the immobilization of enzymes, the reaction of glutaraldehyde with the amino groups of the enzyme. In this case, an aldehyde group of the glutaraldehyde reacts with an amino group to form an imine and the other aldehyde group can react with the polymeric support material. Figure 4 shows an example for the immobilization of an enzyme with glutaraldehyde and a polymeric amine.

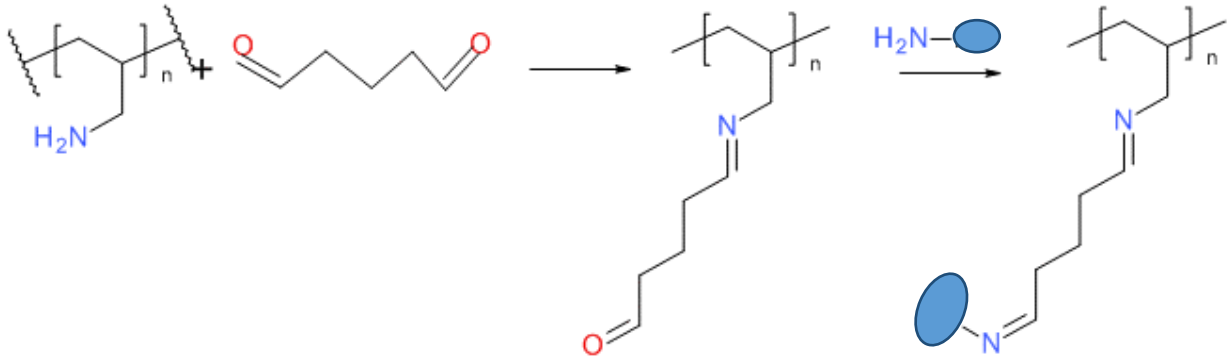
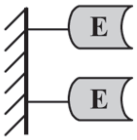


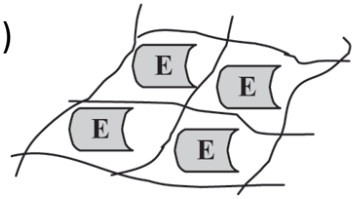
Figure 4. Immobilization of an enzyme with glutaraldehyde and a polymeric amine.

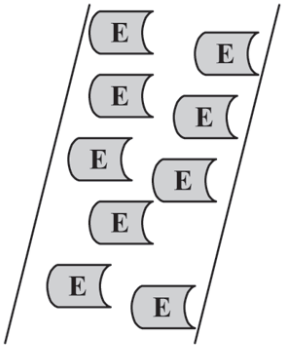
Glutaraldehyde is prone to oxidation and also light sensitive, when it is stored in an air numerous compounds are formed that react in diverse ways with proteins and frequently a wide range of reaction products arise [17]. One way to make use of the reactivity of the amino groups, is the reaction with activated carboxylic acids. These can be done such as in the work of Akkaya et al. with activate carbodiimides. The work of Akkaya et al. also describes immobilization by epoxides, where they also use polymeric material as a support. In the figure 5 different approaches for the immobilization of enzymes are shown [6].

- Covalent binding

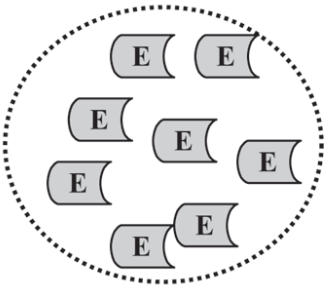


- Entraptment (Beads or fibers)





- Microencapsulation



- Crosslinking

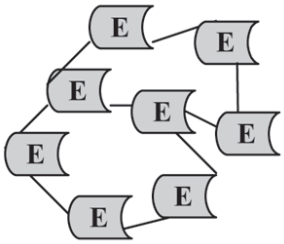


Figure 5. Approaches to enzyme immobilization [6].

1.2.7 Functionalization of protein

Immobilization of enzyme often makes it desirable to process them into solvent other than water. Therefore it would be interesting to make the enzyme soluble and active in organic media which would allow us to study reaction which proceed in hydrophobic environments. For this application it is important that substrate and the product for the reaction both should be poorly water soluble [18].

Enzymes such as amylases, lipases, and proteases, such as chymotrypsin, can also be active in organic solvent, such as benzene, by covalently attaching an amphipathic molecule to the surface of enzyme. The hydrophilic nature of the polyethylene glycol (PEG) makes it possible to modify the enzyme in aqueous solutions (see figure 6).

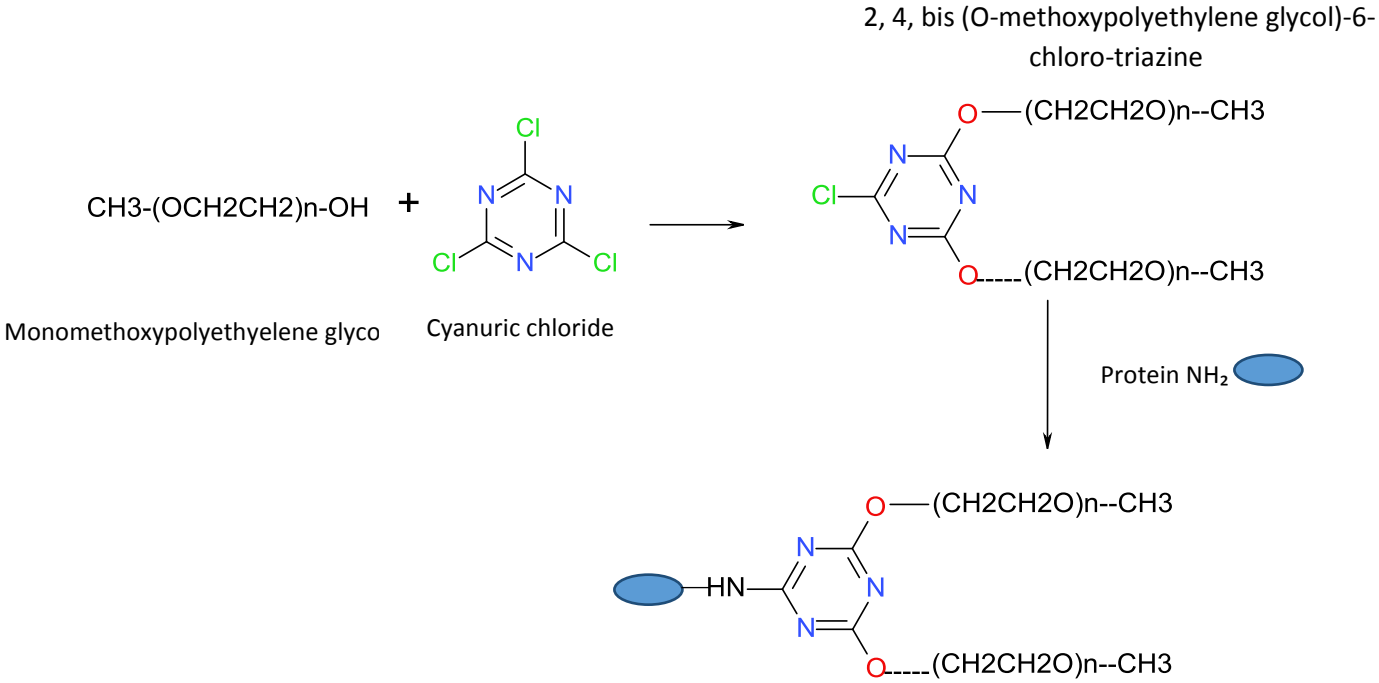


Figure 6. Reaction of a protein with an activated PEG.

Monomethoxypolyethylene glycol (MW 5000) and cyanuric chloride react to 2, 4, bis (O-methoxypolyethytelene-glycol)-6-chloro-triazine which is also called (activated PEG_2). In this molecule two of three chlorine atoms of the cyanuric chloride molecule are replaced with PEG [18, 19]. The residual chlorine atom of the activated PEG_2 can react with N-terminal and/or lysine residual amino group through on the surface of enzyme molecule. Therefore, two chains of PEG can be attached to each amino group through formation of a triazine ring. In generally the coupling reaction can be performed under mild conditions [19].

1.3 Polymers for Protein conjugation

- ### Dextran

Dextran was the first and most studied substrate for protein conjugation [20]. Dextran was conjugated to streptokinase and subsequently used in clinical practice becoming the first protein conjugate to be tested in humans [20]. The polymer was oxidized by periodate yielding aldehyde groups, which in turn, reacted with protein amino groups [21]. Although this method is still currently applied to couple proteins to dextran and polysaccharides in general, it is affected by the multiplicity of binding groups in the polymer backbone that might yield undesired cross-linked bonds if coupling conditions are not well controlled. Moreover two aldehyde groups are generated for each oxidized unit when polysaccharide is treated with sodium periodate [21]. As this approach yields two aldehyde groups (e.g., RO–CH(R′)–CHO and RO–CH(OR′)–CHO) in close proximity, where R and R' are the polysaccharide backbone, it might cause problems due to the differences in the reactivity of aldehydes and to the difficulty in determining which aldehyde group is involved in the protein coupling. That both aldehydes react with the proteins, moreover, is a possibility that is difficult to exclude. As a consequence, if this approach is not properly optimized, there may be constraints in the heterogeneity of the final conjugate. Although dextran is currently commercially available with a wide range of MW and a lower polydispersity index (PI) with respect to that in the past [20, 21].

- ### Hyaluronic Acid (HA)

Hyaluronic acid (HA) is a natural polysaccharide (figure 7) and a key component of extracellular matrix, cartilage and vitreous compartments in vertebrates [22]. The conjugation of biologically active substances to HA is straightforward through a direct activation of the carboxylic acid groups present along the polymer backbone [23]. A chemical approach suitable for amino coupling might present cross-linking limits for HA conjugation to proteins. As a consequence, a different HA activation has been achieved by reacting some HA carboxylic groups with a spacer bearing a protected aldehyde group. Studies on the new aldehyde-HA have produced positive results with regard to protein conjugation with several different proteins and peptides [22, 23, 24].

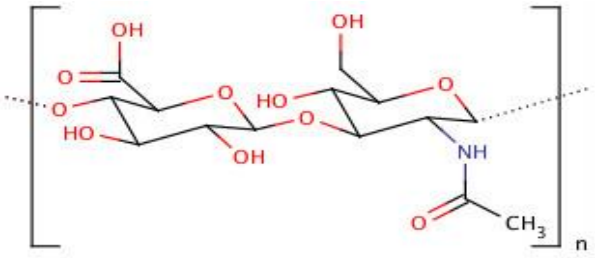


Figure 7. The structure of hyaluronic acid (HA).

- Dextrin

Dextrin is a polymer chain composed of D-glucose units joined together by α-1, 4-glucosidic linkages that form a linear polymer with some degree of branching via 1, 6-glucosidic linkages (figure 8). The polymer was succinylated to achieve conjugation to proteins. This approach links a succinic acid molecule to some hydroxyl groups of dextrin, thus obtaining a carboxyl group suitable for protein conjugation after activation. Dextrin with a degree of succinylation and MWs in the ranges between 9–32% mol and 7–47 kD, respectively, were used to modify different proteins [25].

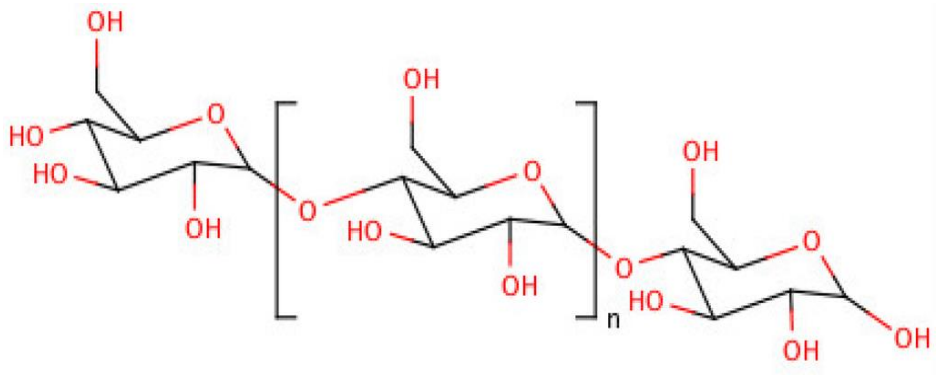


Figure 8. Chemical structure of dextrin.

- Poly(2-ethyl 2-oxazoline) (PEOZ)

Poly (2-ethyl 2-oxazoline) (PEOZ) (figure 9) is a linear synthetic polymer that is considered to be a promising substitute for PEG in view of its stealth properties. PEOZ is soluble in water and in many organic solvents [26]. Like PEG, PEOZ can be

synthesized with low polydispersity and can have a single functional group at one extreme, useful for protein conjugation, which is obtained during the initiation or termination steps of polymerization [26]. PEOZ-protein conjugates have, until now, been investigated only in animal models, but the polymer has produced promising results and no toxicity, even in the PEOZ form, with pendant groups used to conjugate low MW drugs [25, 26].

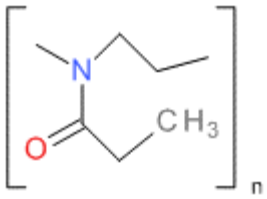


Figure 9. Chemical structure of poly (2-ethyl 2-oxazoline) (PEOZ).

- PEG (Polyethylene Glycol)

Among the polymers studied, PEG (figure 10) has emerged as the most interesting, versatile one, and nine PEG-protein conjugates are, in fact, currently being used in polymer conjugation. The reasons for its success reside in many advantageous properties, such as its hydrophilicity, non-toxicity and non-immunogenicity. Some research also found that PEG also helps to improve the biocompatibility, half-life and safety of proteins, nanoparticles and liposomes [27].

PEG has unique solvation properties that are due to the coordination of 2–3 water molecules per ethylene oxide unit [28]. This high solvation, together with the great flexibility of the polymeric backbone, confers biocompatibility, non-immunogenicity and non-antigenicity to the polymer. Another consequence of solvation is that PEG has an apparent molecular weight 5–10 times higher than that of a globular protein of a comparable mass, as verified by gel permeation chromatography [29]. A single PEG molecule is therefore able to cover an extended surface of a conjugated protein, thus preventing the approach of proteolytic enzymes and antibodies. The chemistry of polymer conjugation to proteins has been mainly developed with PEG, so nowadays, several PEGylating agents are available with different reactivity, MW and structure [27, 28, 29].

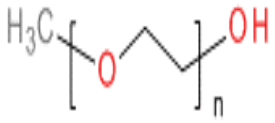


Figure 10. Chemical structure of polyethylene glycol.

1.4 Photochemistry of benzophenone

- General principles of photochemical reactions

The study of chemical reactions and physical processes that occur under the influence of visible and/or ultraviolet light is called photochemistry [30]. Two main classes of photochemical reactions can be distinguished, which depend on whether chemical bonds are formed or cleaved. In photo affinity labelling [31], a new covalent linkage is created between a light-sensitive, detectable ligand and a biopolymer upon irradiation, in a reversibly bound state. As a result of the photo induced coupling reaction, the ligand binding site of the biopolymer (e.g. a receptor protein or an enzyme) becomes irreversibly occupied, and can inactivate or activate the biological function of the biopolymer [32,33]. The photo ligand must contain a readily detectable tag (e.g. radioactive, fluorescent or immune-reactive), which allows ready determination of the macromolecule and in particular, the fragments of the molecule that have been covalently modified [30]. A similar photochemical process is used for photo immobilization of biopolymers or ligands to create modified surfaces or patterned arrays.

One way to generate surface-attached polymer network layers is to use polymers with thermally or photo chemically reactive groups that upon proper excitation react with neighbouring chains to connect them.

Benzophenone containing polymers are often used for such purposes because of the chemical inertness of the BP in the absence of UV light and the well-known photochemistry of this group. Upon irradiation with UV light a transition within the carbonyl group of the benzophenon occurs [33]. Depending on the wavelength this transition is either an n, π* or π, π* transition which is both cases immediately followed by an intersystem crossing to a triplet state. This state is biradicalic in nature.

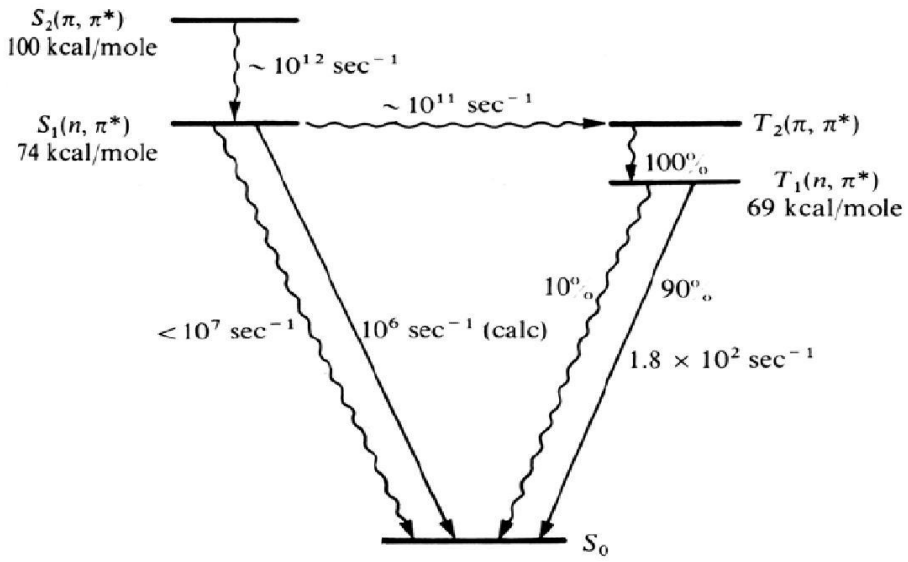


Figure 11. Schematic representation of the electronic transition of benzophenone in a jablonski scheme [31].

The triplet state cannot return to the ground state as such a transition is forbidden then it which yields a very long life time of this photo activated state. It essentially lives until it finds a reaction partner such as a C-H group in its vicinity [34]. This reaction is at first an abstraction of a hydrogen atom by the oxygen cent ered radical which is essentially a transfer of the radical to the carbon from which the hydrogen is taken away. This radical may then recombines with the radical located at the previous carbonyl carbon of benzophenon. Side reactions are also known and these may not always yield insertion reactions [35].

If the BP was originally linked to one polymer chain and inserted into a C-H group of another chain a crosslink is generated [33].

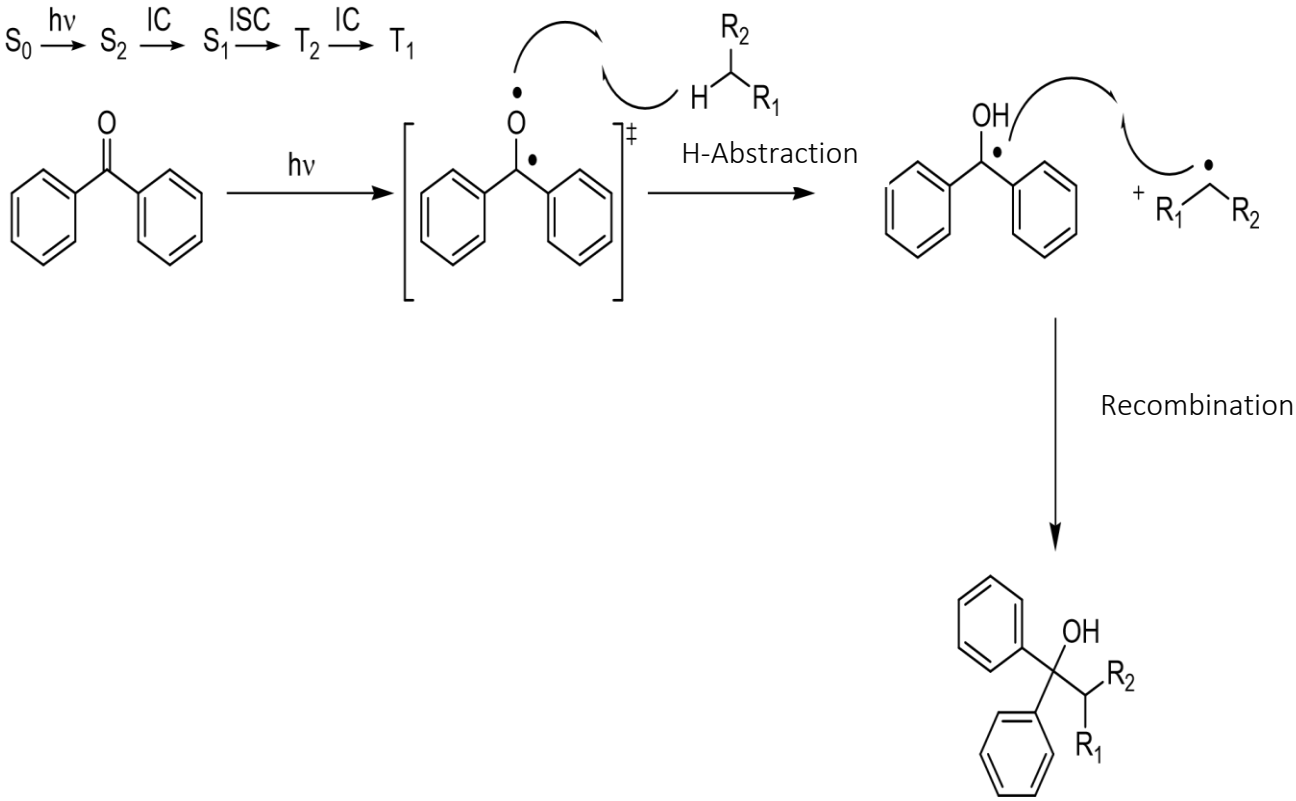


Figure 12. Schematic representation of the light-induced excitation of benzophenone followed by C-H bond insertion.

1.5 Surface attached hydrogel coatings

Hydrogels are polymeric networks, and retain large amounts of water [36]. In the polymeric network hydrophilic groups or domains are present which are hydrated in an aqueous environment thereby creating the hydrogel structure. As the term 'network' implies, crosslinks have to be present to avoid dissolution of the hydrophilic polymer chains segments into the aqueous phase [36]. There are two main methods to develop a hydrogel coating on solid substrate.

- Chemically cross-linked gels
- Physically cross-linked gels

1.5.1 Formation of three-dimensional polymer networks

Polymer hydrogels are highly cross-linked materials having a tridimensional and flexible structure, able to swell when they are immersed in aqueous solutions. Indeed, chemical or physical crosslinking avoids their solubilisation [36]. Tridimensional polymer networks can be formed by either in situ during the polymerization with higher functionalized by using monomers, or by crosslinking of preformed polymers [34]. In one class of reactions low molecular weight cross-linking reagents are used for the formation of the networks [36]. A classic example is sulphur in the vulcanization of natural latex into a rubber. Other examples comprise the reaction of reactive (side) groups on the polymers with each other e.g through condensation or cycloaddition reactions (cinnamic acid or anthracene dimerization).

Another way of network formation is based on preformed polymers. This process can occur through the insertion of a functional group into a C-H bond of the polymer backbone [31]. By the C-H insertion is per functional group is a covalent linkage between the polymer chains is formed, whereas in the aforementioned variant twice as many functional groups are needed to achieve the same degree of linkage. Figure 13 shows the tridimensional structure of polymer hydrogel after swelling.

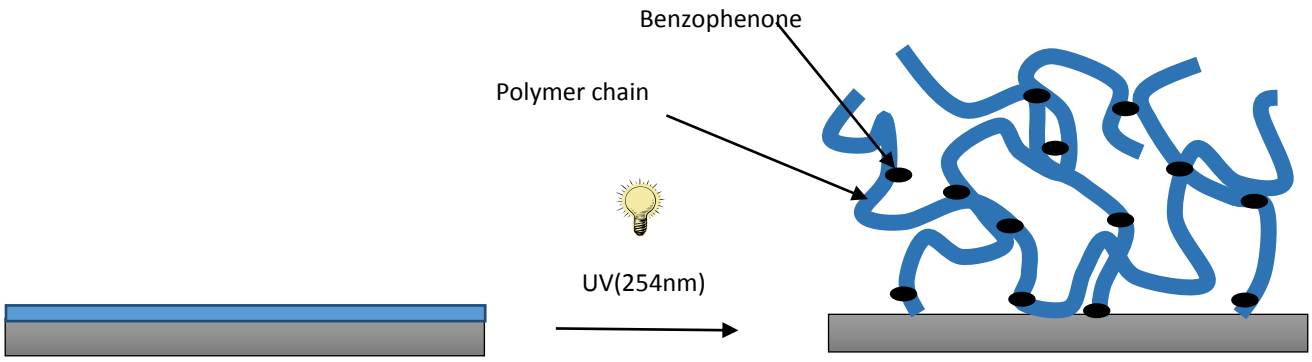


Figure 13. Schematic depiction of the photochemical formation of a surface-attached polymer hydrogel.

1.5.2 Swelling behaviour of polymer networks

It is essential to keep the immobilized enzymes active on the surface to allow for their function in the decomposition of the stains on the surface. A good way to immobilized proteins or enzymes in a functioning way is to embed them into a hydrogel [34]. Depending on the crosslink density this take up of water can be very significant and the mass of a swollen hydrogel is often several tens of the mass of the dry gel.

The swelling process takes place in three different steps: (a) diffusion of water molecules through the matrix, (b) relaxation of polymer chains via hydration, and (c) expansion of polymer network upon relaxation.

- ### Theory of swelling

If such a material is chemically linked to a surface the overall swelling capability is largely reduced because the gel can no longer expand in each direction but only perpendicular to the surface to which it is linked [34]. The results could be analysed using a modified Flory-Rehner theory and showed that the surface-attached gels swell in an anisotropic way. This means that the gels overstretched in the one direction that was available to them [37].

The extent of swelling can be calculated with the help of the Flory-Rehner theory, which describes the swelling behaviour in thermodynamic equilibrium. The total Gibbs free energy of the system ΔG_{total} can be calculated from the Gibbs free energy of mixing ΔG_m and the the free elastic energy ΔG_{el}.

$$\Delta G_{total} = \Delta G_{mixing} + \Delta G_{elastic} \qquad Eq.5$$

In equilibrium, the Gibbs free energy of mixing ΔG_m (Eq. 5) equals the free elastic energy ΔGel (Eq. 6) [37].

$$\Delta G_{mixing} = -\Delta G_{elastic} \qquad Eq.6$$

Where $\Delta G_{elastic}$ is the contribution due to elastic retractive forces developed inside the gel, and ΔG_{mixing} is the result of spontaneous mixing of water molecules with polymer chains [37].

$$\Delta G_{mixing} = kT\, n_1 \ln\varphi_1 + \chi\, n_2\varphi_2 \qquad Eq.7$$

Where, n_1= ratio of solvent molecules,

φ_1= volume fraction of the solvent

n_2= ratio of polymer repeating units

φ_2= volume fraction of the polymer

k = Boltzmann constant

T= absolute temperature

$$\Delta G_{elastic} = kT\frac{ve}{2}(3\lambda^2 - 3 - in\lambda^3) \qquad Eq.8$$

Where, k= Boltzmann constant

T= absolute temperature

ve= the number of links in the network

λ= linear expansion

So if we use linear expansion λ then,

$$\lambda = \sqrt[3]{\left(\frac{v}{vo}\right)} \qquad Eq.9$$

Where, v = volume of the swollen gel

vo= volume of solvent free gel

For calculation of solvent in the gel and solvent outside, we can use the above formula,

$$\mu^1 - \mu_1^0 = \Delta\mu_1 = \left(\frac{\delta\Delta G}{\delta n^1}\right) = \left(\frac{\delta\Delta Gm}{\delta n^1}\right) + \left(\frac{\delta\Delta Gel}{\delta\lambda}\right)\left(\frac{\delta\lambda}{\delta n^1}\right) \qquad Eq\ .10$$

So we can the applies following,

$$\frac{1}{\emptyset^2} = \frac{v}{vo} = \lambda^3 = \frac{vo + n^1 v^1}{vo} \qquad Eq.11$$

Where, v^1= molar volume of the solvent

So now if we differentiate the equations number 7 and 11 and equations number 8 and 11 which give a Flory-Rehner equation.

$$\ln(1-\emptyset^2) + \emptyset^2 + x\emptyset_2^2 = \frac{ve}{NA}\ \frac{V^1}{vo}\ \left(\frac{\emptyset^2}{2} - \sqrt[3]{\emptyset_2}\right) \qquad Eq.12$$

N_A is the Avogadro number and the $\frac{ve}{vo}$ is the crosslinking density of the polymer network,

$$\frac{ve}{vo} = \frac{pNA}{MX}\left(1 - \frac{2Mx}{M}\right) \qquad Eq.13$$

Where Mx is the molecular weight between the two point of attachment and the M is the molecular weight of the polymer before crosslinking. Therefore, we can measure swelling with known interaction parameter χ, and the molecular weight charge between two points of attachment Mx.

This is because of the fixation to the surface reduces the degrees of freedom of the volumetric swelling from three to one. So the degree of swelling S is given by above equation 14.

$$S = \lambda d = \left[\sqrt[3]{\frac{v}{vo}}\right] d \qquad Eq.14$$

Here d is the dimension in which the network can expand [34]. The swelling behaviour of surface-bound and surface free poly (dimethylacrylamide) (P (DMAA)) has been examined and the dependency of the degree of swelling and linear expansion of the number of dimensions experimentally confirmed [33].

1.6 Diffusion of molecules into hydrogels

The diffusion of molecules in hydrogel gels depends on various factors, like the molecular size and the mesh size [38]. Moreover, permeability as well as mechanical properties of radically cross-linked hydrogels depend not only on the size of polymers, but also on the polymer concentration in the reaction mixture [39]. The mesh size and the density of the gel can be change by the initial concentrations of the PEG diacrylate (PEG-DA) polymer and the solute uptake in equilibrium through the interaction with the PEG chain. This increased uptake can be characterized by an enhancement factor determined by partition ratio analysis [38, 39].

2. Aim and Strategy of the work

2.1 Aim

The main objective of the work was to generate self-cleaning surfaces through the immobilization of the enzymes in coatings. Therefore we aimed to develop an enzyme based coating that can be applied on solid surfaces to target the hydrolysis of ester/fat based compounds. This overall goal can be subdivided into a number of specific aims.

Specific aims and problems

- The ultimate goal of the thesis was to generate bioactive surfaces coatings with a covalently immobilized detergent enzyme as an active component. The enzyme amylase, which degrades starch, was chosen to generate such coatings.
- Compatibility between enzyme and coating material needs to be achieved in order to keep the enzymes active. To allow good access of the substrate molecules to the coating it should be quite strongly swollen in water. However, in aqueous solutions amylase can only be solubilized when rather large amounts of salt are introduced into the solution as a previous study has shown [14]. However, large amounts of salts in the coating solution cause a reduction of the crosslink density and the generation of strongly porous structures. This in turn will cause a possible rupture of the network during swelling or due to mechanical stress, which producing to loss of the enzyme (see figure 14).
- Chemical modification of enzyme on its thermo stability, activity and reusability needs to be elucidated.

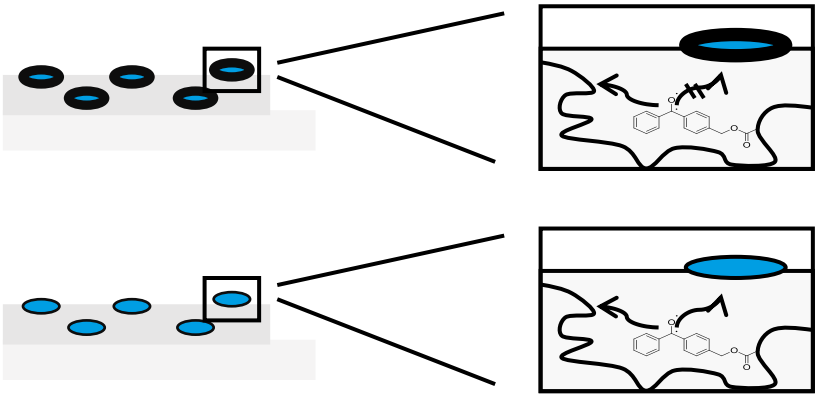


Figure 14. Schematic representation of the effect of high salt concentrations in coating solutions which is responsible for the low efficiency of a following crosslinking process.

2.2 Strategy

To circumvent this problem of film de-attachment we planned to modify the physical properties of enzyme so it could be soluble in non-aqueous solvent (organic solvent) without losing their catalytic behavior [19].

We planned to follow strategies known from the literature in this regards and attach hydrophilic polymers to the enzymes. Such polymers could be PEG or dextran. There are various linker chemistries available from which we plan to choose one that can be readily applied for our system.

While such modification reactions are widely known, it remains uncertain as to whether such approaches lead to a retention of the enzyme activity. It is, hence, crucial to characterize the modified enzyme with regard to the success of the reaction and with regard to its activity as compared to the native biocatalyst.

The modified enzyme will then be immobilized with a polymer that carries a photo reactive group through which it can be cross-linked and attached to a polymeric or organically modified surface. Here, we will use a polymer which has already been used to generate such layers and to immobilize biomolecules on a surface. It is a copolymer of dimethyl acrylamide and a methacrylate carrying a BP unit. The photo

reactive component will make up a few percent in the copolymer and can be cross-linked at either 254 or 365 nm.

It will be crucial to find deposition ways that allow for the generation of homogenous coatings with a low roughness. This can only be achieved in the absence of salt and if an organic solvent is used. Spin or dip coating procedures are in general suitable in this regard.

The most important question is then directed towards the activity of the enzymes in the layer and their self-cleaning behaviour. Figure 15 shows conjugation of enzymes with PEG.

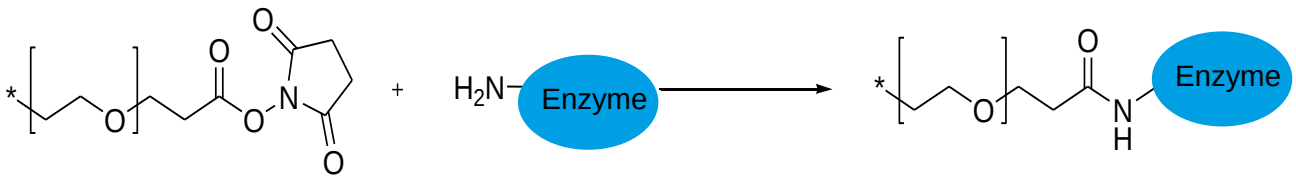


Figure 15. Schematic representation of PEGylation process.

3. Theory of Modification

3.1 Chemical modification: a tool to increase enzyme stability

Enzyme function as well as its stability is governed by its amino acid sequence, which in turn defines the collective non-covalent interactions leading to its specific conformation [3]. While the thermal stability is generally rather low and already longer exposure to temperatures slightly above body temperature can lead to denaturing of the enzymes. Certain so-called thermophilic proteins show a lesser tendency to unfold at high temperatures. Such enzymes have more intramolecular interactions, such as hydrophobic interactions, hydrogen bonding, disulphide bonds and metal binding sites. These stronger interactions produce enzymes with greater conformational stability manifested as better rigidity, higher packing efficiency and higher activation energy for unfolding. Chemical modifications which allow such increased interaction are thus pathways that can be employed to achieve these interactions in less stable mesophilic enzymes.

Many amino acids carries side chains that have reactive functional groups such as the thiol group of cysteine or the amino group of lysine residues (Table 3.1). At least 9 amino acid side chains (Cys, Lys, Asp and Glu, Arg, His, Trp, Tyr, and Met) which are incorporated into proteins can react under mild conditions with specific reagents to yield chemically modified protein derivatives with altered properties [40].The range of suitable reagents has broadened in the past years due to the need for efficient conjugation or immobilization procedures. Two chemical modification strategies in particular have been found to benefit protein stability [40]. These are surface group modification, and covalent coupling of enzymes to polymers such as PEG or polysaccharides such as dextran and sucrose. One note of caution is that, like many other modification techniques, the chemical modifications in many instances have resulted in the reduced catalytic activity of the modified enzymes [41].

Table 3.1 Protein side chain functional group and reagent for their modification [42].

Residue	R-group	Reagent	Reaction
Aspartic Glutamic (C-terminus)	Carbonyl	Carbodiimides	Amidation
		Trialkyloxonium salt	Esterification
Lysine (n-terminus)	Amino	o-Methyl isourea	Guanidination
		Imidates	Amidination
		Borohydrides	Reductive
		Carbonyl compound	Alkynation
		Acid anhydrides	Acylation
		PEG	PEG coupling
Cysteine	Thiol	Maleimido compound	Addition
		Iodoacetic acid	Reduction
		N-ehtylmaleimide	Alkylation
Methionine	Thioether	Hydrogen peroxide	Oxidation
Tryptophan	Indole	N-Bromosuccinimide	Oxidation

3.2 Covalent attachment of polymers to enzymes

This approach involves the attachment of soluble polymers to multiple sites on the enzyme of interest [41]. Alteration of enzyme immunogenicity and prolonging of clearance times have been achieved by attachment to hydrophilic polymers such as PEG [43]. PEG coupling was also found to improve the solubility and activities of enzymes in organic solvents. PEG_{5000} coupled lysine retained about 50% activity at 80 °C while native lysine was completely inactivated at 70°C [43]. The PEG_{5000} derivative was also more resistant to the denaturation effects of sodium dodecyl sulphate (SDS).

During thermal unfolding, polymer modified papain unfolded to a lesser extent than the native papain, and the modified enzyme readily regained its active conformation [41]. Therefore, the structural rigidity found in thermophilic proteins was reproduced in papain by covalent modification with polymers. However, unlike the thermophilic enzymes, where catalytic efficiency is significantly sacrificed [26] for elevated thermal stability, the polymer-modified enzymes seemed to acquire thermal stability without loss of their activity.

Polymers such as dextran and PEG can be used to wrap around enzymes by multi-point attachment to exterior residues of many enzymes [41].These polymer attachments cause a reduction in interactions between the surface residues and the exterior water molecules, hence resulting in more rigid and stable structures that are able to withstand changes in their surroundings [41, 43].

3.3 Enzyme Modification with Poly (ethylene glycol) (PEG)

PEGylation is the chemical modification of enzymes with PEG derivatives, and has generated a lot of interest in the fields of drug delivery and enzyme chemistry [44]. PEG is a non-toxic polymer and when bound to enzymes, they mask the surface residues without altering the enzymatic activity [45]. The PEGylated enzymes are normally soluble, active and highly stable in various organic solvents such as benzene, toluene and trichloroethane. PEGylation was also found to enhance the stability of enzymes in emulsion systems [44].

The site of PEGylation varies depending on the reaction chemistry used. Normally, PEGylation modifies the amine of such residues as lysine on a protein surface at high pH (around 8.0) [45]. This non-site specific reaction results in multiple attachments, which can cause a dramatic decrease in the enzyme activity [44]. An amine specific PEGylation (also known as site-specific PEGylation) is achieved under acidic conditions. The yield of this method is much lower than the conventional PEGylation at higher pH. This observation was further supported by Kim et al. [11]. Who found that site specific PEGylation of lipase yielded much lower concentrations compared to the random PEGylation of lipase reported earlier [44, 45].

In some cases PEG is attached to amino group of enzymes and N-terminal amino acid groups, prior to conjugation with enzymes. When proteins are conjugated the reactions between electrophilically activated PEG and nucleophilic amino acids typically induces substitution of several amines [27]. When multiple lysine have been modified, a heterogeneous mixture is produced that is composed of a population of enzymes where varying numbers of PEG molecules are attached to a single enzyme. This population could range from molecules having zero to the total number of α-amine groups in the enzymes [27]. Figure 16 shows some of the PEG derivatives that are commonly used for enzyme modification.

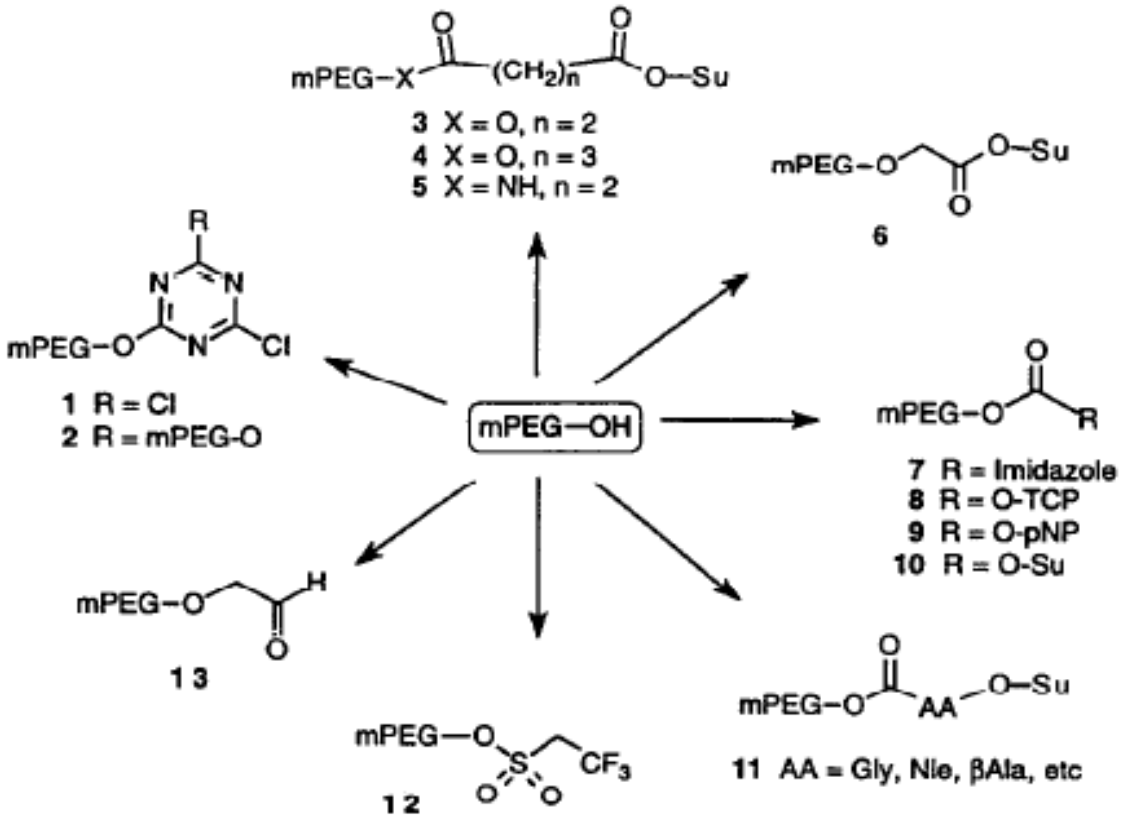


Figure 16. Commonly used PEG derivative for enzyme modification [27].

3.4 Characterization of modified enzymes

3.4.1 Protein-gel electrophoresis

Protein-gel electrophoresis is an analytical method for analysis of the molecular weight of proteins through the movement of these proteins through a swollen polymer gel within an electric field [46]. The charge to mass ratio of each protein determines its migration rate through the gel. Because protein molecules are not strongly charged and the number of charges they carry is more related to the chemical structure of the protein and not so much to the molecular weight of the protein, the proteins are denatured in presence of a strong detergent. When a mixture of SDS and protein is heated to 100°C, the detergent binds strongly physically to the protein and causes unfolding of the protein. The amount of SDS bound to each protein is proportional to its molecular weight, and the rate of migration through the gel is proportional to the molecular weight by a log-linear relationship. However, because very small proteins, i.e. those with less than about 10 kD, do not bind SDS well, small proteins are more difficult to resolve, and therefore require modified electrophoresis conditions. Electrophoresis of covalently joined protein-nucleic acid fusions can also require modified conditions for optimal resolution of different species. This arises from the fact that the nucleic acid component often contributes the vast majority of both the molecular weight and negative charge of fusion molecules, thereby altering the electrophoretic properties of these molecules. Another important features in the separation process is the type and concentration of the buffer in the electrophoresis process. After separation of the proteins according to their charge/mass ratio, they have to be made visible. Figure 17 shows the common procedure for protein gel electrophoresis.

Electrophoresis is a straightforward technique. However, problems may occasionally arise during the various steps in the electrophoresis workflow (eg. current zero or less than zero, gel runs faster than expected, leaking of buffer in the chamber, less band visibility, etc.). The procedure requires considerable time. And due to its rather manual procedure it can hardly be automated.

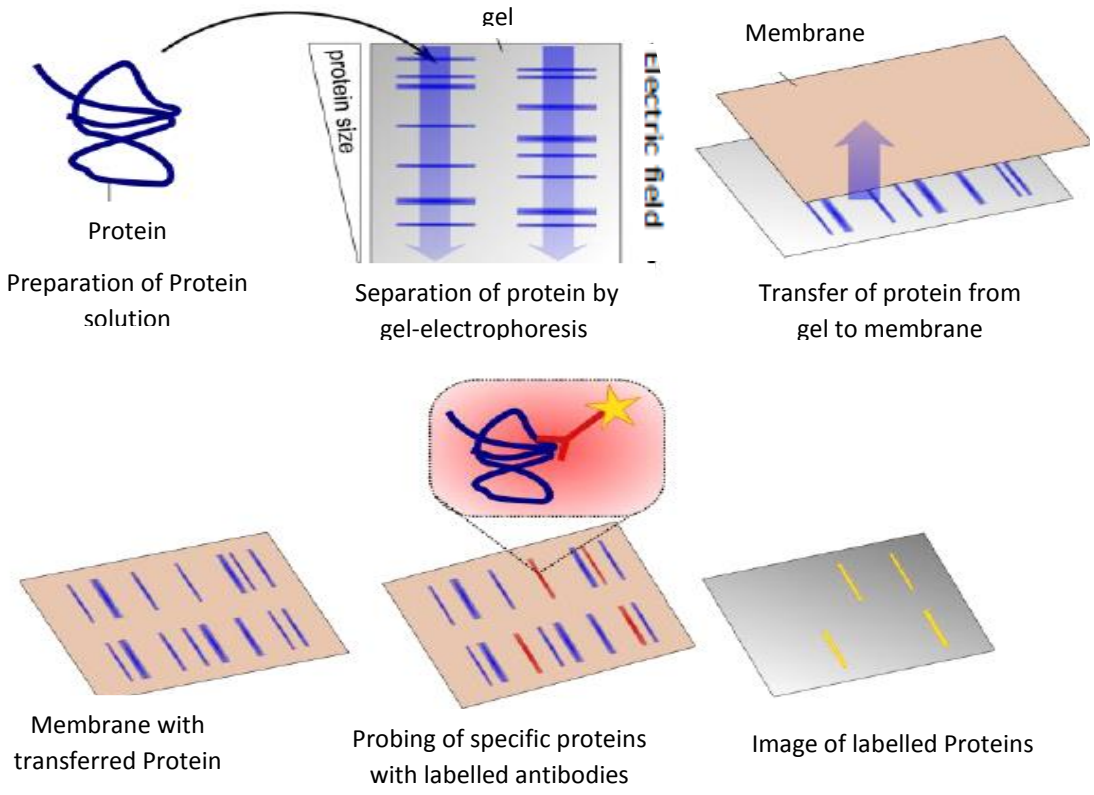


Figure 17. Schematic illustration of work-flow of protein gel electrophoresis [47].

3.5 Quantitative analysis of enzyme activity

Enzyme activity is a measurement of the quantity of active enzyme present and is thus dependent on conditions, The SI unit of enzyme activity is the katal, 1 kat = 1 mol s^{-1}.

Enzyme activity as given in katal generally refers to that of the assumed natural target substrate of the enzyme. An increased amount of substrate will increase the rate of reaction with enzymes, however once past a certain point, the rate of reaction will level out because the amount of active sites available has stayed constant.

3.5.1 Activity of α-amylase on surface

In the activity measurement of α-amylase we used starch as a substrate and then determines the resulting amount of converting starch into maltose using 3, 5-dinitrosalicylic acid [48].

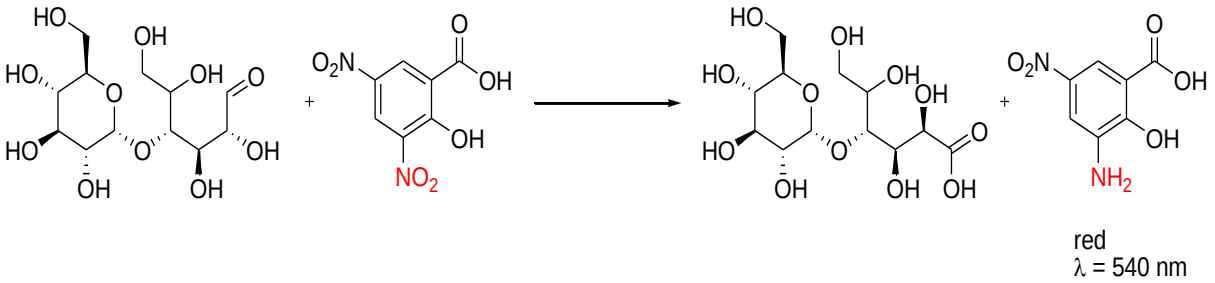


Figure 18. Schematic representation of the oxidation of maltose using 3, 5-dinitrosalicylic acid.

4. Polymer synthesis

Figure 19 depicts the structure of the polymer which is used in this work. The base material consists of dimethylacrlyamide units, which is a rather polymer. In the uncrosslinked form it is soluble in alcohols and water and other polar solvents. To enhance the hydrophilicity in some of the polymers a charged repeat unit, composed of styrene sulfonic acid, is incorporated. To make the polymer photosensitive and to allow photochemical crosslinking BP units are incorporated by using methyacrylolybenzophenone (MABP) as a comonomer in the polymerization process.

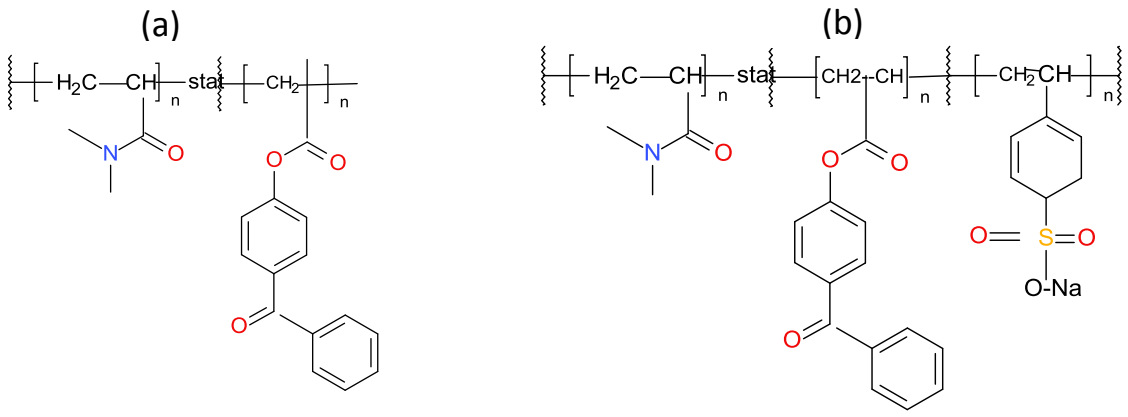


Figure 19. Chemical structure of the polymers which are used for immobilization of enzyme. (a) P (DMAA-MABP5%) and (b) P (DMAA-MABP5%-SSNa2.5%).

4.1 Preparation of polymers

The polymers were generated by free-radical polymerisation using Azobisisobutyronitrile (AIBN) as initiator and DMF or methanol as a solvent. The reactions were performed at a temperature of 60˚C for 20 hs. After stopping the reaction by cooling down to room temperature the product was isolated by precipitation in diethylether and dried under the high vacuum. Afterward the polymer was redissolved in methanol and it was again precipitated in diethyl ether. Table 4.1 shows the polymers used in this study in respect to variation of polymer with a different amount of MABP and SSNa.

Table 4.1 Composition of the generated polymers as a function of the MABP and SSNa contents.

Polymer	DMAA (%)	MABP (%)	SSNa (%)
P (DMAA-MABP5%)	95	5	-
P (DMAA-MABP5%-SSNa2.5%)	92.5	5	2.5

After collecting the final product the polymers were characterized by ^{1}H-NMR and FTIR spectroscopy. The (relative) molecular weights were determined by GPC.

4.2 Characterisation of the polymer

Figure 20 shows the ^{1}H-NMR spectrum of the polymer P (DMAA-MABP5%-SSNa2.5%). The signals in the range of δ =0.9 to 3.3 ppm can be attributed to the DMAA repeat units whereas the signals around 8 ppm are caused by the aromatic hydrogens of the MABP units.

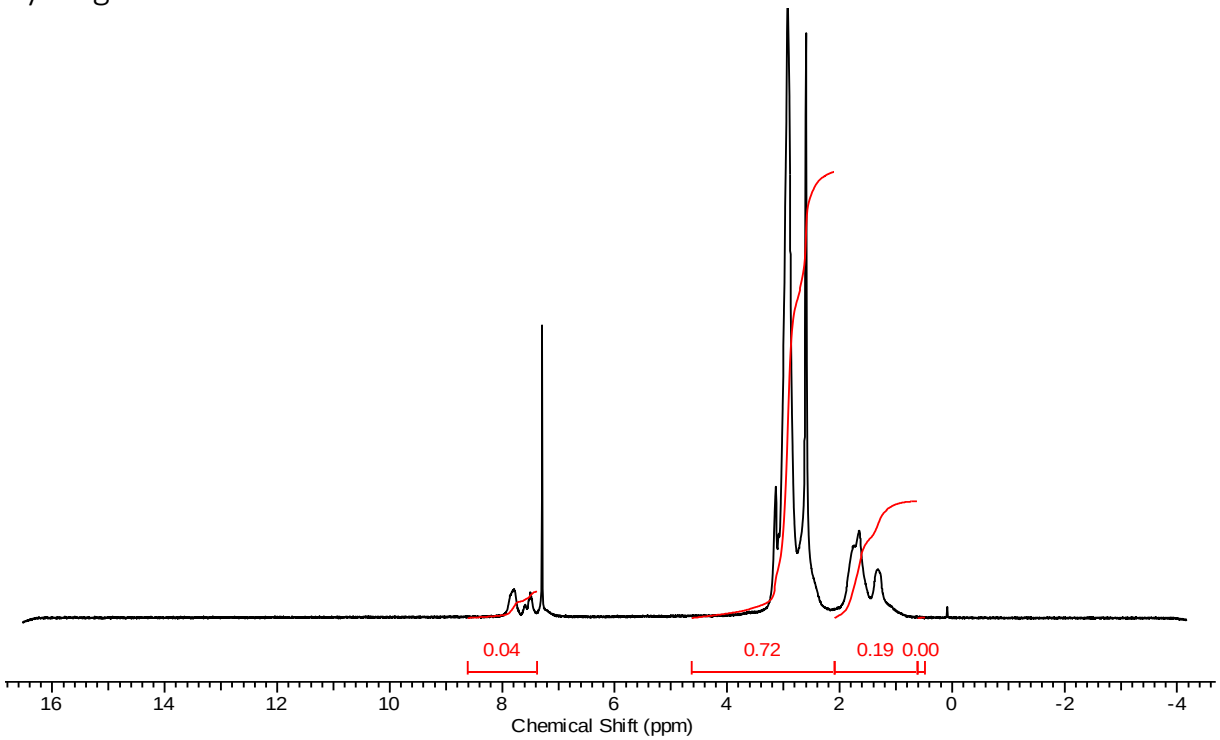


Figure 20. ^{1}HNMR spectrum of polymer (DMAA-MABP5%-SSNa2.5%); solvent ($CDCl_3$).

The amount of MABP incorporated in the polymer was determined by FTIR spectroscopy. To this the ratio of the carbonyl bands of the two components MABP and DMAA was determined as a function of the MABP content in the polymer. The absorption band at a wavenumber around 1739 cm^{-1} can be assigned to the carbonyl group of the ester in MABP whereas the signal at 1641 cm^{-1} results from the carbonyl group of the amide group in the DMAA (see figure 21).

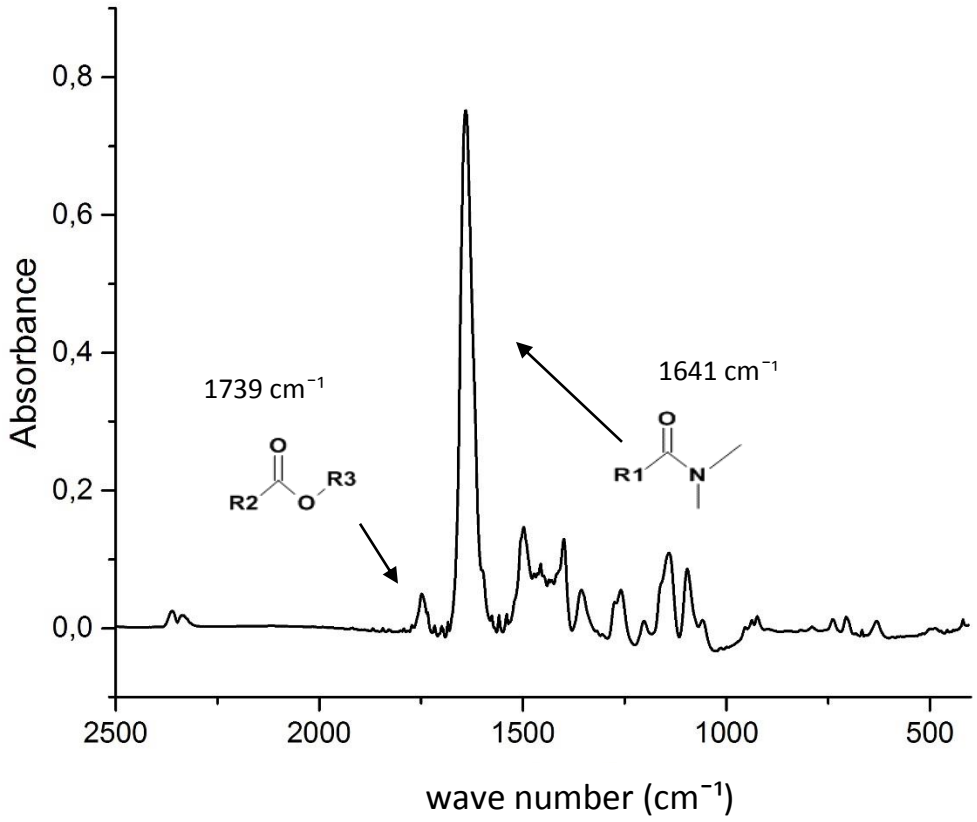


Figure 21. FTIR spectrum of polymer P (DMAA-5%MABP).

In order to obtain a calibration curve, P (DMAA) and MABP were mixed in different ratios and the intensities of the carbonyl absorption bands were determined. This calibration allows to determine the ratio of the molar absorption coefficients of the two components.

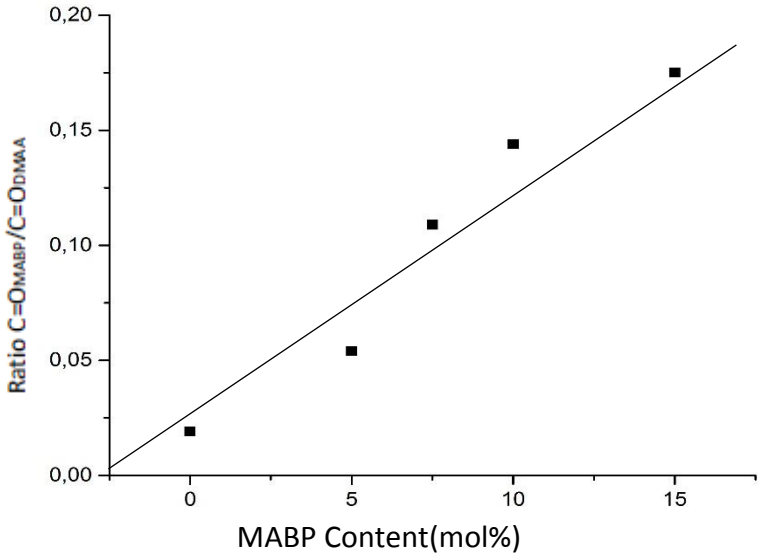


Figure 22. Calibration curve of mixtures with different concentration of MABP and P (DMAA) obtained by FTIR spectroscopy; depicted is the ratio of the two carbonyl absorption bands as a function of composition of the mixtures.

Table 4.2 shows the MABP contents of the polymers used in this project. It can see that the MABP content of the polymer is slightly lower than that of the amount of MABP used in the polymerisation reaction.

Table 4.2 MABP contents of copolymer determined by FTIR

Polymer	MABP Content (mol %)
P(DMAA-5%MABP)	3.6
P(DMAA-5%MABP-2.5%SSNa)	3.1

For the determination of molecular weight of the polymer, GPC (gel permeation chromatography) was used. In these experiments DMF was used as the solvent and narrow polydispersity Poly (methyl methacrylate) (PMMA) samples were used as standards. The molecular weight distribution of the polymers by GPC are summarized in table 4.3.

Table 4.3 Molecular weight and polydispersity index (PDI) of the copolymers synthesized in this study.

Polymer	M_n (g/mol)	MW(g/mol)	PDI
P (DMAA-MABP5%)	118,000	382,000	3.2
P (DMAA-MABP5%SSNa2.5%)	102,000	379,000	3.7

The data shows that the followed procedure generated relatively high molecular weight copolymers.

4.3 Discussion

A series of PDMAA copolymers containing photo cross-linker (MABP) were successfully synthesized through free radical polymerization. These polymers were then deposited onto a solid substrate and FTIR spectroscopy measurements were performed to calculate the MABP contents in the polymers.

The actual amount of BP was slightly lower than expected from the monomer composition. The BP contents of the polymer chains was sufficient to crosslink the polymer and attach the enzymes.

5. PEGylation of α-amylase

To enhance the solubility of the enzymes in aqueous solutions and to allow a better compatibility with the polymer, α-amylase was conjugated with PEG chains. Linear PEG-NHS was applied as an efficient modifier for the enzyme through reaction with lysine residues.

5.1 Conjugation of α-amylase with PEG (Polyethylene glycol)

Native α-amylase enzyme was purified by ultrafiltration with a regenerated cellulose membrane (MW cut-off 30-50 kD) before modification. For the modification reaction first enzyme solution was added to a 5-ml glass vial and incubated in an ice bath with magnetic stirring. PEG conjugation was initiated by adding a desired amount (depending on modification ratio) of PEG that was pre-dissolved in DMSO (dimethyl sulphoxide). The reaction was performed in sodium phosphate buffer at a pH value of pH=8.0. At this pH, a significant percentage of amino groups exists in a non-protonated state, leading to efficient reactions with the NHS groups of the NHS active ester. After 4 h of reaction, ultrafiltration centrifugation was conducted to remove free PEG modifier.

The total amount of recovered product showed a typical yield of 72% based on total amount of enzyme added in the reaction. After purification, the enzyme concentration was adjusted back to 0.9 mg/ml for the preparation of coatings.

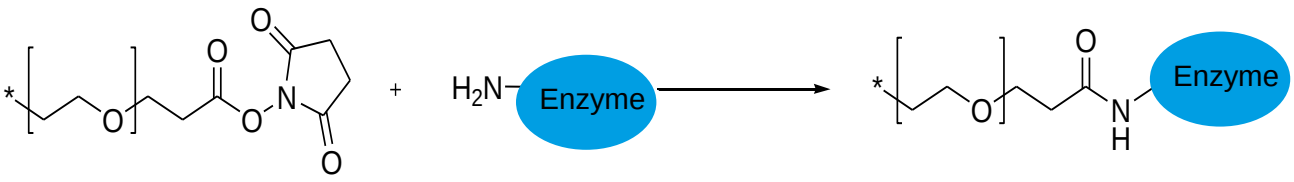


Figure 23. Schematic representation of PEGylation process of α-amylase.

5.2 SDS PAGE Characterisation of modified enzyme

After the unreacted PEG modifier was removed via ultrafiltration, the degree of pegylation of the conjugated enzyme was determined by SDS polyacrylamide gel electrophoresis. Basis of this determination is that the molecular weight of the enzyme increases significantly through addition of one or more PEG chains. An example for a result obtained in such measurements is given in figure 24.

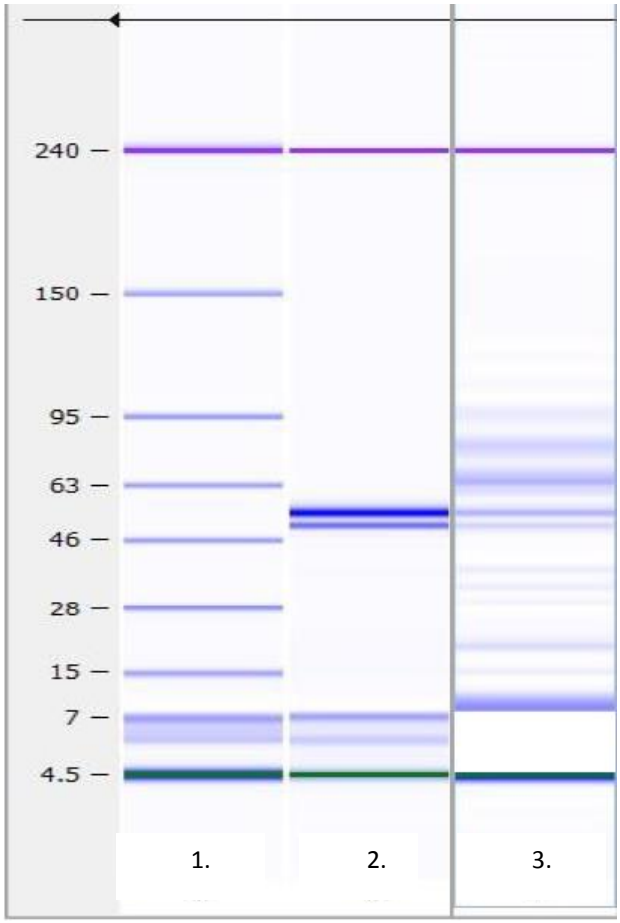


Figure 24. Electrophoresis (SDS-PAGE) analysis of PEG-conjugated α-amylase lane 1 shows standard protein markers, lane 2: native α-amylase, lane 3: PEG conjugated α-amylase.

The protein gel electrophoresis analysis shows that the modification is efficient and almost no native enzyme is left in the sample, when the molar ratio between PEG and the native enzyme was 5:1. The molecular weight of the modified amylase fell

in the range up to 95 KD, compared to the native amylase shows MW band at 50-55 kD.

5.3 Discussion

The extent of chemical modification was determined by SDS-PAGE gel electrophoresis. PEG modified amylase is clearly detected on PAGE through an additional band at higher molecular weight. The MW of the enzyme increases by 20-45 kD in addition to the MW of the native amylase, which was 50-55 kD.

This increase in the molecular weight of the enzyme indicates binding of 4-9 PEG molecules per amylase according to above equation,

$$pegylation\ ratio = \frac{Avg\ MW\ of\ Pegylated\ enzyme}{Avg\ MW\ of\ native\ enzyme}$$

Therefore, the reaction between the PEG and amylase was successful in sodium phosphate buffer, pH 8.0. At this pH, a significant percentage of amino groups exists in a un-protonated state. Figure 25 shows the schematic illustration of attachment of PEG with α-amylase.

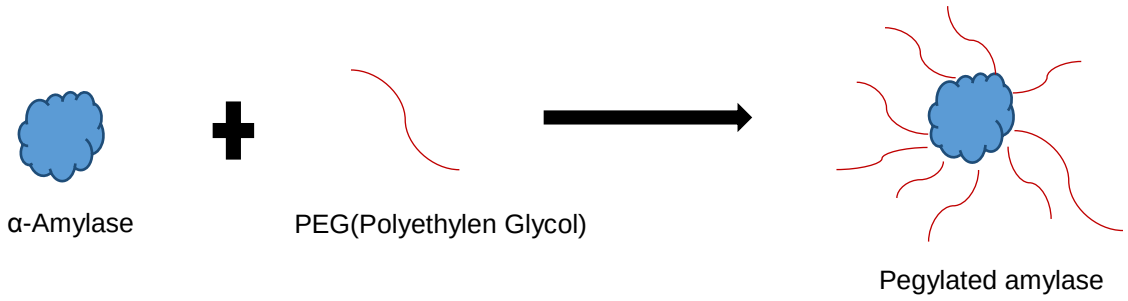


Figure 25. Schematic illustration attachment of PEG with enzyme.

Being a glycoprotein, amylase also possesses 19 lysine residues at the molecular outer surface which providing point for modification with surface sugar group. However, the number of PEG attachment is quite reasonable if we consider the number of surface lysine groups of amylase. Number of attachment also indicates the heterogeneity of the modification reaction which resulted in at random PEG attachment.

6. Functional hydrogel coating with α-amylase

6.1 Coating Preparation

Silicon wafers were selected as a substrate because many analytical tools are available for this substrate for surface analysis. Coating solutions containing native amylase or pegylated amylase were prepared. For the preparation of native amylase solutions, α-amylase (porcine pancreas; manufacturer's specification: 1800 U/mg was dissolved in a concentration of 1 mg/ml in previously prepared 0.01M; 7.5 pH PBS buffer in which 5 mg/ml of P (DMAA-MABP5%-SSNa2.5%) polymer had been dissolved. In another experiment PEG modified amylase was dissolved in 99.9% ethanol at a concentration of 0.9 mg/ml together with 5 mg/ml of P (DMAA-MABP5%) polymer. The coatings were prepared by dip coating. The resulting layer was then irradiated under UV light in order to crosslink the coating at λ= 254 nm for different exposure times. The coated layers were then washed with ethanol or PBS buffer to remove non-attached enzyme on the polymer surface. Then the activity was measured in order to evaluate the catalytic behaviour of the immobilized enzyme on the surface. We found that PEG conjugation enhances the enzyme retention in the film and less material was washed out during extraction.

6.2 Characterization of enzyme containing coatings

6.2.1 Characterization of enzyme containing coatings with SEM

An important question is the homogeneity and roughness of the enzyme containing surface. Scanning electron microscope (SEM) was used to study this question and some typical micrographs are shown in figure 26.

The image obtained from the native amylase without PEG modification shows a very rough surface with visible crystal salts on top of the coating. This may result from the salt needed in the coating solution in order to make enzyme soluble. During drying of the polymer film the salt starts to crystallize. Thus the photo crosslinking leads to porous networks which are not homogenous upon drying. The more strongly porous structure also allows the removal of more entrapped enzyme, so that more enzyme is removed during the washing process.

In contrast to the native amylase, the PEG conjugated amylase containing polymer coatings form very smooth surfaces as shown in the figure 26(b). This can be attributed to the salt-free conditions applied during deposition.

(a)

(b)

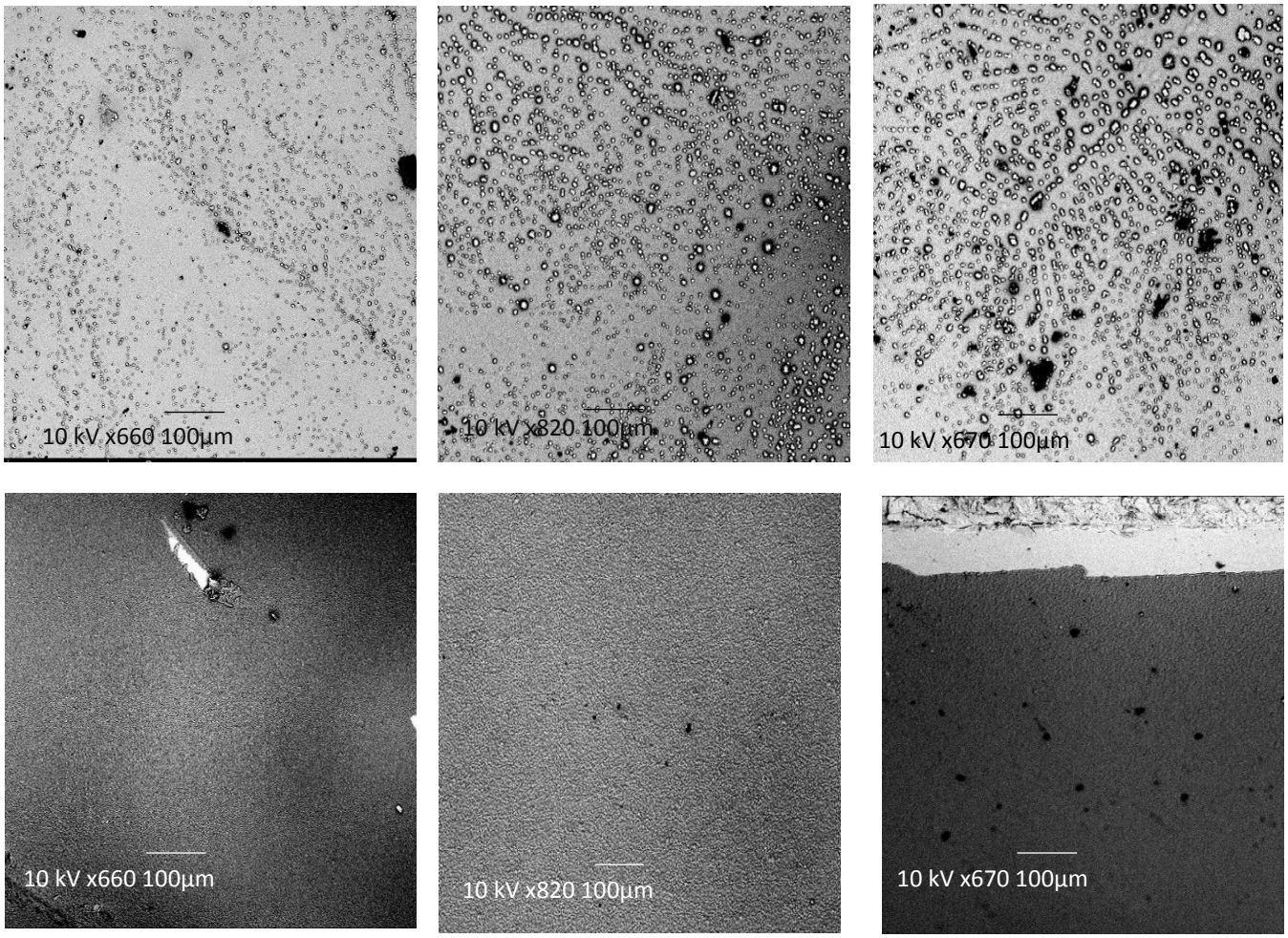


Figure 26. SEM micrographs obtained from hydrogel coatings with (a); native amylase and (b) PEG conjugated amylase.

6.2.2 Characterization of enzyme containing coatings with fluorescence microscopy

Based on above mentioned finding the distribution of α-amylase was further investigated by using inverted fluorescence microscopy. For the preparation of coatings suitable for fluorescence microscopy isothiocyanate was mixed with the coating solution and incubated for 2 h. After that the solution was directly used for coating purposes.

The image obtained from the native amylase in figure 27(a) shows a very rough surface with salt crystals. The distribution of the enzyme is not uniform.
In contrast to this the PEGylated amylase containing coating figure (27b) shows a much more homogeneous surface with a uniform distribution of the enzyme.

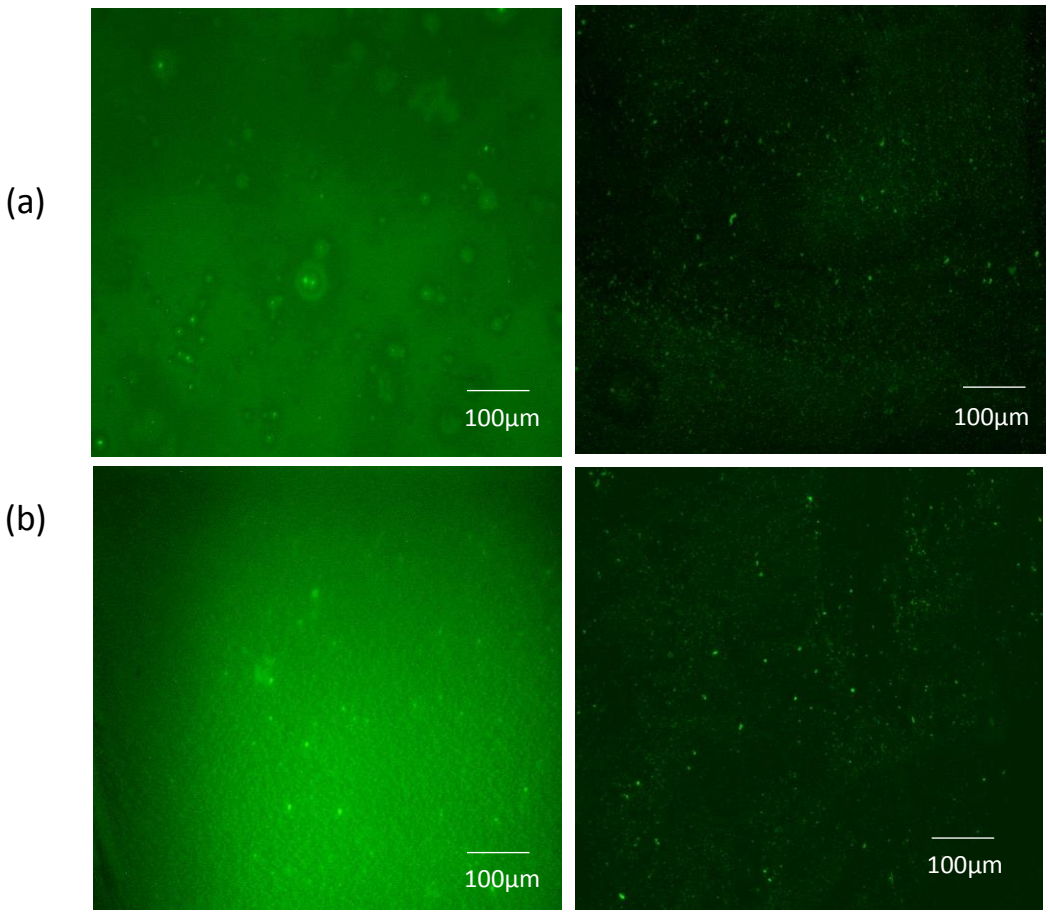


Figure 27. Fluorescence microscopy image obtained from hydrogel coating of native amylase (a); PEG conjugated amylase (b).

6.2.3 Topography or Roughness of the coatings

Topography or roughness may also influence the interaction of surface with biomolecules. In addition, the thickness of the layers is also an important parameter which influences the activity of the coated surfaces.

AFM (Atomic Force Microscopy; Nanoscope IIIa, Digital instruments) was used to examine the roughness of enzyme coated surface, the microscope was operated in

tapping mode, using a commercial tip with a resonance frequency of 300 kHz, and a spring constant of 42 N/m. The AFM micrograph were recorded in air at room temperature. The figure 28 shows the roughness measurement of the PEG conjugated enzyme and native amylase.

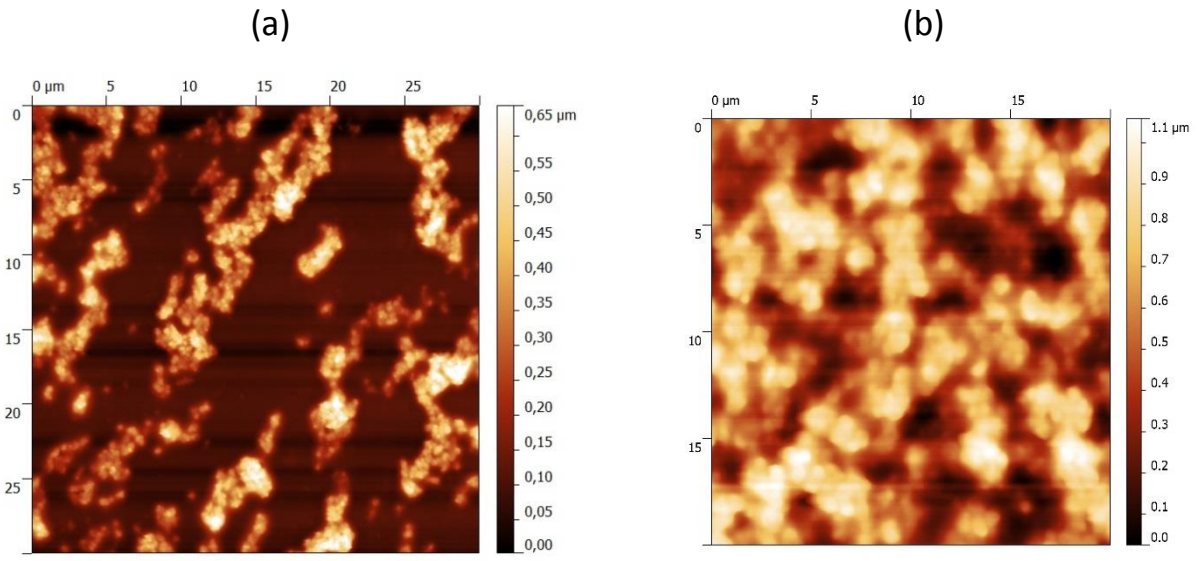


Figure 28. AFM images of (a) PEG conjugated enzyme containing coating; (b) native amylase containing coating.

The PEG conjugated enzyme containing coating shows the average roughness (Ra) of around 95 nm when the coating with native amylase shows average roughness with 140 nm.

Thickness of enzyme and polymer containing layer were measured by using auto-nulling ellipsometry from nano-films (EP3) which was operated at λ=532nm at variable angular modes from 55-75˚. In table 6.1 the different values for the roughness and thickness for PEG conjugated enzyme and native enzyme are shown.

Table 6.1 Thickness and roughness of the PEG conjugated amylase and native amylase

Samples	Thickness (d)	Roughness (Ra)
PEG conjugated Amylase	87nm	99.1nm
PEG conjugated Amylase	81nm	94.4nm
PEG conjugated Amylase	74nm	110 nm
Native Amylase	95nm	123 nm
Native Amylase	91nm	116.6nm
Native Amylase	114nm	180.4nm

The thickness of the deposited film for PEG conjugated amylase ranged between 70-90 nm, while for native amylase it ranged between 90-120 nm, indicating that there are no significant changes in the layer thickness in the different deposition processes. The AFM results give roughness values between 90 and 110 nm for PEG conjugated amylase while the native amylase containing coatings had rougher surfaces compare to PEG conjugated amylase. If we compare the results for both amylase we can say that the PEG conjugated amylase allows to generate more homogeneous layers compare to the native amylase. The salt present into the native amylase coating increases the roughness of samples.

6.3 Enzyme activity in the coating

6.3.1 Colorimetric determination of α-Amylase

The activity of the native amylase and the PEG conjugated amylase in the coating was measured by using a colorimetric method with the help of 3, 5-dinitrosalicylic acid as the chromophore. Amylase belongs to the family of glycoside hydrolase enzymes that break down starch into glucose molecules by acting on α-1, 4-glycosidic bonds. The α-amylases cleave at random locations in the starch chain, ultimately yielding malt triose and maltose, glucose and "limit dextrin" from amylase. α-amylase is a major digestive enzyme in which due to the amyl lytic activity reducing groups are released from starch and reacts with 3, 5-dinitrosalicylic acid (see figure 29).

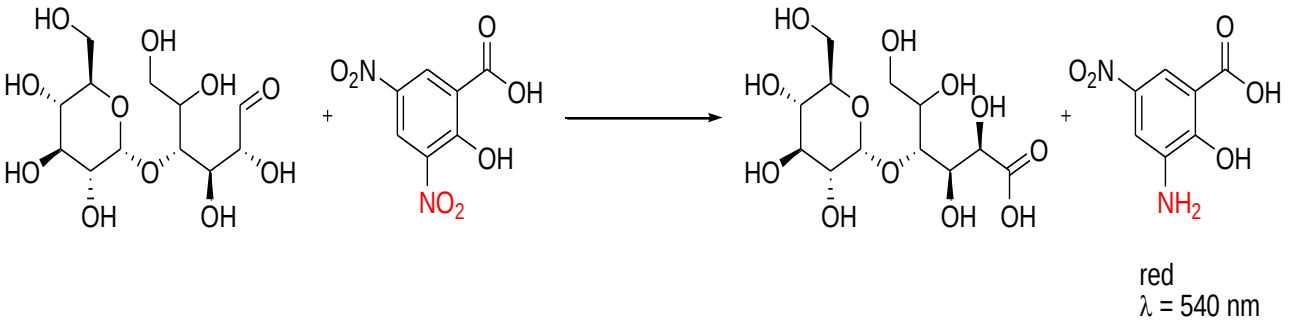
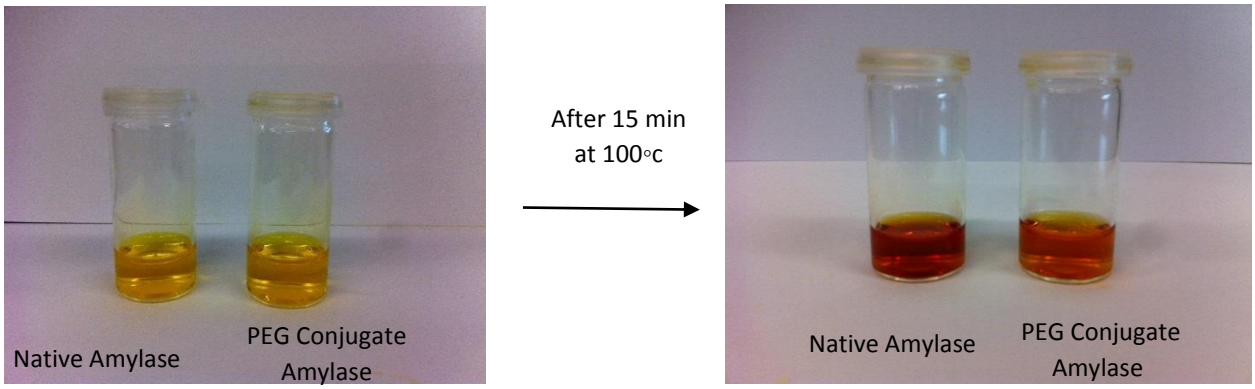


Figure 29. Schematic representation of the oxidation of maltose using 3, 5-dinitrosalicylic acid

The substrate solution comprised of 1% w/v potato starch in 20 mM sodium phosphate buffer with 6.7 mM sodium chloride (pH 6.9)[48]. Coated panels were cut into smaller pieces of 1.5 cm × 1.6 cm before being tested, with 1 ml of substrate solution applied for one coating specimen, incubated under room temperature for 3 min. For native enzyme and PEG conjugated enzyme, 10 µl of enzyme sample solution was added into 1 ml of substrate solution and tested under the same conditions. At the end of the reaction, 1 ml of 96 mM 3, 5-dinitrosalicylic acid was added to the reaction solution and incubated in boiling water for 15 min before being cooled down in an ice bath. The equivalent of reducing sugar was determined with a Cary 50- Varian UV–Vis spectrometer at 540 nm. One unit of α-amylase activity is defined as the amount of enzyme that produces 1.0 mg of reducing sugar from starch in 3 min at room temperature. Thermo stability and enzyme retention were examined by measuring residual enzyme activities (through the above procedure) of concerned samples as a function of different condition i.e. time, temperature (see figure 30).

(a)



(b)

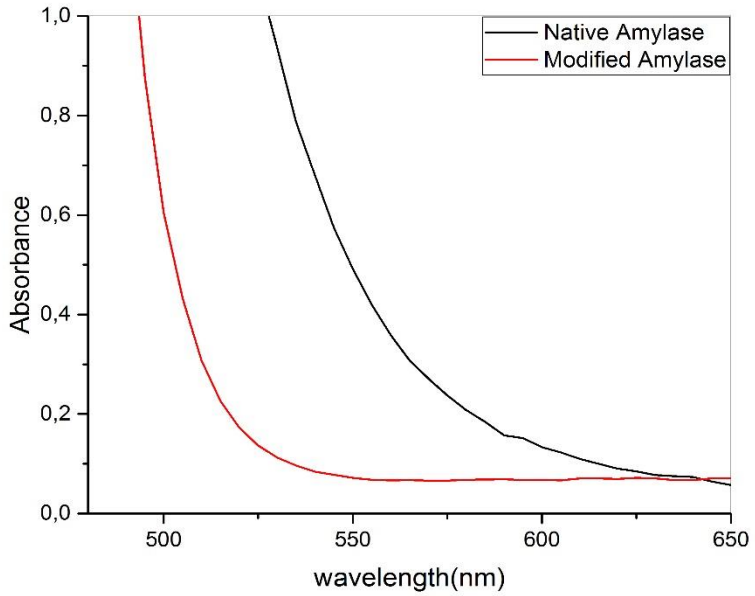


Figure 30. (a) Measurement of the activity of amylase containing coating after an Incubation time of 10 min at room temperature (b) UV absorbance of the washing solution of coatings contain native amylase and PEG conjugated amylase.

For the activity measurements of the native amylase and the PEG conjugated amylase, calibration curve (with maltose as standard) was used to calculate the concentration of the reducing sugar generated by the coating. From these measurements the activity of the native amylase and the PEG conjugated amylase containing coatings was derived.

With the help of the Lambert-Beer law the concentration of protein solution was calculated.

$$A = log\left(\frac{Io}{I}\right) = \varepsilon Cl \qquad Eq.15$$

Where, A = absorbance

ε = molar extinction co-efficient ($M^{-1}cm^{-1}$)

C = concentration of solution (mg/ml)

l = path length of the light passing through solution

ε was calculated from the maltose standard curve and l (path length) is always 1 cm, the absorbance of the washing solution was measured by UV/vis spectroscopy. With those values the activity of the native amylase and PEG conjugated amylase were calculated and finally the concentration was derived. Here we found that activity of the native enzyme was much higher compared to PEG conjugated amylase. From the activity measurement of 3 samples right after preparation of the coating, the PEG conjugated enzyme had an average activity of about 90 % of the starting material whereas the native amylase had average activity around 140 % as shown in figure 31.

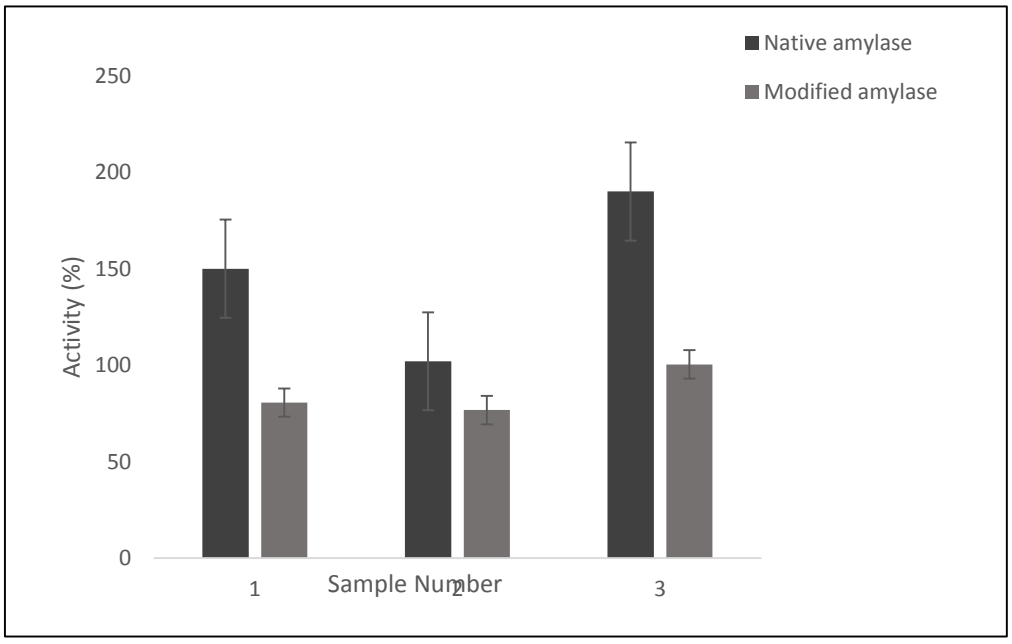


Figure 31. Activity of washing solution of PEG conjugated amylase and native amylase.

6.3.2 Influence of UV-Irradiation on enzyme activity

Enzymes have a tendency to degrade under UV irradiation, but many enzymes can also survive under such conditions. Therefore we evaluated the activity of native amylase and PEG conjugated amylase in UV irradiated coatings. The concentration of the native amylase was 1 mg/ml in 0.01M, PBS buffer (pH 7.5) and 5 mg/ml of P (DMAA-MABP5%-SSNa2.5%). The concentration of PEG conjugated amylase was 0.9 mg/ml in 99.9% ethanol with 5 mg/ml of P (DMAA-MABP5%). The resulting layers were irradiated with UV at a wavelength of λ= 254nm. The UV irradiation times selected were from 10 to 50 min and the activity of enzyme was measured. We still found remaining activity for the native amylase even after long irradiation times and PEG conjugated amylase (figure 32). The results show that the PEGylated amylase was in general more stable against UV irradiation in comparison to the native amylase.

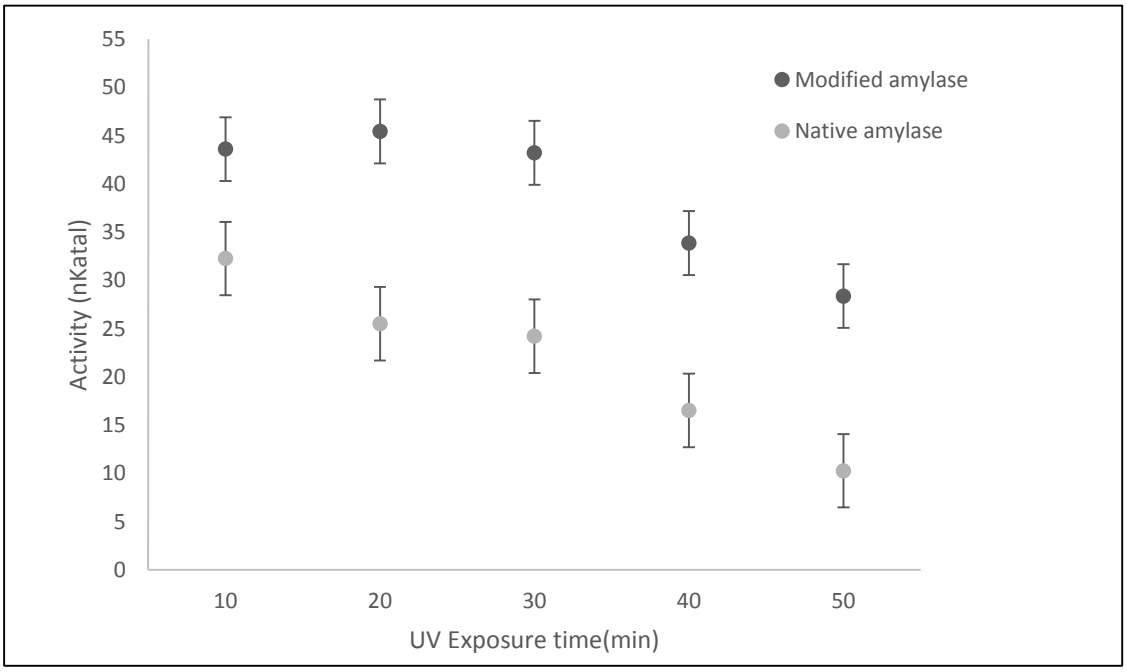


Figure 32. Activity of coating with native amylase and PEG conjugated amylase as function of exposure time at λ= 254nm.

6.3.3 Thermal stability test

The thermal stability is one of the most important properties for biomolecule based materials in practical applications [8]. Enzymes are most active at an optimum temperature (around 37˚C), they shows little activity at low temperature and lose the activity at high temperature as denaturation occurs [2]. The thermal stability of native amylase and PEG conjugated amylase was investigated by heating the coated substrate in a water bath at different temperatures. Then the residual bioactivity of the coated substrate was measured as a function of heating time at temperatures between 20 and 70˚C.

Also against higher temperatures PEG conjugated amylase retained a higher enzyme activity compared to the native amylase. The PEG conjugated amylase lose only about of 40% of the original activity at maximum in the studied temperature range and was even more active at higher temperatures (70˚C) where native amylase shows significant loss of activity (figure 33).

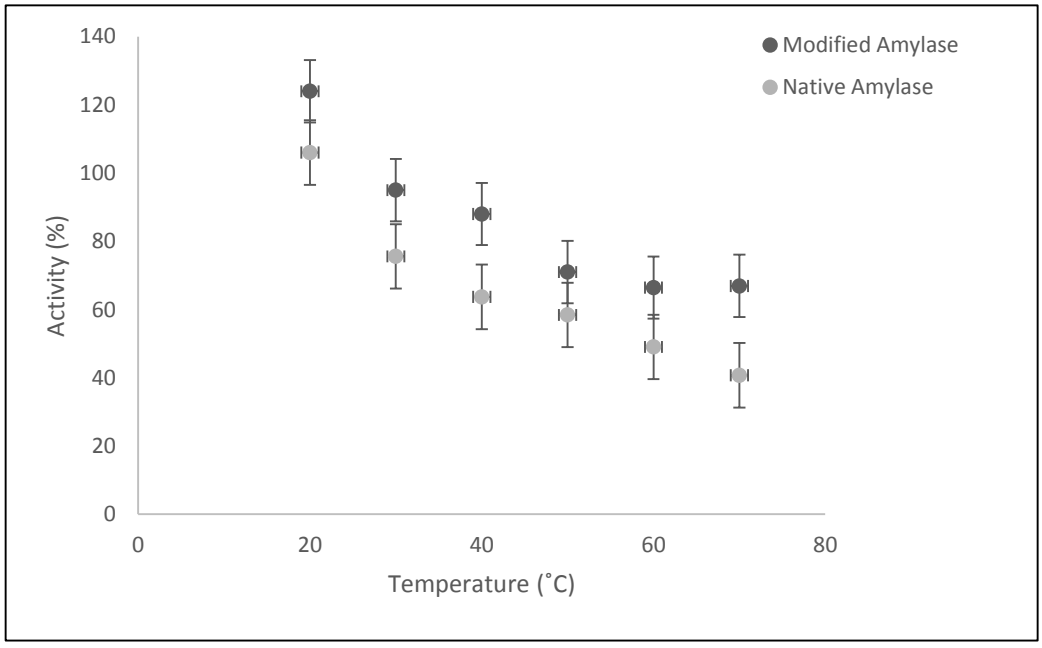


Figure 33. Effect of temperature on the activity of PEG conjugated amylase and native amylase

To measure the wettability of the coated surface at different temperatures we measured the contact angle of PEG conjugated contained coated surface. The static contact angle was measured at different temperatures (20-100˚C) while the substrate was immersed into a water bath. A water droplet around was brought into contact with the coating and the contact angle was directly measured. Figure 34 shows the static contact angles of PEG conjugated amylase contained coated surface at different temperatures. It is observed that no change in the water contact angle, which indicate the thermal stability of the PEG conjugated containing coating.

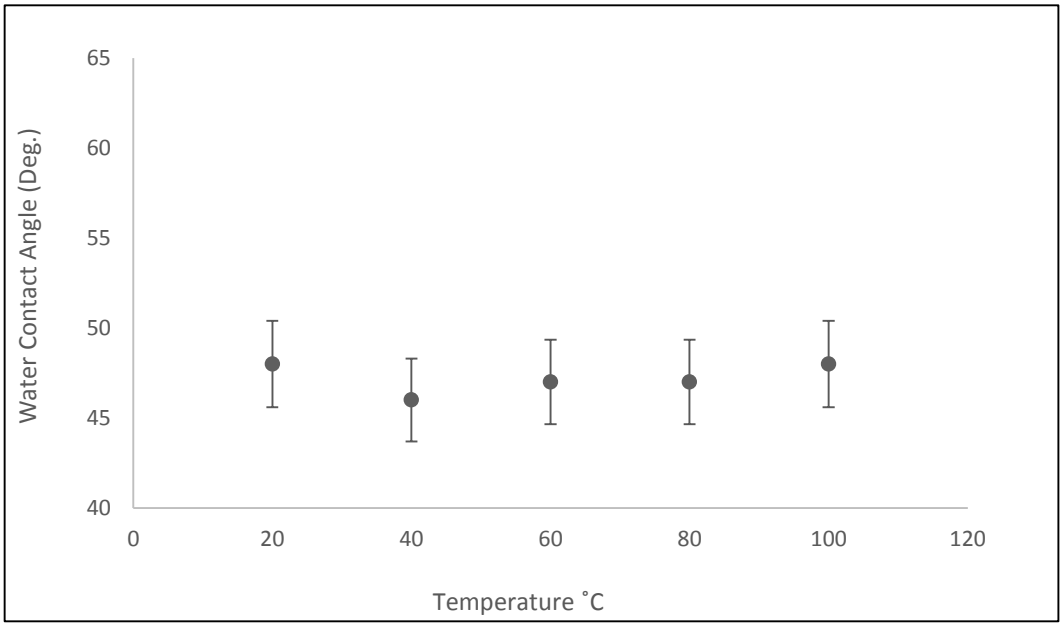


Figure 34. Variation of contact angle at different temperature.

6.3.4 Long term stability

The long term activity of the enzyme containing coatings is a crucial question if they are used as a self-cleaning surfaces. The results for PEG conjugated amylase and native amylase coatings as a function of time are shown in figure 35. After different times, the residual activity of the coated surface was measured and then we observed that the native amylase has a half-life time around 13 days while PEG conjugated enzyme still shows 60% activity even after 22 days. It also shows that coating with native amylase significantly lose their activity while PEG conjugated

amylase loses less activity. The longer half-life time of the PEG conjugated amylase coating may be attributed to a stability enhancement in addition, it could be possible that the polymer network of the coating may also helps the enzyme to protect against protein chain unfolding.

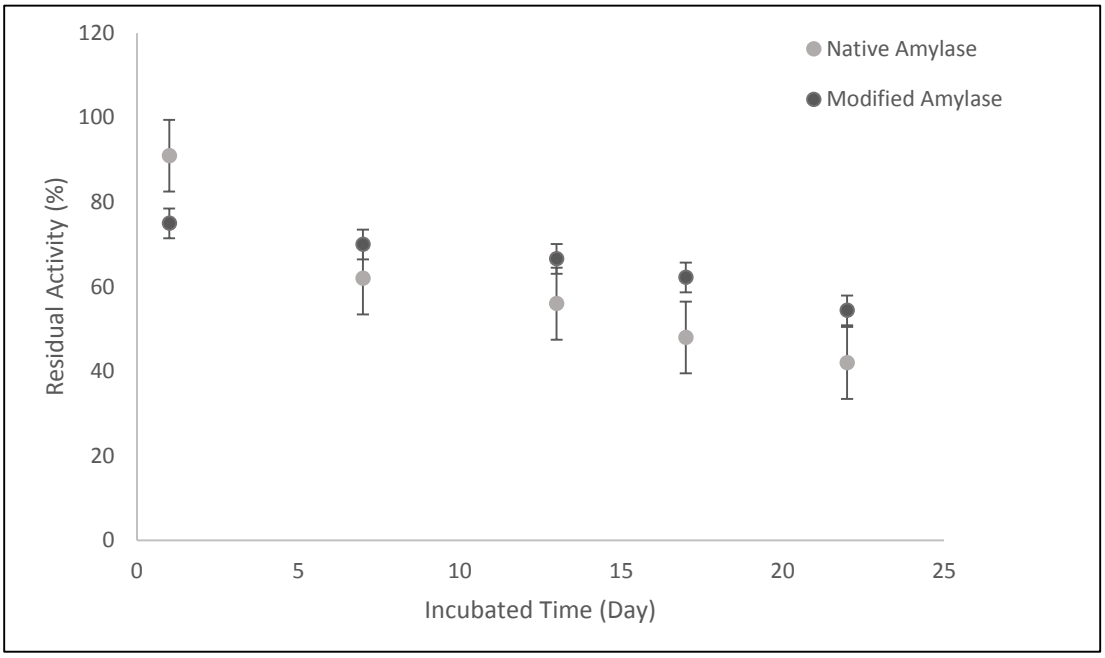


Figure 35. Activity of enzyme containing coatings as a function of time.

6.3.5 Self-Cleaning Functionality Test

To illustrate the self-cleaning surface properties of the coating, staining of the surfaces by mayonnaise was used as a model. In addition to egg yolk, vegetable oil, water, salt, vinegar, sugar, etc., "light" mayonnaises contain starch as a thickening agent. Coatings were prepared on silicon substrates containing the different amylases (PEG conjugated amylase, native amylase), and a drop of mayonnaise was applied at one end of the substrate. The substrate was then placed in an upright position and the result was monitored as a function of time. In figure 36 a, b and c the image taken after 0, 10 and 30 min are shown. Sample 1 is coated with only polymer without any enzyme. Samples 2 and 3 are coated with native amylase and PEG conjugated amylase respectively.

On the enzyme free coating the no movement of the drop can be observed as a function of time. In the two other cases the decomposition of starch reduces strongly the viscosity of the mayonnaise and it begins to slide down the substrate. There was no significant difference between the sample containing the native amylase and the PEG conjugated amylase.

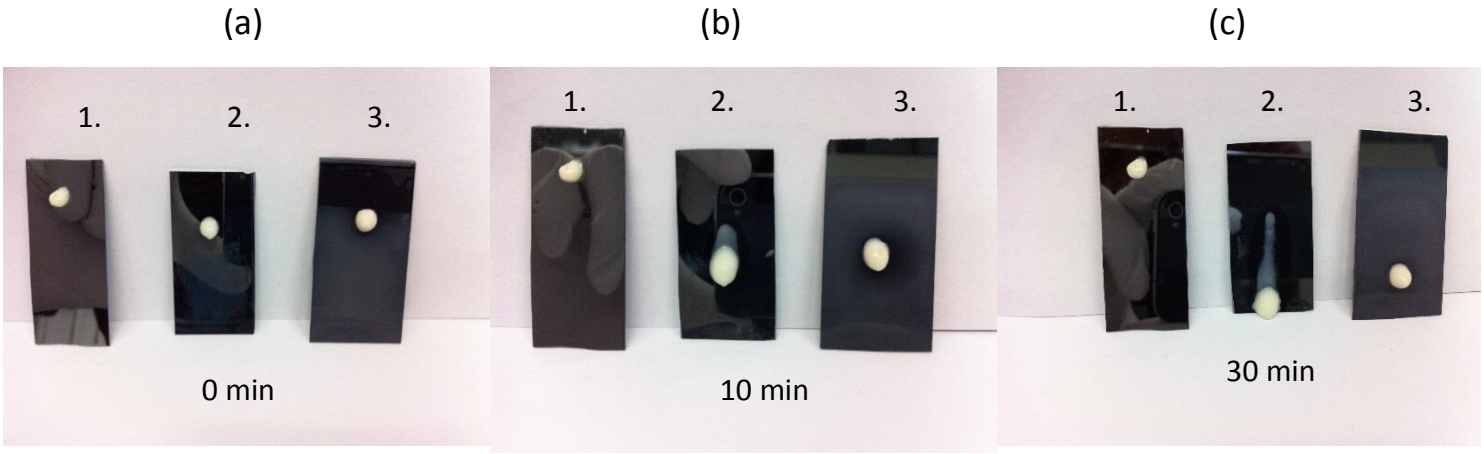


Figure 36. Self-cleaning functionality test (a) test time at 0 min when the all coated substrate tilted vertically. 1. Silicon substrate without any enzyme 2. Coating with native amylase 3. Coating with PEG conjugated amylase (b) test time at 10 min (c) test time at 30 min.

The coated substrates were then washed with 0.01 M PBS buffer for 24 hs and reused in an identical test, to check the stability and activity of both enzyme coated substrates. Figure 37 shows the result of this repeated test. The sample numbers are identical to those given above for the first test.

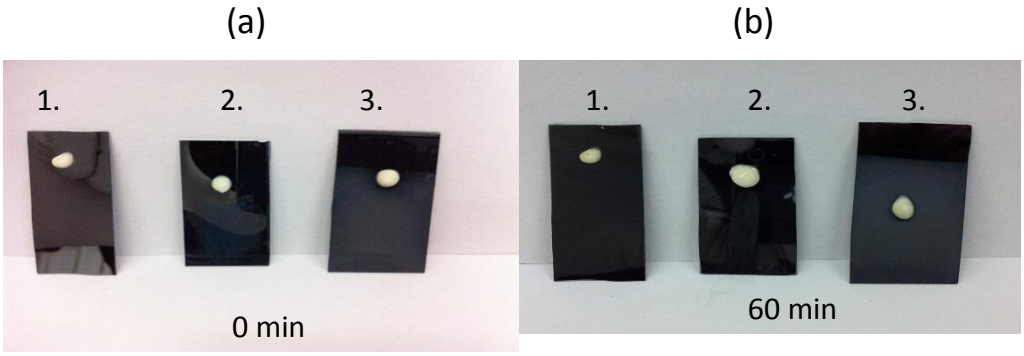


Figure 37. Repeated self-cleaning functionality test after the was washed with 0.01 M PBS buffer for 24 h (a) test time at 0 min when the all coated substrate were tilted vertically. 1. Silicon substrate without any enzyme coating 2. Coating with native amylase 3. Coating with PEG conjugated amylase (b) test time at 60 min.

This second test shows very different results for the two enzyme containing coating samples. The coating with the native amylase almost lost all its activity. In contrast to this the PEG conjugated amylase performed better and after 60 min visible motion of the drop was found. This indicates that the surface is still capable to decompose the starch.

6.4 Discussion

In the present section the immobilization of native amylase and PEG conjugated amylase into surface-attached hydrogel films and the characterization of the obtained enzyme containing coatings were described. Both enzymes were mixed with either copolymer P (DMAA-MABP5%-SSNa2.5%) or copolymer P (DMAA-MABP5%). The native enzyme/polymer mixture was deposited from an aqueous 0.01M PBS solution while the pegylated enzyme and the copolymers were dissolved in 99.9% ethanol. After coating the substrates were exposed to UV light (λ= 254nm), in order to excite the benzophenone units, so that the polymer becomes cross-linked. The enzyme becomes attached to the polymer and the forming network becomes covalently attached to the substrate.

The activity of native amylase and PEG conjugated amylase on coated surface was measured by using a colorimetric method taking 3, 5-dinitrosalicylic acid as the chromophore. The pegylation process reduced the activity of the enzyme only slightly and even after pegylation the enzyme was still highly active. However, the PEG conjugation improved the compatibility between enzyme and synthetic polymer significantly and allowed the deposition from a salt free (alcoholic) solution. This resulted in a more stable network structure and also in more stable catalytic behaviour, thus helping to disperse and retain the enzyme in the thin film coating. The coated enzyme shows selective surface bioactivity which allows the coating to selectivity degrade stain-forming biomolecules thus avoiding formation of a stain on the coated surface.

The PEG conjugated amylase demonstrate good retention of enzyme activity. Compared to native amylase the PEG conjugated amylase shows stable activity at a relatively high temperature (70˚C).

After UV exposure the PEG conjugated amylase showed much more stable activity compare to native amylase. Even after 50 min of UV exposure at **λ**=254nm the enzyme containing coating shows activity of 30 nkatal which was much higher than that of the coating containing native amylase.

This is supported by the result of SEM and fluorescence microscopy which shows a much smoother topography and more homogeneous deposition of PEGylated enzymes within the coating. We attribute these difference to a better compatibility

of the enzyme with the coating material due to the polymer conjugation, but even more to the fact of that film was deposited from an organic solvent rather than from buffer solution. These solutions leaves behind the large amount of salt in which the enzyme may be captured and which also generated the high surface roughness.

The homogeneous dispersion of the PEGylated enzyme in the coating also lead to in general, better performance of such layers as compared to coating with native enzyme. The layers are more stable under UV illumination, they retain more activity if heated to higher temperature, and they also show an improved long term stability.

Both, layers with native and PEGylated amylase show initially a self-cleaning behaviour. The decomposition of the starch reduced the viscosity of the mayonnaise and the stain can be rinsed away. However, only the layer with a PEGylated enzyme retains this ability after repeating the experiments on the same spot. This shows that most of the native enzyme was not really incorporated into the layers but only loosely attached at the surface. The conjugation with PEG and hydrogel material however the provided a good environment to keep enzyme stable for longer time.

7. Summary and Outlook

The project focused on the development of enzyme-based functional coatings with optimized protein-material interactions for best performance in terms of compatibility, activity and stability. We focused on amylase as an example of enzyme which is used frequently in detergent formulas. The enzyme was then immobilized on the surface as a surface attached hydrogel coating. These coatings were generated from polydimethylacrylamide copolymers containing a photocrosslinker (P (DMAA-MABP5%-SSNa2.5%) and P (DMAA-MABP5%)). The precursor polymers were synthesized from the respective monomers and characterised with regarding to the chemical composition, molecular weight with the help of GPC; ^{1}H-NMR and FTIR spectroscopy, respectively.

As known from the previous research, the production of such a bioactive coating faces the critical challenge to maintain compatibility between the enzyme molecule and the coating material. Earlier research also was shows that the conjugation of the enzyme with different polymers can stabilize them. So, inspired by that, we investigated the polymer-conjugated enzyme for the preparation of self-cleaning coatings. We have chosen to use PEG (polyethylene glycol) for conjugation with α-amylase (detergent enzyme) as the active material in the coating. After conjugation the enzyme is also soluble in ethanolic solutions. The coated layer were prepared from aqueous 0.01M PBS buffer (native enzyme) or 99.9 % ethanol (conjugated enzyme). The layers were generated by dip coating and then subsequently cross-linked and surface attached using UV irradiation at 254 nm.

The enzyme functionalised coatings were analysed by using SEM (Scanning Electron Microscopy) and inverted fluorescence microscope. In the latter case fluorescein isothyocynate was used as fluorescent dye which was mixed with the coating solution. From the microscopy measurements we found that the protein distribution in case of coating prepared from PEG conjugated amylase is much more uniform compared to those from native amylase. In the latter case we also saw salt crystals on the coated surface which will lead to a decrease of the crosslinking efficiency of the coating and might subsequently lead to leakage of the enzyme from the surface.

Summary and Outlook

The catalytic behaviour of the immobilized enzyme on the surface was measured under different conditions. It was observed that the PEGylated enzyme stayed more active under UV light, and retained higher activity if exposed to higher temperature. The self-cleaning properties were initially comparable for both systems but only layer with the PEGylated amylase retained activity in repeated tests on the same spot. However, more research is needed to clearly identify the active components in these surface architectures and to generate truly long term stable self-cleaning surface such as degree of crosslinking and illumination time during crosslinking. Also other enzymes such as lipase or other protease can be immobilized. As a consequence, we believe that that the combination of polymer conjugation of the enzyme and the embedding of these conjugates into surface attached hydrogels is a promising pathway towards self-cleaning surfaces.

8. Experimental section

8.1 Materials

Table 8.1 List of the reagent and other materials.

Reagent & Material	Quality	Manufacturer
α-Amylase from Aspergillus oryzae	30 U/mg	Sigma-Aldrich
α-Amylase from porcine pancreas	1821 U/mg	Sigma-Aldrich
AIBN	98%	Fluka
BSA	98%	Sigma-Aldrich
Centrifugal filter unit	30 & 50 kD	Merck Millipore
Coomasie-Brilliant-Blue G250	90%	Sigma-Aldrich
Dichloromethane	99.5%	Roth
Diethyl ether	99.5%	Roth
Dimethyl acrylamide	99%	Sigma-Aldrich
3,5-Dinitrosalicylic acid	98%	Roth
Ethanol	99%	Sigma-Aldrich
Fluorescein isothiocyanate	99%	Sigma-Aldrich
β-Galactosidase from Aspergillus oryzae	8 U/mg	Sigma-Aldrich
Hydrochloric acid (HCl)	32%	Sigma-Aldrich
Starch		Sigma-Aldrich

Maltose		Sigma-Aldrich
Methanol	99.5%	Sigma-Aldrich
PBS-Buffer		Sigma-Aldrich
PEG (polyethylene glycol)	5 kD	Sigma-Aldrich
Pottasium Sodium Tartrate	99%	Sigma-Aldrich
Si-Wafer		Si-Mat
Sodium Carbonate	99.9%	Fluka
Sodium Styrene Sulfonate (SSNa)	90%	Sigma-Aldrich

8.2 Instrumentation

1. Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H-NMR (Proton NMR) spectra were acquired using Avance 250 MHz spectrometer from Bruker. For ^{1}HNMR, the sample was dissolve in $CDCl_3$ or DMSO with concentration of 10-20 mg/ml and Tetramethylsilane used as a standard.

(NMR Measurement was performed by Mrs. Daniela Mössner (IMTEK: CPI: University of Freiburg))

2. Gel Permeation Chromatography (GPC)

The molecular weight (MW) of the synthesized polymer were investigated by using GPC with the model Agilent 1100 from polymer standard service (PSS). Respected polymer standard were employed with the concentration of 4 mg/ml in DMF at the flow of 1 ml/min.

(GPC Measurement was performed by Mrs. Natalia schatz (IMTEK: CPI: University of Freiburg))

3. Fourier Transform –Infrared spectroscopy (FTIR)

FTIR spectra were recorded using BioRad Excalibur FTS 3000 spectrometer. Potassium bromide (KBr) pallets were used for solid sample as a background. The spectra were recorded in the range of 4000-450 cm^{-1} with the resolution of 2 cm^{-1}.

4. UV Spectroscopy

The UV/Vis spectra were recorded with a Cary Bio 50 - spectrometer from Varian. For the measurements in solution were used quartz cuvettes with the layer thickness of 1 cm. The measurements with Bradford reagent were carried out using disposable cuvettes.

5. Dip Coater

The samples were prepared through dip coating using the Z 2.5 tension testing machine from Zwicki GmbH, which allowed immersion into and withdrawal from solution with a precisely chosen rate. The coating solutions were prepared using the chosen solvent with an appropriate concentration. The substrate sample was immersed at the velocity of 100 mm/min and also withdrawn at 100 mm/min. All dip coating experiments were performed at an ambient room condition with the temperature of around 25˚C.

6. Contact Angle measurement

Static contact angle of the surface attached network were measured using the sessile drop method though contact angle meter (OCA 20) setup (Dataphysics, Filderstadt, Germany), with liquid dispenser and an image processing program which provides an automatic determination of contact angle.

7. UV-Cross linker

All photochemical reactions were performed using light source Strata-linker 2400 Stratagene, California, USA, at a wavelength of $\lambda = 254$ nm.

8. Fluorescence Microscopy

All the fluorescence microscopy images were taken using a fluorescence microscope (Microscope TS 100 (Nikon) lamp: HBO 50W/AC L1 Osram from mercato AG, Köthen, Germany.

9. Atomic force Microscopy (AFM)

AFM micrographs were recorded using a Nanoscope IIIa microscope (digital instrument Santa Barbara, CA, USA) and the microscope was operated in tapping mode, using commercial tip with a frequency of 300 kHz.

(All the AFM measurement was performed with the help of Ms.Wibke Hartleb; CPI; IMTEK)

10. Ellipsometry

The film thickness of the deposited layers were measured using auto-nulling ellipsometry from Nanofilm (EP3) operated at the wavelength of λ=532 nm at variable angles from 55-75˚.

11. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy was used in order to measure the top surface and enzyme distribution on coating substrate. SEM-EDX analysis was used to study the enzyme distribution.

(All the SEM measurement was performed with the help of MS. Melanie Eichhorn; CPI; IMTEK)

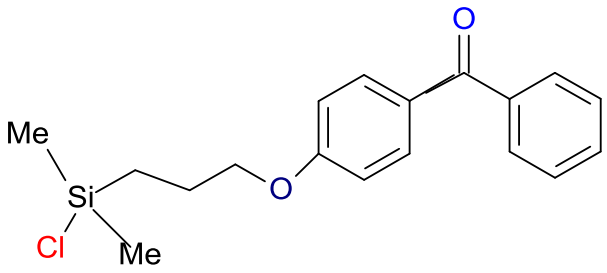
12. Ultrafiltration

Eppendorf centrifuge 5840R was used for the purification of enzymes.

8.3 Substrate preparation

- **Silanization of silicon-wafer with 4- (3'-chlorodimethylsilyl) propyloxybenzophenone**

Silicon substrate had to be silanized before applying a hydrogel coating. For the surface modification with BP (benzophenone)-silane, the substrates were dried in vacuum down to a 1 mbar and afterwards kept under nitrogen. They were immersed in dry toluene (50 ml) and 5 to 10 droplets of triethylamine were added together with 1 ml BP-silane solution. The vessel was sealed and kept in the dark for at least 24 h before the wafer were cleaned with toluene and methanol. The BP-silanized wafer were protected from light to avoid photoreaction of the benzophenone.



Chemical structure of 4- (3'-chlorodimethylsilyl) propyloxybenzophenone.

8.4 Synthesis

- ### Synthesis protocol for P (DMAA-MABP 5%)

For the synthesis of P (DMAA-MABP5%) MABP (0.67 g, 2.52 mmol, 5 mol %) AIBN (0.009 g, 0.05 mmol, 0.1 mol %) and DMAA (4.89 mL, 4.71 g, 47.5 mmol) were dissolved under nitrogen in a Schlenk flask in 20 ml of DMF. Prior to mixing the stabilizer was removed from the DMAA through column chromatography. From a stock solution of AIBN (6.52 mg/mol) in DMF 1 ml was added and the reaction vessel was sealed. The solution was degassed through 3 freeze thaw cycles. The polymerization was carried out for 20 h at 60 ° C. The polymer is precipitated in diethyl ether (300 ml), filtered off and briefly dried under high vacuum and again precipitated in diethyl ether. For re-precipitation the polymer was again dissolved in diethyl ether. After the filtration polymer was again dried in a high vacuum and freeze-dried for final purification.

Yield: 5.42g, 64%

GPC (Calibration: PMMA-DMF-LiCl): M_n= 118 000 gm/mol; MW 382 000 gm/mol; PDI=3.2

FTIR: Over the ratio of carbonyl bands of ($C=O_{Amide}$) = 1641 cm^{-1}, ($C=O_{Ester}$) =1739cm^{-1}, MABP content: 3.6 %.

^{1}H-NMR (250 MHz, $CDCl_3$, impurities δ= 2.05 to 2.09 ppm**) δ (ppm):** 1.2-3.2 (m, 293H; -C-H), 7.2-8.0 (m, 9H; -C-H $_{Aromatic}$)

- **Synthesis protocol for P (DMAA-MABP 5%-SSNa 2.5%)**

The reaction conditions were the same as described above, only a different composition was chosen. MABP (0.25g, 2.02 mmol), SSNa (0.28 g, 1.10mmol), AIBN (0.26g, 0.4mmol) and DMAA (3.8ml, 3.1g, 36.8 mmol).

Yield: 2.4 g, 61%

GPC (Calibration: PMMA-DMF-LiCl): M_n=101 000 gm/mol, MW= 379 000 gm/mol; PDI=3.7.

FTIR: Over the ratio of carbonyl bands of ($C=O_{Amide}$) = 1641 cm^{-1}, ($C=O_{Ester}$) =1739cm^{-1}, MABP content: 3.1 %.

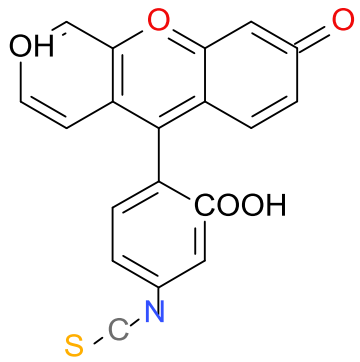
^{1}H-NMR (250 MHz, $CDCl_3$) δ (ppm): 0.9-3.3 (m, 165H; -C-H), 7.2-8.0 (m, 7.2H; -C-H $_{Aromatic}$).

- **Conjugation of α-amylase with PEG**

Native α-amylase enzyme was purified by ultrafiltration with a regenerated cellulose membrane (MW cut-off of 30 and 50 kD) before use. The ultrafiltration was conducted with a 5 ml cell, with each load of 160 mg of enzyme purified with 3 refills of 10 mM pH 7.5 PBS buffer solution. Enzyme solution was first added to a glass vial and incubated in an ice bath with magnetic stirring. PEG conjugation was initialized by adding the desired amount (depending on modification ratio) of PEGNHS that was pre-dissolved in DMSO (dimethyl sulphoxide). After 4 h of reaction, ultrafiltration centrifugation was conducted to remove free PEG modifier (five rounds of purification with fresh buffer of the same volume of the original sample for each round) with a 50 kD MW cut-off membrane.

- **Labelling of the enzyme with fluorescein isothiocyanate**

A solution of fluorescein isothiocyanate 1 mg/ml was prepared in 0.01 M PBS buffer. This solution (0.5 ml) was added to respective coating solution which contained enzymes with either native amylase or PEG conjugated amylase. After incubattion at room temperature for around 45 min the solution was directly used for coating.



Chemical structure of fluorescein isothiocyanate.

- Preparation for protein gel electrophoresis

The enzymes were analyzed by SDS-polyacrylamide gel electrophoresis using SDS-PAGE gels. The amylases were transferred to a nitrocellulose membrane, and blocked with a blocking solution (5% bovine serum albumin (BSA)) overnight at 4˚C. Incubation with the primary antibody was performed overnight at 4˚C. The blots were washed 3 times with PBS buffer and incubated with secondary antibodies for 2 hs. After 3 times washing with PBS the blots were incubated with enzyme and HRP (1:1000 diluted) and washed 3 times with PBS before detection [46].

HRP was detected by adding 3 ml of TMB substrate solution onto membrane. After 10 min incubation the membrane was washed 3 times with ddH_2O, and image was analyzed.

(All experiment of protein gel electrophoresis was done with the help of Dr. Thomas Brandstetter, Bio-chip group: CPI: IMTEK)

8.5 Sample preparation

For the preparation for samples, first silicon wafers were cut into a uniform size (2.0 x 2.5 cm), and treated with 4- (3'-chlorodimethylsilyl) propyloxybenzophenone. After completion of the formation of the samples the thicknesses of the layers were measured by ellipsometry. After generation of the BP-saline monolayers the substrates were coated with a solution containing the photopolymer P (DMAA, MABP 5%) (5 mg/ml) and the respective enzymes (modified α-amylase (porcine

pancreas, pp)) or (Aspergillus oryzae) with different concentration in ethanol by dip coating. For covalent fixation this coating was irradiated for 3 to 5 min with UV light (λ = 250 nm). After exposure to UV light the substrate was washed again with ethanol and dried under N_2 or in vacuum.

Table 8.2 Composition of coating solution containing modified amylase.

Concentration of PEG modified amylase (mg/ml)	Polymer concentration (mg/ml)	Solvent
Pegylated amylase (0.9)	P(DMAA-MABP5%) 5	Ethanol (99%)
Pegylated amylase (1.0)	P(DMAA-MABP5%) 5	Ethanol (99%)

On the other hand for native amylase, after the silicon substrate was treated with Bp-Si, the substrate was coated via dip coating (v = 100 mm/min, immersion depth =2-4 cm) with coating solution which contains (α-amylase) (porcine pancreas) or (Aspergillus oryzae) and synthetic polymer P (DMAA-MABP5%-SSNa2.5) in a PBS buffer with a different concentration. Composition of coating solution containing native amylase are mention in above table.

Table 8.3 Composition of coating solution containing native amylase.

Concentration of native amylase (mg/ml)	Polymer concentration (mg/ml)	Solvent (mol/l)
Native amylase- 1	P(DMAA-MABP5%-SSNa2.5%) -5	PBS buffer 0.1
Native amylase -1	P(DMAA-MABP5%-SSNa2.5%) -5	PBS buffer 0.01
Native amylase -1	P(DMAA-MABP5%-SSNa2.5%) -5	PBS buffer 0.001
Native amylase -5	P(DMAA-MABP5%-SSNa2.5%) -5	PBS buffer 0.2

Fluorescein isothiocyanate (dye) was prepared in PBS buffer (0.001 mol/l) for the preparation of a sample for fluorescent microscopy. Prepared solution was directly used (50 µl) for measurement.

8.6 Activity Measurement

For activity measurement of the enzyme 1 g of potato starch was dissolved in dd$H_2$0 water (100ml) with constant stirring on a heating plate until it started boiling and kept for 15 min at that temperature. Then it was cooled down at room temperature. Sodium potassium tartrate solution (12 g) was mixed with previously heated PBS buffer (50-70˚C) and kept on a heating plate with constant stirring until it became dissolved. 96 mmoles of 3.5- dinitrosalicylic acid was prepared in 20 ml purified water on heating plate with constant stirring. The two reagents were mixed together. With the help of this prepared reagent (chromophore) the amount of reducing sugar was measured in enzyme solution [48].

For the activity of conjugated amylase and modified amylase was dissolved (10 µl) in PBS buffer (100 ml, 0.01M, pH=7.8) were dissolved.

Table 8.4 Sample composition for enzyme activity measurement.

	Sample 1	Sample 2	Sample 3	Sample 4	Reference
Starch solution (ml)	1.00	1.00	1.00	1.00	1.00
Enzyme solution (ml)	0.20	0.40	0.60	0.80	0.00
Color reagent (ml)	1.00	1.00	1.00	1.00	1.00
Enzyme solution (ml)	0.80	0.60	0.40	0.20	1.00
dd$H_2$0 water (ml)	9.00	9.00	9.00	9.00	9.00

Finally, the absorbance was measured at 540 nm and the concentration of maltose was deduced by using the calibration curve. From this, the α-amylase (modified-native) activity was calculated. To measure the activity of amylase containing layers starch solution (0.5 ml, 1%) was applied on the enzyme-containing layer. After an incubation time of 15 min at 37 °C, the sample was removed and replaced with PBS buffer (0.01M), so that a total volume of 2 ml was present. After addition of the

cooler reagent (0.3 ml), the solution was heated for 15 min to 100 °C. After the solution was cooled ddH_20 water (1.7 ml) added and the absorbance at 540 nm. With the aid of a reference sample and calibration maltose concentration was determined, from which the activity of the coating can be determined taking into account the incubation period.

8.7 Preparation of Calibration curves

- FTIR Calibration Curve

To prepare a calibration curve for measuring a MABP content into the copolymer, different amount of P (DMAA) and MABP were prepared separately into iso-propanol with concentration of 10 mg/ml. The different composition of the mixtures of P (DMAA) and MABP are depicted into the table 8.5.

Table 8.5 Preparation of mixtures with different amounts of P (DMAA) and MABP.

MABP Content (%)	P(DMAA) (ml)	MABP (ml)	Iso-propanol (ml)
0	0.5	0	4.5
2.5	0.49	0.03	4.48
5	0.48	0.07	4.46
7.5	0.46	0.10	4.44
10	0.45	0.13	4.42
15	0.43	0.20	4.37

- Maltose standard curve

Preparation for maltose standard calibration curve

10 mM phosphate buffer (PBS) was prepared in purified water, then after the pH was adjusted to 7, starch solution was prepare in 25 ml PBS by heating the starch solution in a beaker with constant stirring on a heating plate. The solution was heated until it get started to boiled and was maintained at this temperature for 15

min. Afterwards the starch solution was cooled at room temperature with constant stirring, and the starch solution was returned on its original volume 25 ml.

Preparation of colour reagent solution

12 g of sodium potassium tartrate were dissolved in 8 ml of PBS buffer and the solution was heated directly on heating plate with constant stirring until everything dissolved. 96 mmoles 3-5, Dinitrosalicylic acid were dissolved in 20 ml purified water with a constant stirring on heating plate at 50-60˚C, and then the two solution was mixed.

To prepare a calibration curve for the detection of the reducing sugars in the solutions of various concentrations with the previously prepared 3, 5-dinitrosalicylic acid solution was reacted at 100 ° C for 15 min. Subsequently, the absorbance was measured at 540 nm. The concentration of protein was plotted against the corresponding absorbance which resulted into a standard curve where linear fit give straight line with the equation (figure.38).

$$y = 1.31x - 0.1699$$

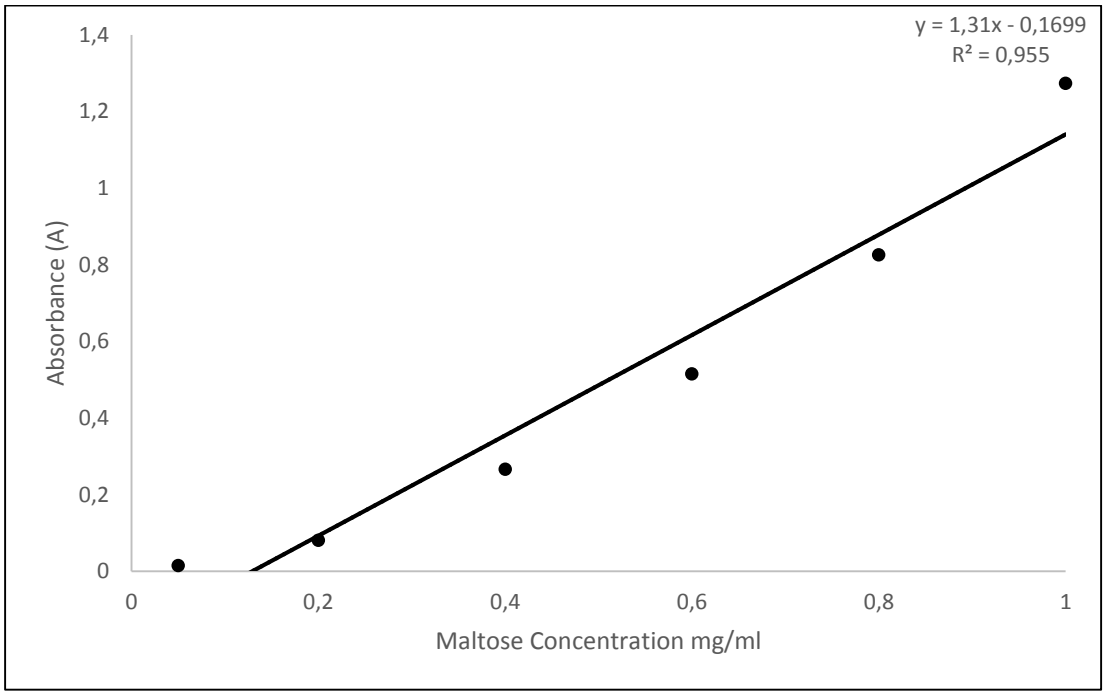


Figure 38. Absorbance at 540 nm for different concentrations of maltose after reaction with 3-5 dinitrosalicylic acid.

- Preparation of Bradford reagent and calibration curve for protein assay

Preparation of the protein reagent

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 l water. Bovine Serum Albumin (BSA) solutions are used as standard.

Protein assay method

BSA solution containing 10 to 100 μg protein in a volume up to 0.1 ml was pipetted into 12 x 100 mm test tubes. The volume in the test tube was adjusted to 0.1 ml with appropriate buffer. Five millilitres of protein reagent was added to the test tube and the contents were mixed either by inversion or vortexing. The absorbance at 595 nm was measured after 2 min against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent. The amount of protein was plotted against the corresponding absorbance which resulting in a standard curve where linear fit give straight line with the equation.

$$y = 5.1541x + 0.0211$$

The measurement of the protein concentration in the unknown solution was carried out with by mix the Bradford reagent (2.5 ml) with the protein solution (0.25 ml) and measuring the absorbance at λ = 595 nm.

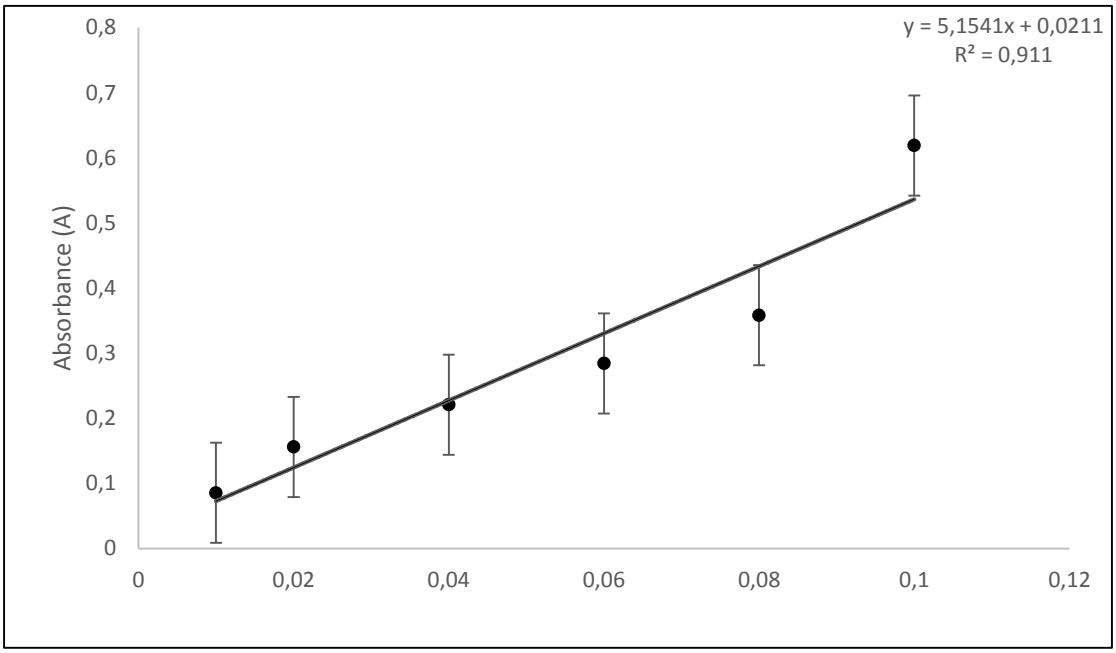


Figure 39. BSA calibration curve obtained by reaction with Bradford reagent.

9. References

1. Ganesh A, Raut H, Nair S, Ramakrishna S. A review on self-cleaning coatings. Journal of Material Chemistry **21**, 16-30, 2011.

2. Liu K, Jiang L. Bio-inspired self-cleaning surfaces. Annual Review of Material Research **42**, 231–263, 2012.

3. Zhang L, Wu S, Buthe A, Zhao X, Wang P. Poly (ethylene glycol) conjugated enzyme with enhanced hydrophobic compatibility for self-cleaning coatings. Advance Materials **7**, 59-81, 2012.

4. Nakajima A, Fujishima A, Hashimoto K, Watanabe T. Preparation of transparent super hydrophobic silica films by sublimation of aluminium acetylacetonate. Advance Material **11**, 8-13, 1999.

5. Bhushan B. Bioinspired super hydrophobic self-cleaning and low drag surfaces. Proceedings of SPIE-The International Society for Optical Engineering **8818**, 802-881, 2013.

6. Verma G. Hydrophobic self-cleaning coating based on siloxane modified waterborne polyester. International Journal of Scientific Engineering and Technology **2**, 192-220, 2013.

7. Yao L, He J. Recent progress in antireflection and self-cleaning technology from surface engineering to functional surfaces. Progress in Materials Science **61**, 94–143, 2014.

8. Frank SN, Bard AJ. Heterogeneous photo catalytic oxidation of cyanide ion in aqueous-solutions in TiO_2 powder. Journal of American Chemical Society **99**, 303–304, 1977.

9. Yao L. Recent developments in superhydrophobic surfaces with unique structural and functional properties. Soft Matter **8**, 11-21, 2012.

10. Klibanov AM. Approaches to enzyme stabilization. Biochemical Society Transactions **11**, 19-20, 1983.

References

11. Kirk O, Torbe J, Borchert V, Fuglsang C. Industrial enzyme applications. Current Opinion in Biotechnology **13**, 345–351, 2004.

12. Newhouse ML, Tagg B, Pocock S, McEwan A. An epidemiological study of workers producing enzyme washing powders. Lancet **1**, 689-693, 1970.

13. Wong C, Whitesides GM. Enzymes in synthetic organic chemistry. Elsevier Science Ltd, Oxford, UK, 1994.

14. Schwärzle D. Auf dem Weg zu selbstreinigenden Oberflächen, Photochemische Herstellung von enzymhaltigen Hydrogelbeschichtungen, Master Thesis, University of Freiburg, Germany, 2014.

15. Iyer P, Ananthanarayan L. Enzyme stability and stabilization in aqueous and non-aqueous environment. Process Biochemistry **43,** 1019–1032, 2008.

16. Ogino H, Ishikawa H, Enzymes which are stable in the presence of organic solvents. Journal of Bioscience and Bioengineering **91**, 109–116, 2001.

17. Brena BM, Viera FB. Immobilization of Enzymes and cells. Springer **22**, 15-30, 2006.

18. Inada Y, Takahashi K, Saito Y, Yoshimoto T, Ajima A, Matsushima A. Application of polyethylene glycol- modified enzyme in biotechnological process: organic solvent soluble enzyme. Trend in Biotechnology **7**, 190-194, 1982.

19. Klibanov A. Improving enzymes by using them in organic solvents. Nature **409**, 241-246, 2001.

20. Larsen C. Dextran prodrugs-structure and stability in relation to therapeutic activity. Advanced Drug Delivery Reviews **3**, 103–154, 1989.

21. Mehvar R, Shepard TL. Molecular weight dependent pharmacokinetics of fluorescein-labelled dextran in rats. Journal of Pharmaceutical Science **81**, 908–912, 1992.

22. Evered D, Whelan J. The Biology of Hyluronan, Wiley & Sons, Chichester, UK, 1989.

23. Almond A. Hyaluronan. Cellular and Molecular Life Science **64**, 1591–1596, 2007.

24. Laurent TC, Fraser JR. Hyaluronan. FASEB J **6**, 2397–2404, 1992.

References

25. Pasut G. Polymers for protein conjugation. Polymers **6**, 160-178, 2014.

26. Mero A, Pasut G, Fijten L, Schubert M, Hoogenboom R, Veronese FM. Synthesis and characterization of poly (2-ethyl 2-oxazoline)-conjugates with proteins and drugs: Suitable alternatives to PEG-conjugates. Journal of Control Release **125**, 87–95, 2008.

27. Zalipsky S. Chemistry of polyethylene glycol conjugates with biologically active molecules. Advanced Drug Delivery Reviews **16**, 157-182, 1995.

28. Nakajima A, Fujishima A, Hashimoto K, Watanabe T. Preparation of transparent super hydrophobic boehmite and silica films by sublimation of aluminium acetylacetonate. Advance Material **11**, 1365–1368, 1999.

29. Manjula B, Tsai A, Upadhya R, Perumalsamy K, Smith P, Malaval A, Vandegriff K, Winslow R, Intaglietta M, Prabhakaran M, Site-specific PEGylation of hemoglobin at Cys-93(β). Correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. Bioconjugate Chemistry **14**, 464–472, 2003.

30. Dormán G and Glenn D. Prestwich, using photo labile ligands in drug discovery and development, Trends in Biotechnology **11,** 25-48, 2000.

31. Turro, N. J. Modern Molecular Photochemistry. University Science Books, 2010

32. Osterwinter G, Navarro J, Crespo R, Prucker O, Rühe J. Photocrosslinked surface attached polymers networks. ACS Spring **8**, 25-29, 2012.

33. Perrez M, Navvaro R, Prucker O, Rühe J. Binding of functionalized polymers to surface-attached polymer networks containing reactive groups. Macromolecules **47**, 2695-2702, 2014.

34. Toomey R, Freidank D, Rühe J. Swelling behaviour of thin surface-attached polymer networks. Macromolecules **37**, 882–887, 2004.

35. Wörz A, Barchtold B, Moosmann K, Prucker O, Rühe J. Protein-resistant polymer surfaces. Journal of Material Chemistry **22**, 19547-19561, 2012.

36. Nostrum CF, Hennink WE. Novel crosslinking methods to design hydrogels. Advanced Drug Delivery Reviews **64**, 223-236, 2012.

References

37. Pandiyarajan CK, The interaction of blood protein and platelets on surface attached poly (alkyl acrylamide) network, PhD Thesis, University of Freiburg, IMTEK, CPI, 2013.

38. Hagel V, Haraszti T, Heike B. Diffusion and interaction in PEG-DA hydrogels. Bio-interphases Journal **8**, 36, 2013.

39. Zaks A, Klibanov A. Enzyme-catalysed processes in organic solvents. Proceeding of National Academy of Science **82**, 3192-3196, 1985.

40. Stein K. The amino acid composition of α-amylase from Aspergillus oryzae. Journal of Bio-Chemistry **235**, 452-520, 1960.

41. Yamagata Y, Arakawa K, Yamaguchi M, Kobayashi M, Ichishima E. Functional changes of dextran-modified alkaline proteinase from alkalophilic Bacillus sp. Enzyme Microbiology Technology **16**, 99-103, 1994.

42. Jayawardena MB. Stability and activity of self-cleaning lipase based Paints. PhD thesis, University of Sydney, 2009.

43. Benjaporn S. Effect of PEG molecular weight and linking chemistry on the biological activity and thermal stability of PEGylated trypsin. International Journal of Pharmaceutics **357**, 252–259, 2008.

44. Yao L. Preparation of interpenetrating polymer network composed of poly (ethylene glycol) and poly (acrylamide) hydrogels as a support of enzyme immobilization. Progress in Material Science **7**, 852–858, 2008.

45. Kadajji V, Guru V. Betageri A, Water soluble polymers for pharmaceutical applications. Polymers **3**, 1972-2009, 2011.

46. A Guide to Polyacrylamide Gel Electrophoresis and Detection-Bio-Rad. Bio-Rad Laboratories Inc. Bulletin **6040** Rev, 45-92, 2012.

47. Rendl M. Time-resolved analysis of biological reactions using liquid plugs of nanoliter volume, PhD Thesis, University of Freiburg, IMTEK, CPI, 2014.

48. Enzymatic assay of α-amylase, Sigma Aldrich (EC 3.2.1.1).

49. Akkaya B, Sahin F, Demirel G, Tumturk H. Functional polymeric support for immobilization of cholesterol oxidase. Journal of Biochemical Engineering **43**, 333-337, 2009.

References

50. Wu S. Enzyme-enabled responsive surfaces for anti-contamination materials. Journal of Biotechnology and Bioengineering 110, 1805–1810, 2013.

51. Rendl M, Bonisch A, Mader A, Schuh k, Prucker O, Brandstetter T, Rühe J. Simple one-step process for immobilization of biomolecules on polymer substrates based on surface-attached polymer networks. Langmuir 27, 6116–6123, 2011.

52. Prucker O, Naumann A, Knoll W, Frank CW, Rühe J. Photochemical attachment of polymer films to solid surfaces via monolayers of benzophenone derivatives. Langmuir 121, 8766-8770, 1999.

53. Jung Y. Natural and Biomimetic Artificial Surfaces for Superhydrophobicity, Self-Cleaning, Low Adhesion, and Drag Reduction, PhD thesis, The Ohio State University, 2009.

54. Spori D. Structural Influences on Self-cleaning Surfaces, PhD thesis, ETH ZURICH, 1979.

55. Bixler G. Bioinspired Surface for Low Drag, Self-Cleaning, and Antifouling: Shark Skin, Butterfly and Rice Leaf Effects, PhD thesis, The Ohio State University 2013.

56. Sheldon R. Enzyme immobilization-the quest for optimum performance. Advanced Synthesis and Catalysis 349, 1289-1307, 2007.

57. Pandiyarajan CK, Prucker O, Zieger B., Rühe J. Influence of the molecular structure of surface-attached poly (N-alkyl acrylamide) coatings on the interaction of surfaces with proteins, cells and blood platelets. Macromolecules 13, 873–884, 2013.

58. Fürstner R, Barthlott W, Neinhuis C, Walzel P. Wetting and self-cleaning properties of artificial superhydrophobic surfaces. Langmuir 21, 956-961, 2005.

59. Katre N. The conjugation of protein with polyethylene glycol and other polymer to enhance their therapeutic potential. Advance Drug Delivery Reviews 10, 91-114, 1992.

60. Bradford M, A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254, 1976.

61. Yang S, Marchio J, Yen JW. A Dynamic light scattering study of galactosidase-Environmental effects on protein conformation and enzyme activity. Biotechnology Progress 10, 525-531, 1994.

References

62. Silva C, Zhang Q, Shen J, Paulo A. Immobilization of proteases with a water soluble–insoluble reversible polymer for treatment of wool. Enzyme and Microbial Technology **39**, 634–640, 2005.

63. Castro G. Enzymatic activities of proteases dissolved in organic solvents. Enzyme and Microbial Technology **25**, 689–694, 1999.

64. Prucker O, Naumann CA, Knoll W, Rühe, J, Frank CW. Photochemical attachment of polymer films to solid surfaces via monolayers of benzophenone derivatives. Journal of American Chemical Society **121**, 8766–8770, 1999.

65. Atkins PW, Paula J de. Physiochemical chemistry, 8th edition, W.H. Freeman, New York, USA, 2006.

66. Moschallski M, Evers A, Brandstetter T, Rühe J. Sensitivity of microarray based immunoassays using surface-attached hydrogels. Analytica Chimica Acta **781**, 72–79, 2013.

67. Schuh K, Prucker O, Rühe J. Tailor-made polymer multilayers. Advance Materials **23**, 6019–6023, 2013.

68. Seiji M, Itakura H, Enzyme inactivation by ultraviolet irradiation and protective effect of melanin in vitro. Journal of Investigative Dermatology **47**, 507-511, 1996.

Acknowledgement

First and foremost I would like to express my sincere gratitude to Prof. Dr. Jürgen Rühe for giving me opportunity to work in his lab and supported me throughout my master thesis with his patience. It was truly a rewarding experience to work among a group of creative individuals. One simply could not wish for a better and friendlier supervisor like him.

I would also like to express my gratitude to Prof. Dr. Monica Galleano for all her support and giving me a time during my master work. I also thankful to her to accept my work as a second supervisor.

Many thanks to Dr. Oswald Prucker "Ossi" for your supervision, encouragement and friendship. Thanks for been there at all times, and also for taking the time to proofread my thesis, I think you are a backbone of CPI.

Special thanks to Dr. Thomas Brandstetter I greatly appreciate your support in my research work and some fruitful suggestion.

Very special thanks to Mr. David Schwärzle, I truly appreciate both of your help and sharing an office with me.

Special thanks to Frau. Petra Hettich (IMTEK; CPI), Frau and Yvonne Weigand (IMBS) for always looking after the administrative things, special thanks to frau. Petra for helping many international students without you all of us at the CPI could not function.

To Alex, Marcel, Mathias, I would like to also thank them for being a great friend throughout my time here. All the members of the CPI lab for your support and great working atmosphere, a big thank you! It has been rewarding working with people from different scientific backgrounds and with whom I managed to share many fun times.

Finally, to my mother, father, my brother and Prachi, I am forever indebted. Thank you for your never ending encouragement and support shown to me in the last some years. You all have instilled in me the ethic of always completing what's begun and given me the ability to overcome hardships and carryon when life gets tough.

Affirmation

I hereby affirm that this Master thesis is my own work and to the best of my knowledge it contains no materials previously published or written by another person.

All passages, which are quoted from publication or paraphrased from these sources are indicated as such.

Any contribution made to the research by others, with whom I have worked at IMTEK, CPI is explicitly acknowledged in the thesis.

This thesis was not submitted in the same or in a substantially similar version not even partially, to another examination board and was not published elsewhere.

Freiburg, May 4th, 2015 — Urmil Shah

..... — .....

Place, Date — Signature