CHALMERS

Spectroscopic studies of immobilized proteins

Investigating immobilization kinetics and enzyme activity

Master of Science Thesis

KASSAM ABDEL MALLAK

Department of Chemical and Biological Engineering
Division of Chemistry and Biochemistry
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden, 2013

Spectroscopic studies of immobilized proteins
Investigating immobilization kinetics and enzyme activity
Kassam Abdel Mallak

© Kassam Abdel Mallak, 2013.

Department of Chemical and Biological Engineering
Chalmers University of Technology

SE-412 96 Göteborg
Sweden
Telephone + 46 (0)31-772 1000

ABSTRACT

In this report studies of immobilized proteins with spectroscopy are presented. The proteins are immobilized into mesoporous silica particles. Two main aspects are studied.

First the immobilization kinetics has been studied using fluorescence spectrometry. This novel model worked well for studying the immobilization. The proteins are stained with the fluorophore Epicocconone. The results show that lipase immobilize faster than Bovine serum albumin (BSA) due to their smaller size.

The second main aspect of the project is to investigate availability of enzymes immobilized into particles to substrate. Lipase is the enzyme in question and the substrate is 4-nitrophenyl acetate. Lipase hydrolyze 4-nitrophenyl acetate to 4-nitrophenol which can be detected with absorbance spectrometry at 400nm. The lipase is immobilized over 24 hours and the activity assay is conducted at 37°C. Three types of experiments were conducted. First the lipase concentration was kept constant and the particle concentration was varied, secondly the particle concentration was kept constant and the protein concentration constant and thirdly both lipase and BSA were co-immobilized to the particles.

When the particle concentration was varied a linear increase of specific activity (mol product/g protein/min) was seen until a plateau is reached at a particle to protein ratio of 96. One explanation to this linear increase is that with more particles more lipase can immobilize and faster. A second explanation could be that at lower particle concentrations the environment inside the particle is too crowded. One of the explanations can be confirmed by determining the free lipase concentration in the supernatant. The reason for the plateau could be that above a ratio of 96 all lipase that can be immobilized has been immobilized and therefore no increase in specific activity.

Looking at the experiments where the protein concentration was varied the results indicated that immobilizing to much lipase could lead to a too crowded environment inside the particle pores and this lead in return to lower specific activity. But this explanation also has to be confirmed by determining the amount immobilized lipase.

The experiments with both lipase and BSA showed some interesting results but these has to be further investigated.

KEY WORDS

Mesoporous-silica particle, lipase, BSA, immobilization-kinetics, fluorescence-spectroscopy, epicocconone, activity-assay, availability, co-immobilization

Table of contents

1. INTRODUCTION

The introduction section consists of a general description of the use of microorganisms and enzymes in the chemical industry. Further the aspects of immobilized proteins are introduced and finally the purposes of this report are presented.

1.1. Green Chemistry

Cell factories have gained interest in the quest for a more sustainable and green society by using green feedstock and by moving away from today's oil based civilization. Cell factories are and will be important aids to solve the problems with energy crisis, climate change and making the chemistry industry greener. Microorganisms, plant cells and mammalian cells have been used for different applications. Examples of these applications are seen in fuel production, food production and the pharmaceutical industry where therapeutic proteins are expressed. Microorganism used is different species of yeast (Gerngross 2004), bacteria (Murphy 2012). Plant cells (McDonald and Huang 2012) and mammalian cells (Wurm 2004) are more and more used today. All these hosts or cell factories have different advantages and disadvantages in terms of product quality, yield, safety and costs.

Enzymes can by themselves be applied to various applications as biocatalysts. They have good catalytic efficiency and they could be very specific to substrates and also show specificity in terms of the products stereo-and regioselectivity. The most important feature of the use of enzymes is that they function very well under mild and environmentally clean reaction conditions (Vulfson 1994).

There is a future aspect of the continued use of enzymes in industrial applications. Firstly there are many undiscovered enzymes in various organisms that could have relevant applications in the future. Secondly enzymes properties and their expression can be improved with protein engineering and metabolic engineering. In both of these fields of research there are fast advances in the quest for a greener society.

1.2. Immobilized proteins

In this project spectroscopic studies will be carried out on immobilized enzymes. Upon immobilization into particles the enzymes stability and activity may increase. These features depend on the particle and enzyme properties. One general explanation to the increased activity and stability upon confinement inside a particle is that the protein is packed tightly, similarly as in the dense environment of the cell. In this environment the proteins are in a more active state. The main advantage of immobilized enzymes is however that the particles can be easily recovered and reused in several cycles of reactions. This is very advantageous since many enzymes are expensive and in this way they could be reused (Thörn et al 2011).

Different proteins bind with different strength and different ways to the particle. The pH in protein solutions can be varied and this could make the proteins more or less charged. Since the surfaces of the particles are negative the affinity of the protein for the particles can be increased. The proteins bind to the particles in three ways; by covalent bonding, physical adsorption and electrostatic interaction (Gustafsson 2012).

The particle properties can also be varied. Particle size, pore width, pore length and pore wall properties has shown to have effect on the immobilized proteins activity and stability. Looking at enzymes their availability to substrate molecules is very much affected by particle properties (Gustafsson 2012). One problem with this approach is leakage of the immobilized proteins from the particles. This can be resolved by covalently bind proteins or partially

closing the pores. These solutions could however affect the enzymes activity negatively (Thörn et al 2011).

There are three main aspects of this topic, firstly the enzymes themselves has to be expressed and purified, secondly the particles onto which the enzymes will be immobilized have to be produced and finally evaluation of the concentrations (Carlsson 2011) and activity of the immobilized proteins must be performed. In the latter area of research not so much is known about the immobilization process itself and its kinetics. How fast do proteins with different properties immobilize into particles? Also the availability of the enzymes that has diffused deep into the particles has not been investigated systematically. The main question is whether the enzymes disturb their neighboring enzymes and whether the substrate is as available to every enzyme immobilize including the enzymes bound deep into the particle? The answer to the last question has an economical aspect since it will say which particle to protein ration that give as high specific enzyme activity as possible. At the same time this particle to protein ratio has to be compared to the material costs of particles and enzymes. These economic aspects are of importance for future industrial use of immobilized enzymes. The main approach in this project is to use biophysical methods such as fluorescence spectroscopy and absorption spectroscopy to answer questions about the immobilization process and availability.

Investigating the immobilization kinetics with fluorescence is a new approach that will be further described in the methods section. Investigating if this novel method works is an important part of the project. Physical adsorption and immobilization of protein and other biomolecules to particles and surfaces has earlier been studied by for example ellipsometry (Malmsten 1994), which is an optical method recording changes in polarization of elliptically polarized light. Absorbance spectroscopy has also been used where the amount proteins not immobilized is measured and this indirect method gives an idea on how much and how fast the proteins have immobilized (Gustafsson et al 2011). More recently quartz crystal microbalance (QCM) (Liu et al 2001) has been used as well as surface plasmon resonance (Tamiya et al 2005 and 2006). These methods will not be further described.

1.3. Purpose of report

The purpose of the report is to investigate if the immobilization kinetics can be studied using fluorescence spectroscopy and to understand the process of immobilization of the enzymes into mesoporous silica particles. The goal has been that this understanding will be concluded with presentation of the kinetics of the immobilization process.

The second main purpose is increasing the understanding about how available enzymes imbedded deep in the particles are to the substrate. Activity and concentration analyses with absorbance spectroscopy have been conducted and results from these experiments have partially increased the understanding for the availability.

2. THEORY

The theory part consists of all relevant theory around the concepts and methods that was the basis for the experimental work used in this report. First the general theory that is the basis for the methods and equipment used is presented. Further the specific enzyme and dye used during this project is described. Finally a brief overview of general kinetics and the assumed specific kinetic model for the immobilization kinetics is defined.

2.1. Light and Matter

Light is electromagnetic radiation that consists of two components: one electric component and one magnetic component. The two components can be described as two sinusoidal waves that are perpendicular to each other. In vacuum these waves propagate through space at a constant speed (c) of approximately$3 \times 10^8\, m/s$.

The relation between wavelength (λ), frequency (ν) and speed of light (c) is seen in equation 1.

$$\lambda\nu = c \tag{1}$$

Light as well as all electromagnetic radiation has besides wave properties also particle properties. Electromagnetic radiation can be described as energy packets that are more commonly known as photons.

Light can interact with matter under the right conditions. Matter in this case could be molecule whose distribution of electrons is disturbed upon the interaction with light. By excitation of the molecule means that it reaches a higher energy level. For this excitation to occur the energy of the photon must match the energy gap between the initial (i) and the final state (f). This gap is described by equation 2 and is called the Bohr frequency condition. In figure 1 this is visualized as an energy gap between two electronic states that have to match for the molecule to be excited.

$$\Delta E = E_f - E_i = h\nu \tag{2}$$

In equation 2, h is Planck's constant. This transition of states can be measured in the laboratory by measuring the light not absorbed by the molecule or sample in question (Atkins 2009).

2.2. Fluorescence Spectroscopy

There are several paths for the molecule to move up to higher electronic states and to go back to the ground state. Excitation through absorbance is the dominating process. Fluorescence is the radiative process where the molecule relaxes to a ground state by emitting light. These paths can be illustrated in Jablonski diagrams as in figure 1. In the process of fluorescence there are several steps. First the molecule absorbs light; this absorption brings the molecule to an excited (an excited electronic state) high energy state (Atkins 2009). It is not preferable for the molecule to stay in this high energy state. The molecule can go back to the ground state by transferring energy to higher vibrational states. The molecule can also spread this energy to other molecules, like oxygen, when colliding with them (radiation less decay or collision quenching) however all energy that is needed to be redistributed cannot be accepted by colliding molecules and the only way for the molecule to reach its ground state is to go through radiation of energy (see figure 2) and this is called fluorescence (Atkins 2009).

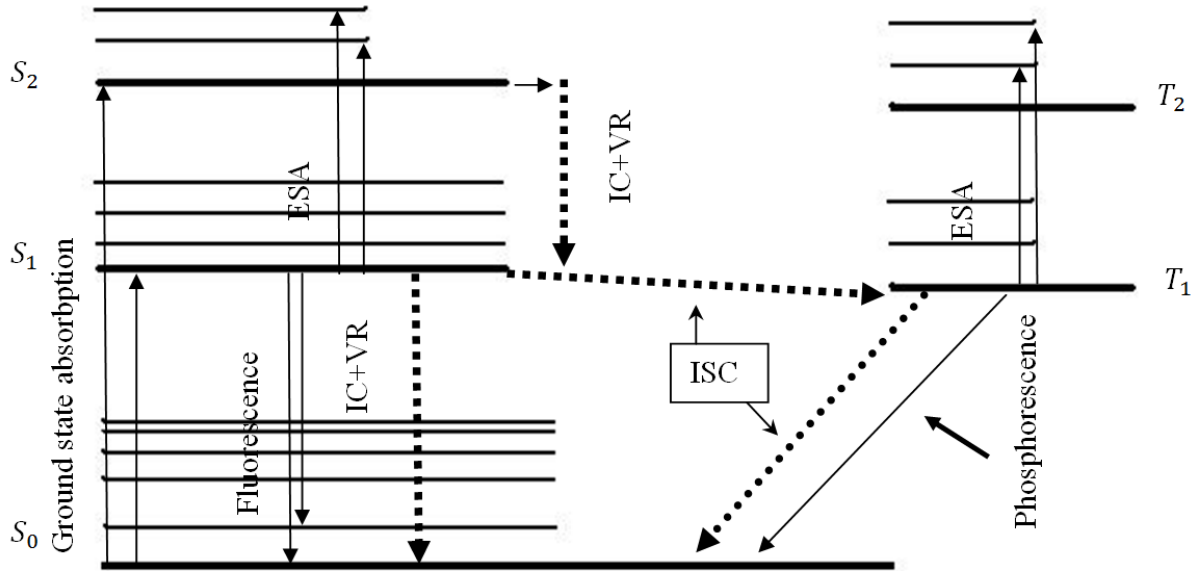

Figure 1. Jablonski diagram with all electronic states of a molecule and the transitions between them. The radiative processes are indicated by solid arrows and the non-radiative processes by dashed arrows. IC is internal conversion, VR is vibrational relaxation and ISC is intersystem crossing. ESA is excited state absorption. S_n are singlet states and T_n are triplet states.

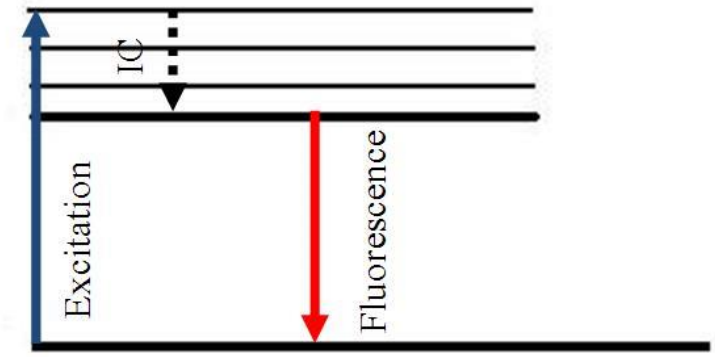

Figure 2. Jablonski diagram specific for fluorescence. The molecule in question first absorbs energy (blue arrow) and then fluoresce light (red arrow) to reach the ground state S_0.

Besides radiative relaxation there is non-radiative relaxation. This relaxation is faster than radiative relaxation and this explains why most molecules don't fluoresce. Looking at figure 1 the solid arrows represents radiative processes and the dashed arrows represent non-radiative processes. The non-radiative processes internal conversion (IC) and intersystem crossing (ISC) are ways for the molecule to change state without emitting light or a photon. By IC the molecule can go from an excited state (S_n) to a lower state (S_{n-1}) if these states have the same vibrational energy. This process doesn't lead to lower energy in the molecule but can be continued by vibrational relaxation within the lower state. In figure 1 both these processes are represented by a short horizontal arrow (IC) and a dashed arrow (VR) or a single dashed arrow (S_1 to S_0). In ISC the excited molecule changes state by moving from a singlet (S_n) to a triplet (T_n) state and further to the singlet ground state (S_0). When moving from triplet state to the singlet ground state phosphorescence can occur. Phosphorescence is related to fluorescence where the difference is that phosphorescent molecule doesn't directly emit the radiation it absorbs (Atkins 2009). However fluorescence is also emission of radiation.

For fluorescence spectroscopy experiments a spectrofluorometer is used. This is an instrument that can record both excitation and emission spectra for the fluorophore that is used. With this

type of instrument it is possible to record excitation and emission spectra over several wavelengths but also kinetic studies are possible where only one wavelength (monochromatic light) is used and the intensity of the fluorescence can be recorded over time (Lakowicz 2006). A fluorophore is a fluorescent molecule that emits light upon excitation. Fluorophores are e.g. used for staining and coloring of biological molecules such as DNA, RNA, and proteins to determine their concentration, activity (in the case of enzymes) or only for visualizing them on separation gels. Important features for a fluorophore is that its fluorescence (intensity) increases upon binding to the biological macromolecule, which is good for determination of concentration, that it has specific and strong binding and that it generally has strong fluorescence so small amount of the fluorophore is needed regardless of purpose. There are two important factors for describing a fluorophore. These are the quantum yield (Φ) and the lifetime (τ). The quantum yield describes the relationship between the amount of photons emitted by a molecule through fluorescence and the amount of photons absorbed by the same molecule (Lakowicz 2006). This is relationship is defined in equation 3:

$$\Phi = \frac{Photons\ emitted}{Photons\ absorbed} = \frac{k_F}{\sum_i k_i} \quad (3)$$

where the numerator is the fluorescence rate constant and the denominator is the sum of rate constants for all processes in the transition to the ground state from the excited state. The fluorescence rate constant in the relationship represent the photons emitted whilst the sum of rate constants make up for all transitions (Lakowicz 2006).

The average time in which a molecule occupies an excite state is referred to as the fluorescence lifetime. The lifetime is inversely proportional to the sum of rate constants involved in the transition from the excited state to the ground state; this relationship is seen in equation 4 (Lakowicz 2006).

$$\tau = \frac{1}{\sum_i k_i} \quad (4)$$

Finally a good fluorophore should also have a large Stokes shift. The Stokes shift is the difference in wavelength between the maxima of the absorption and the emission spectra. This simplifies the measurement and analysis in experiments where the fluorophore is used (Lakowicz 2006).

A more detailed description of the fluorophore Epicocconone that was used in this project will follow in section 2.4.

2.3. Absorption Spectroscopy

Absorption spectroscopy is based on the absorption of light or radiation and recording or determining of which wavelength that was absorbed (Atkins 2009). By knowing at which wavelength a compound absorbs this wavelength can be used to excite the fluorophore in a fluorescence spectroscopy experiment.

Absorption spectroscopy can also be used for concentration determination. When using absorption of light to determine concentrations of a compound it should absorb light and this absorbed light should uniquely be absorbed by said compound in the solution. Since most compounds absorb in the UV range in the electromagnetic spectrum it is preferable to do the analysis in the visible range. Proteins and DNA can themselves absorb light in the ultraviolet region. For example proteins absorb light at 280 nm due to the aromatic side chains and this

can be used to determine protein concentration in a solution but these solutions of protein need to be very pure.

The definition of absorbance is seen in equation 5:

$$A(\lambda) = \log\left(\frac{I_0}{I}\right) \quad (5)$$

I_0 is the intensity of light before passing the sample and I is the intensity after passing the sample. The difference between I_0 and I is due to the absorption of light.

The basis of concentration analysis is the Beer-Lambert law which is seen in equation 6:

$$A(\lambda) = \varepsilon(\lambda)\, cl \quad (6)$$

in this case the Beer-Lambert law statuses that absorbance is proportional to the concentration c of the sample (Atkins 2009), where ε(λ) is the extinction coefficient (or molar absorptivity) that is unique for the compound and specific for one wavelength λ. The molar extinction coefficient most often has the unit $M^{-1}cm^{-1}$. The sample is contained in a cuvette that is put into the absorption spectrophotometer and l is the pathlength (of the cuvette) with centimeters as the most common unit. The concentration c is in M (Harris 2010). The experimental equipment for absorption experiments is a spectrometer. In figure 3 a simple description of the analysis is illustrated.

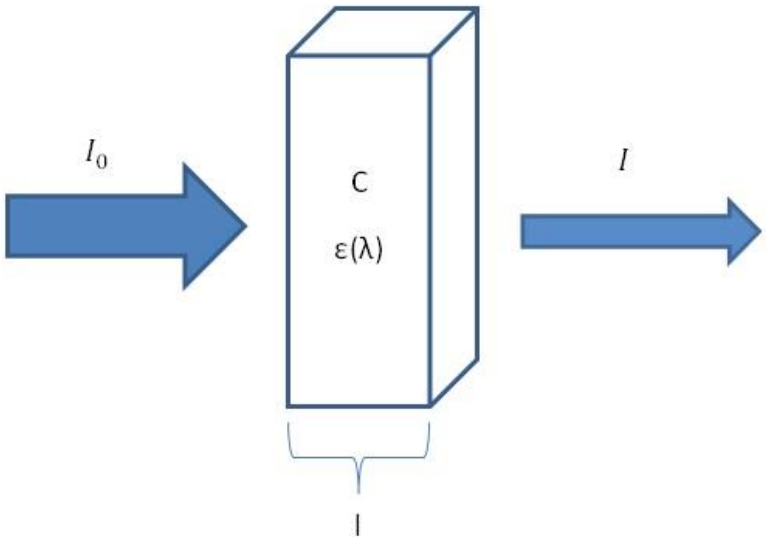

Figure 3. Schematic illustration of the absorption of light when it passes a sample. I_0 is the intensity of light before and I is the intensity after passing the sample. C is the concentration of the sample and ε(λ) is the molar extinction coefficient specific for the wavelength λ and l is the path length.

2.4. Epicocconone

The only dye that was used throughout this project is Epicocconone. Epicocconone is a natural product that first was isolated and purified from the fungus *Epicoccum nigrum* (Bell and Karuso 2003). Epicocconone is a dye which binds to the protein (see mechanism in figure 4) forming amide binding (Karuso et al 2005). Upon binding to protein it has an orange fluorescence with an excitation range of 300-550 nm. More specifically upon binding to protein Epicocconone have local excitation maxima at 390 and 520 nm and an emission maximum at 605 nm. There is a change in emission maximum upon binding to protein is from

520 to 610 nm. Epicocconone have a large Stokes shift (Mackintosh et al 2005). The large Stokes shift means that quenching is reduced when two fluorophores comes close to each other (within Förster distance, see Fluorescence Resonance Energy Transfer or FRET) and exchange less energy to each other.

Another important feature is that the intensity from Epicocconone bound to protein increases when the protein (the protein of interest) is in the particle (personal communication Nils Carlsson 2013[1]) which is a useful phenomenon in the experiments which gives the possibility to investigate the kinetics of immobilization. A possible explanation for this phenomenon could be that there is less quenching inside the particles since there is less oxygen inside the particles. The increased intensity upon immobilization is the bases for the investigation of the immobilization kinetics.

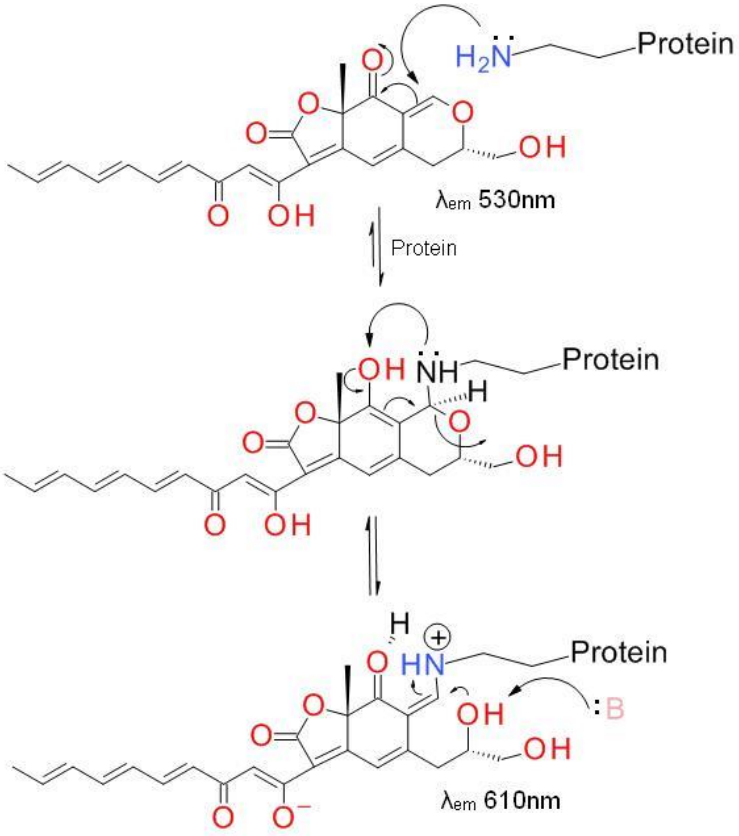

Figure 4. The reversible mechanism for binding of epicocconone to a protein. The emission wavelength changes from 530 to 610 nm. This reaction mechanism is from Karuso et al 2005.

In figures 5 and 6 are experimental data where emission and excitation spectra presented. In figure 5 an absorbance spectrum for epicocconone bound to butyl amine is seen. There are two peaks, one at approximately 340nm and one at approximately 530nm. These peaks can be seen as excitation maxima. In figure 6 an emission spectrum is seen. This spectrum is more specifically of the fluorescence from epicocconone bound to BSA. The epicocconone in this case is excited at 520nm and this result in an emission peak at approximately 605nm. This type of measurement is conducted for every sample ahead of the immobilization kinetics experiment described in section 3.2.3.

[1] Nils Carlsson (PhD student, Chalmers University of Technology, Department of Chemical and Biological Engineering, Division of Chemistry and Biochemistry), personal communication with the author December 2012.

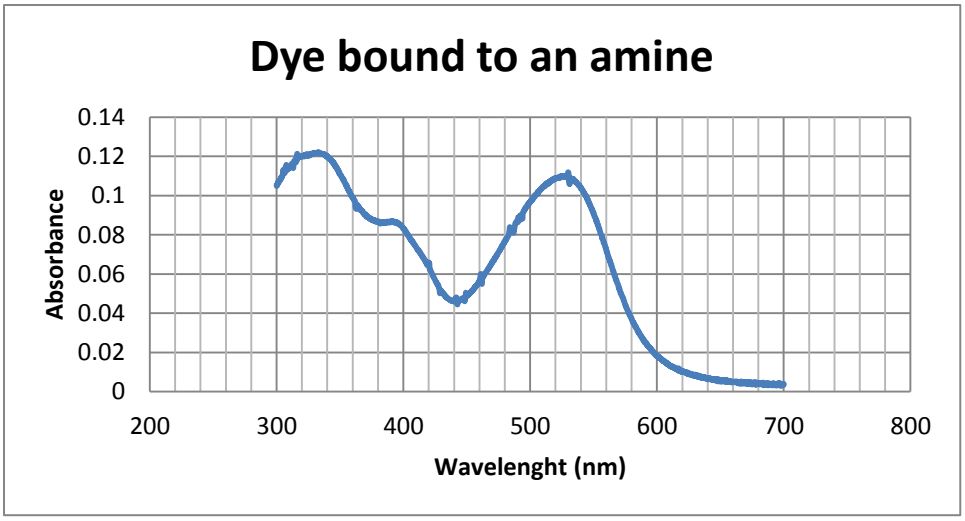

Figure 5. The absorbance spectrum for epicocconone when it is bound to butyl amine. There are two peals one at approximately 340nm and one peak at approximately 530nm. Butyl amine is a small primary amine.

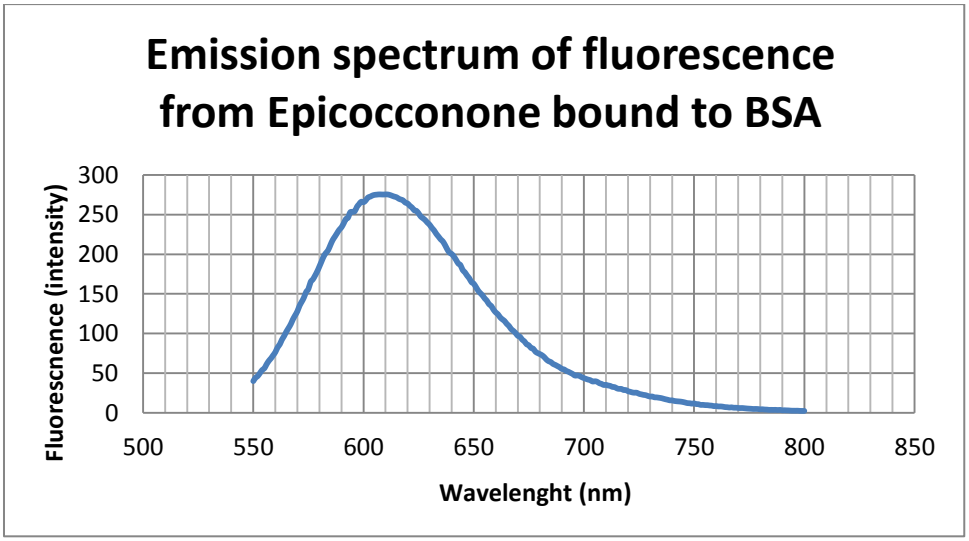

Figure 6. The emission spectrum of fluorescence for epicocconone bound to BSA. A peak is seen at approximately 605nm. This type of measurement was done for every sample in the immobilization kinetics experiments described in section 3.2.3.

2.5. Proteins & enzymes

Proteins are what the genes code for. They make up life and help the cell to function, are built from amino acids and have different functions as: catalyzing metabolic reactions, transporting molecules, replicating DNA or acting as receptors for signals from outside the cells and forwarding and enhancing the stimuli from outside. Enzymes are proteins expressed in nature that are used as catalyst in various reactions (metabolic reactions) in the cell of the organism. As catalysts enzymes are selective and they accelerate the rate and specificity of metabolic reactions. Enzymes as proteins are large biological molecules that can be produced in cell factories (in yeast or bacterium) and later purified for use in other purposes than the natural purpose.

2.5.1. Lipase catalyzed reactions

Triacylglycerol acylhydrolases or more commonly known as lipases are types of enzymes that are translated in many different organisms. They catalyze the hydrolysis of glycerol ester bonds. Lipases can also catalyze esterification and transesterification reactions under certain

conditions (Linko et al, 1996). Lipases are activated when adsorbed to an oil-water interface. This means they do not catalyze the hydrolysis of relevant substrates in the bulk. However the hydrolysis of triacylglycerol to glycerol and free fatty acids can occur in aqueous solution. In nature lipases are for example involved in lipid metabolism and thus important for fat digestion and lipoprotein metabolism in eukaryotes. Lipases are both used and have potential to be used in various industrial applications. They could be used in more environmentally clean organic chemical processing, in production of biosurfactants, in the oleochemical industry, paper industry, production of pharmaceuticals and the food industry (Banerjee et al, 2001). Over the last 20 years examples of industrial applications of lipases are in detergents where they are involved in hydrolysis of fats, in dairy industry where there action is hydrolysis of milk fat, cheese ripening and modification of butter fat. Looking at applications in chemical, pharmaceutical and cosmetics the lipases are involved in enantioselectivity, synthesis, transesterification and hydrolysis (Vulfson 1994).The three main reactions catalyzed by lipases are:

Hydrolysis:

$RCOOR' + H_2O \rightarrow$ RCOOH + R'OH

Esterification:

$RCOOH + R'OH \rightarrow RCOOR' + H_2O$

Transesterification:

$RCOOR' + R''COOR* \rightarrow RCOOR* + R''COOR'$

2.6. Mesoporous silica particles

Mesoporous silica (MPS) is a material that is cheap to make, robust and its properties can be altered (pore size and surface modifications). The pore sizes available vary between 2 and 50 nm but it is more usual with smaller sizes in the range 2-15nm. Very simply described the MPS particles are prepared by mixing a structuring agent that is an organic compound that acts as a template (e.g. a polymer) whose length decides the pore diameter with a silica source that will build up the actual particle by polymerization of the silica. After polymerization the organic template is burnt away or flushed away using solvents (Gustafsson et al 2011). Both the particles and the pore-arrangement can have different shapes (cubic, hexagonal pore arrangement) depending on the concentration of the structuring agent and its shape defined by the critical packing parameter (cpp). The particles can be looked as like monolith channels used in gas catalysts but instead of metals (Platinum or Palladium) acting as catalysts enzymes immobilized on the internal surface of the particles could be used.

The silanol groups at the surface of the MPS have a large effect on the immobilization kinetics and the amount of immobilized proteins. The surface of the MPS is negatively charged at pH over 2.7 (Gustafsson 2012). Amino acid residues can bind to the surface through electrostatic forces, hydrophobic interactions and hydrogen bonding. These intermolecular interactions have different strengths in relation to each other and to the MPS surface depending on solution properties of for example pH, protein shape, protein size, protein properties in terms of primary, secondary, tertiary structure and protein charge.

2.7. Kinetics

To simplify the understanding for the process, in this project it is assumed that the immobilization of proteins onto particles follows either first order or second order kinetics.

Upon immobilization the intensity from the dye bound to a protein increases (see section 2.4 about Epicocconone). By utilizing this feature the kinetics can be studies where an exponential curve describes the process.

In first order kinetics only one reactant is relevant for the rate ν (eq. 7):

$$v = -\frac{d[A]}{dt} = k[A]^1 \quad (7)$$

where *k* is the rate constant. The general equation (eq.8) for a reactant A is

$$[A] = [A]_0 \times e^{-kt} \quad (8)$$

where $[A]_0$ is the starting concentration of reactant A and t is the time. The same equation is seen in logarithmic form in eq.9:

$$\ln[A] = \ln[A]_0 - kt \quad (9)$$

For a second order reaction two reactants of one kind (eq.10a) or of two kinds (eq.10b) are relevant for the reaction rate:

$$v = k[A]^2 \quad (10a)$$

$$v = k[A][B] \quad (10b)$$

The general equation for a reactant A or two reactants A and B in a second order reaction is seen in eq. 11a and 11b:

$$\frac{1}{[A]} = \frac{1}{[A]_0} + kt \quad (11a)$$

$$\ln\frac{[B][A]_0}{[A][B]_0} = k([B]_0 - [A]_0)\,t \quad (11b)$$

The half times for a first order (eq. 12) and a second order (eq. 13) (for A, the same for B) reactions are:

$$t_{1/2} = \frac{\ln 2}{k} \quad (12)$$

$$t_{1/2} = \frac{1}{[A]_0 k} \quad (13)$$

The temperature dependence of the reaction rate constant is taken into account in the Arrhenius equation (eq. 14):

$$k = A \times e^{E_a/RT} \quad (14)$$

where E_a is the activation energy, R is the gas constant and T is the temperature.

This temperature dependence will not be investigated during this project. The data from the kinetic experiments are corrected for volume increase upon adding of particles and normalized. This is further described in section 3.2.3. After correlating and adjusting data it is fitted to exponential curves with the following equation (eq. 15):

$$y = y_0 + A_1(1 - e^{\frac{-x}{t_1}}) \quad (15)$$

The exponential intensity curve from the experiments follows equation 15 where the intensity increases with time and levels out after a certain amount of time. The corrected intensity values (see section4.1) are plotted against time. From the equation for this curve (eq. 15) a time value t_1 is calculated. This can be seen as a half time for the process in question.

Equation (15) is the result when a simple model is assumed for the immobilization process, which is of first order with respect to both the particle concentration (g/l) and the protein concentration (g/l):

$$\frac{d[Particle+Protein]}{dt} = k(T)[Protein]^1[Particle]^1 \quad (16)$$

From equation 16 the interesting constant $k(T)$ ($\left(\frac{g}{l}\right)^{-1} \times min^{-1}$) can be extracted, this is the rate constant for the immobilization process with regard to the protein used and general for particle concentration. There is a relation between k, the rate constant specific for each particle concentration (k_n) and the particle concentration for each experiment:

$$k_n = k(T) \times [Particle] \quad (17)$$

Each k_n (min^{-1}) value can be calculated from each t_1 value from this relationship:

$$k_n = \frac{1}{t_1} \quad (18)$$

By plotting k_n versus the particle concentration a straight line should appear and the slope would be k(T) (see equation 17) which is the rate constant for the assumed model (see equation 16.

3. MATERIALS & METHODS

Firstly the actual proteins, mesoporous particles and the dye used in this project are described more specifically in the materials section 3.1. In the methods section 3.2 a description of how the experiments were executed is presented.

3.1. Materials

Here in in the materials section the main chemicals and the proteins are described. In the methods section the buffers used in each experiment will be mentioned but not further described. The instrumentation used will also be stated. The particles that the proteins immobilize onto were not synthesized during this project and were kindly offered by Hanna Gustafsson from the Surface Chemistry department at Chalmers University of Technology.

3.1.1. BSA

One of the proteins used in the immobilization kinetics study (described in section 3.2.3.) and briefly used in the availability studies (described in section 3.2.4) is Bovine Serum Albumin (BSA). BSA is a serum albumin that is expressed in cows. In the field of biochemistry examples of applications for BSA is its use in immunoblots, as nutrient for cell culture and in Bradford protein assays for measuring protein concentration in solution. Naturally Albumin is important for keeping the oncotic pressure for body fluids. BSA has 583 amino acid residues and the molecular weight of 66,463Da. It has the hydrodynamic structure of a prolate ellipsoid (Wright and Thompson 1975) with a hydrodynamic radius of approximately 3.5nm (Yohannesa et al 2010). The isoelectric point of BSA in water at 25°C is 4.7 (Ge et al 1998). The Extinction coefficient is $43{,}824 M^{-1} cm^{-1}$ at 279nm (Peters 1975). Another important feature of BSA is that it prefers to bind to surfaces. To study the process of immobilization of the protein onto the MPS particles the protein will be stained with the dye Epicocconone that will be described in section 3.1.3. The BSA used in this project was purchased from *SIGMA-ALDRICH*.

3.1.2. Lipase

The lipase that was used in the experiments comes from the fungi *Rhizomucor miehei*. *Rhizomucor miehei* is a species of fungus that is commercially used for production of enzymes. Structurally lipase from *Rhizomucor miehei* has a lid covering the active site. This lid changes conformation and exposes the active site to the surroundings when the lipase is activated by a hydrophobic interface (Skagerlind et al 1995). This enzyme has a size of approximately 30 kDa and has a globular shape in its native form (Mathews 2000). This lipase has a hydrodynamic radius of approximately 2.25nm and an isoelectric point of 3.8 (Gustafsson 2012). From now on lipase from *Rhizomucor miehei* will simply be referred to as lipase.

3.1.3. Epicocconone

The only dye that was used in this project was Epicocconone. It was ordered from *SIGMA-ALDRICH* as a part of the *Fluoroprofile kit* for concentration determination. Its general properties is that it upon binding to an amine or more relevantly to proteins it has local excitation maxima at 390 and 520nm and an emission maximum at approximately 605nm as described in the theory in section 2.4.

3.1.4. Particles

In this project one type of MPS particle called SBA-15 KP04 was used. Its material properties where previously determined: a BJH pore width of 9.3nm, a BET surface area of $502 m^2/g$ and

a total pore volume of 1.18cm^3/g (Gustafsson 2012). The particles have a hexagonal shape with a size (diameter) of 1000nm.

3.1.5. 4-nitrophenyl acetate

For the enzyme activity assays the substrate used was 4-nitrophenyl acetate ordered from *SIGMA-ALDRICH*. It has a molecular weight of 181.15 g/l. When used it was weighed and dissolved in the common solvent DMSO to 1 or 0.1M.

In the lipase activity assay the catalytic hydrolysis of 4-nitrophenyl acetate (pNPA) to 4-nitrophenol (pNP) and acetic acid is studied (seen in figure 7).

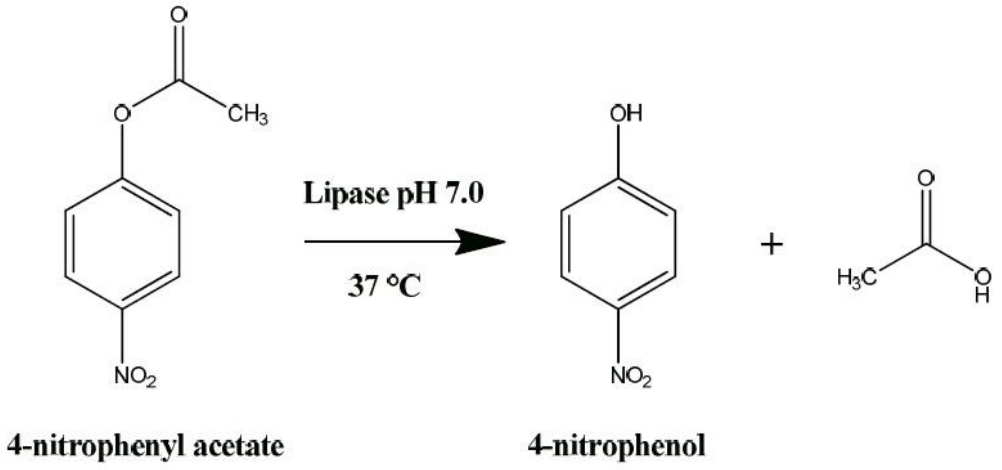

Figure 7. The lipase catalyzed hydrolysis of 4-nitrophenyl acetate into 4-nitrophenol and acetic acid. The reaction occurs at 37°C and pH 7.

pNP is the product that is analyzed in an absorbance spectrophotometer. The product will have bright yellow color with an absorbance at 400 nm with a molar extinction coefficient of 14 200 $M^{-1}cm^{-1}$.

3.1.6. Instrumentation and cuvettes

AThermo scientific NanoDrop 1000 spectrophotometer from Saveen, Weiner was used for determining protein and dye concentrations.

A Cary 50Bio UV-visible spectrophotometer from Varian was used in absorbance measurements and more specifically in the activity and availability experiments described in section 3.2.4.

A Cary Eclipse fluorescence spectrophotometer from Varian was used in the kinetic studies described in section 3.2.3.

A variety of quartz cuvettes of different volumes were used in the experiments.

3.2. Methods

In the methods section a general description of the main experiments conducted during this project will be described.

3.2.1. Staining of proteins with Epicocconone

The staining of the proteins was done at pH 8 where both proteins used are negatively charged. The proteins are also still in their native form with as many amine groups as possible are available (while the protein is still native) for Epicocconone to bind in to. Basically 0.1 M

pH 8 phosphate citrate buffer was mixed with respective protein solution, Epicocconone and a quantification buffer that followed with the dye as part of *Fluoroprofile kit* when ordered from *SIGMA-ALDRICH*. The staining mixture was covered in aluminum foil and kept dark for the entire incubation time of one hour at room temperature. To remove unbound Epicocconone the staining mixture was purified on *illustra NAP-10 columns* from *GE Healthcare Life Sciences*. The column was washed with 0.1 M pH 6 phosphate citrate buffer and after the washing the staining mixture was added to the column and 0.5-1.0 ml fractions was collected in eppendorf tubes. The protein content in relevant eppendorf tube was measured with a nano-drop and the fractions with higher protein concentration was put together and the protein concentration was one again determined in the stock solution of stained protein.

The degree of labeling (DOL, dye-to-protein ratio) was determined by absorption spectroscopy by using the Lambert-Beer law (see equation 6). This was done for lipase and BSA stained with epicocconone. The absorbance of the conjugate solution (stained protein) was first measured after the purification on the *illustra NAP-10 column*. The next step was to determine the absorbance (A_{max}) at the absorption maximum (λ_{abs}) of the dye (epicocconone) and the absorbance (A_{280}) at 280 nm (absorption maximum of proteins). The absorbance of the protein also had to be corrected for epicocconone absorption at 280 nm. The concentration of bound dye was then calculated from equation 19.

$$DOL = \frac{A_{max}/\varepsilon_{max}}{A_{prot}/\varepsilon_{prot}} = \frac{A_{max}\times\varepsilon_{prot}}{(A_{280}-A_{max}\times CF_{280})\times\varepsilon_{max}} \quad (19)$$

Where ε_{max} is the molar extinction coefficient of the dye (11200 $M^{-1}cm^{-1}$) at the absorption maximum, where ε_{prot} is the molar extinction coefficient of the protein at 280 nm (44000 $M^{-1}cm^{-1}$ for BSA and 42800 for $M^{-1}cm^{-1}$) and where CF_{280} (A_{max}*CF_{280} is 0.042) is the correction factor for the contribution of the dye to the absorbance at 280nm. All molar extinction coefficients were determined experimentally but these procedures will not be further described.

3.2.2. Preparation of particle solutions used in experiments

For all experiments with particles a particle solution was prepared. This procedure was simply to weigh an amount of particles and after this the particles was dissolved in 0.1 M pH 6 phosphate citrate buffer to the wanted concentration. A vortex was used to solve the particles and afterwards the particle solution was also sonicated for 10 minutes to prevent particle aggregation. Right before use in relevant experiments the sonicated particle solution was vortexed since particle sedimentation occurred directly at any time when the solution was kept still. The vortex, sonication and vortex procedure was repeated each day the particle solution was used.

3.2.3. Investigation of immobilization kinetics

When the immobilization kinetics was studied a fluorescence spectrophotometer equipped with a four cuvette holder was used. First a protein solution with the stained protein was prepared. The molar concentration of protein was kept constant over the course of the study and it was the same for both lipase and BSA. The molar concentration in the cuvettes for the proteins during the experiments was kept at approximately 4.5×10^{-8}M. The particle concentration during the experiments in the cuvettes was for BSA varied between 0.0185-0.3564g/l and for Lipase also between 0.0185-0.3564g/l. For Lipase also lower particle concentrations was used were the concentrations varied between 0.00185g/l to 0.0644g/l.

A solution with the wanted protein concentration was first prepared in 0.1 M pH 6 phosphate citrate buffer and this solution was then divided into the four cuvettes that was going to be used. The excitation wavelength was set to 520 nm and the emission wavelength was registered at around 605 nm depending on where the peak of the emission spectra occurs on the pre-scan that was always performed before the kinetics was studied (see figure 6). The emission slit was set as low as possible to minimize the photo-bleaching effect and the excitation slit was set as high as possible at 20 nm. The settings of the slit varied from BSA to Lipase since BSA was stained with more Epicocconone due to its larger size and that is why it fluoresced much more.

In the cuvettes small magnets was added and the magnetic stirrer in the instrument was started to minimize the sedimentation effect on the particles. 10 minutes in to the kinetic measurement a certain volume of particles was added to three of the cuvettes. The fourth cuvette was kept as a reference to take the photo-bleaching of the dye into account. Upon the adding of the particles a rise in intensity occurs. The kinetics was then studied over 3-5 hours depending on the particle concentration. The measurement was stopped when the residual kinetic curve had reached a plateau. The residual kinetic curve is simply the difference between the sample intensity curve and the reference sample.

After the measurement the reference sample intensities were subtracted from the each of the other samples intensities. The intensities were also corrected for the volume increase due to the added particle solution and the curves were normalized so they start at zero. The data was corrected and normalized in *Excel* and analyzed in *Origin* where the kinetic curves were adjusted to exponential curves from which the half-time t_1 could be extracted from equation 15. From equations 16-18 the rate constant (k (T)) for the assumed model (first order with regard to both protein and particle concentration) was calculated for respective protein. How this was done is described at the end of section 2.7.

3.2.4. Immobilized Lipase activity assays for studying availability

In this phase of the project different types of assays were performed. In all activity assay experiment unstained proteins were used. At first free lipase was investigated with the substrate 4-nitrophenyl acetate. This was done so that good experimental conditions are established were the substrate is well in surplus so that the maximum enzyme activity is achieved. The results from these preparatory experiments are not presented in the report. Another feature is the problem with auto-hydrolysis of the substrate 4-nitrophenyl acetate that had to be addressed. This was done by investigating the auto-hydrolysis over the course of time at the relevant temperature of 37°C. All free lipase activity and auto-hydrolysis experiments were performed in 50mM pH 7 phosphate buffer. The results for these preparatory experiments are not presented in the results section.

The relevant type of assay is where the activity of immobilized lipase is investigated. The main idea is to see whether the environment inside the particles can be too crowded with lipase. The hypothesis is that this is the case and therefore a good relationship between the particle and protein concentration can result in that the highest possible activity is found. Three types of experiments were performed. First the enzyme concentration was kept constant while the particle concentration was varied. The second type of experiments the particle concentration, where the highest specific activity was seen in the previous experiments, is used and kept constant and the protein concentration was varied instead. In the third type of experiments both lipase and BSA are used in co-immobilization experiments.

The concentrations used for the first two types of experiments in the results section 4.2 are presented in table 1 and 2. These concentrations are the concentrations of the particles and

proteins during immobilization not during the activity assay. In the lipase activity assays the particle solutions were further concentrated.

Table 1. Particle and protein concentrations during immobilization. The particle concentration was varied and the lipase concentration was kept constant.

Particle concentration (g/l)	Lipase concentration (g/l)	Particle to protein ratio
0.113	0.00532	21.3
0.170	0.00532	32.0
0.227	0.00532	42.7
0.284	0.00532	53.3
0.340	0.00532	64.0
0.397	0.00532	74.6
0.454	0.00532	85.3
0.510	0.00532	96.0
0.896	0.00934	96.0
0.995	0.00934	106.6
1.095	0.00934	117.3
1.195	0.00934	128.0
1.294	0.00934	138.6
1.394	0.00934	149.3

Table 2. Particle and protein concentrations during immobilization. The lipase concentration was varied and the particle concentration was kept constant.

Particle concentration (g/l)	Lipase concentration (g/l)	Protein to particle ratio
0.896	0.00933	0.010
0.896	0.01862	0.021
0.896	0.03706	0.042
0.896	0.05532	0.063
0.896	0.07339	0.083
0.896	0.09129	0.104
0.896	0.10901	0.125
0.896	0.12657	0.146
0.896	0.14395	0.167
0.896	0.17823	0.208

The enzymes were immobilized on a thermo-shaker at 25°C and 900 rpm shaking. The buffer used for the immobilization was 0.1 M pH 6 citrate phosphate buffer. The immobilization mixture was left for 24 hours on the thermo-shaker after which equilibrium is assumed to have been reached. After immobilization washing of the particles was conducted. The reaction mixtures were centrifuged (7 min, 16 000 G) then the supernatant with free proteins was removed and the pellet was then re-suspended in 50mM pH 7 phosphate buffer. These washing steps were repeated three times to remove all free protein.

For the activity assay for immobilized lipase the substrate concentration was 0.01M in the reaction tube. The particle solution is concentrated 1.5 times approximately when it used in the activity assay as compared to the particle concentration during the immobilization. In the reaction tubes 4-nitrophenyl acetate (pNPA), the concentrated immobilized protein solution and 50mM pH 7 phosphate buffer were mixed and put on the thermo-shaker at 37°C and 900 rpm shaking. After one hour the samples are put on ice and after cooling down and stopping the lipase catalyzed hydrolysis the samples were centrifuged (7 min, 16 000 G). Since the

samples often had a strong absorbance out of the measurable range 0.1-1 a part of the supernatant is taken and diluted to an appropriate concentration and the product absorbance is measured with an absorbance spectrophotometer. A peak at 400 nm was observed and with the extinction coefficient for the product 4-nitrophenol (pNP) the concentration can be calculated. Finally the product concentrations were corrected for the auto-hydrolysis of pNPA and also the absorbance of pNPA left in the solution. The specific activity was calculated from these corrected product concentrations where amount product was divided by incubation time and amount protein ($\frac{mol}{g \times min}$). The data was treated and the graphs were made in *Excel*.

3.2.4.1. Co-immobilization of Lipase and BSA

The third type of experiments was also used for investigating the availability of the substrate to the immobilized lipase. The idea was that BSA will somehow be in the way and disturb lipase when it hydrolysis the substrate pNPA (see figure 8). In these experiments both lipase and BSA is used. First lipase or BSA is immobilized onto the particles for 24h in 0.1 M pH 6 phosphate citrate buffer. After washing steps with 0.1 M pH 6 phosphate citrate buffer BSA and lipase are added to the test tubes and left to immobilize. Lipase and BSA are added to the opposite test tube where the other protein was first immobilized. At the same time a third test tube with BSA and lipase is prepared were the idea is to let the proteins immobilize at the same time and compete for the binding sites inside the particles. For this third type of experiments one protein and one particle concentration is used. The particle to lipase ratio in these experiments is 96. To conclude, in the first test tube lipase is immobilized over 24 hours, then BSA is added and let to immobilize over 24 hours. In the second tube BSA is immobilized over 24 hours, and then lipase is added. In the third test tube lipase and BSA immobilize at the same time over 24 hours. The lipase concentration used in these experiments was 0.00934g/l and the BSA concentration was 0.021g/l. This means that both have the same molar concentration in the solution. The particle concentration was 0.896g/l in all cases. This translates to a particle to lipase ratio of 96. The activity assay for the co-immobilization experiments were conducted in the same way as described at the end of section 3.2.4.

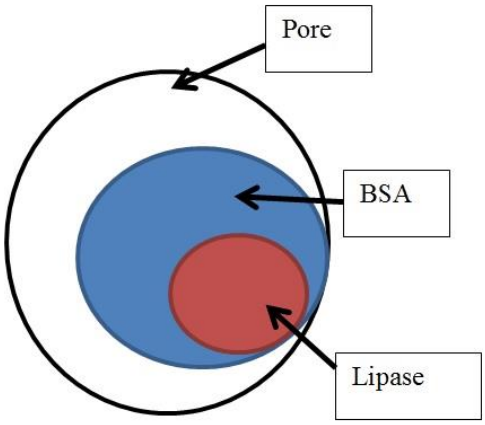

Figure 8. Schematic picture of how BSA could hinder lipase from going further into the pore of a MPS particle. The pore has a diameter of approximately 9nm; BSA has a hydrodynamic radius of approximately 7nm and lipase a hydrodynamic radius of 4.5nm.

4. RESULTS

First the results of the project will be presented. There are mainly two different types of experiments, immobilization kinetic studies and lipase activity assays. In sections 5.1 and 5.2 a discussion of the results will follow, here in the results section no discussion is written, the results are just simply presented.

4.1. Investigation of immobilization kinetics

Here follows the results from the immobilization experiments with BSA and Lipase. One type of particle has been used throughout the experiments and it is SBA-15 KP04. Both proteins will have negative net charge since the experiments are carried out at pH 6. In the immobilization experiments a protein solution is made and divided to 4 cuvettes. The fluorescence measurements is started and after about 10 minutes different amount of particle is added to three of the cuvettes whilst the fourth cuvette is left as a reference sample to take into account the photo bleaching of the dye. The reference sample is used as a baseline and is subtracted from the other measured fluorescence curves. All experiments are carried out at 22°C (room temperature). The degree of labeling (DOL) was successfully determined and gave logical results. The experimental procedure is described in the end of section 3.2.1. The epicocconone to BSA ratio was determined to 8.3 and the epicocconone to lipase ratio was determined to 1.6. When the number of possible binding sites (amine groups) was calculated from the amino-acid sequence for respective protein the results were the following: 117 sites including the N-terminal for BSA and 38 sites including the N-terminal for lipase. The ratios doesn't correspond the ratio between respective amounts of binding sites. However it is the total amount of binding sites that has been determined and not the amount available binding sites for epicocconone if the proteins folding are considered.

Relevant features of the proteins used are seen in table 3.

Table 3. Basic data for the proteins used in the experiments. It is the hydrodynamic radius, the isoelectric point for respective protein and the degree of labeling (DOL) or the dye to protein ratio for BSA and lipase.

Protein	R_H	pI	DOL
BSA	3,48 nm	4,7	8.3
Lipase	2,25 nm	3,8	1.6

The first result is general for all these experiments described in this section. It is possible to investigate the immobilization kinetics with this new method using fluorescence spectroscopy. By changing the settings on the spectrophotometer the immobilization kinetics can be studied both for BSA and lipase. In the following figures (9 and 10) raw data from immobilization experiments with BSA are represented. It is data from two different occasions with different particle concentrations. For the experiments represented in figure 9 the experimental setup was different, in this case particles were weighed 3 separate times and diluted to the same concentration, this was done to investigate the reproducibility of the particle preparation.

The reference sample with no particles is clearly different from the samples where particles were added. This difference is the basis of the analysis of the kinetics. The difference is due to the increase of intensity of the fluorescence from epicocconone when the stained proteins immobilize into the particles.

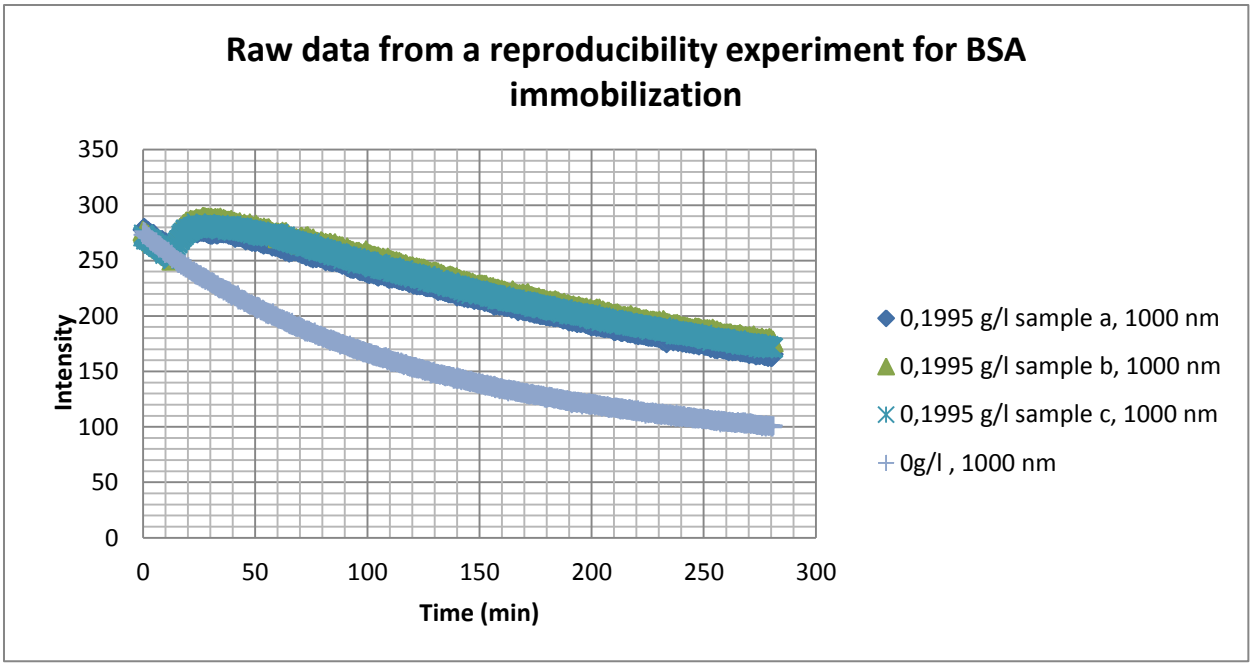

Figure 9. Raw data curves from a reproducibility experiment. Three different particle concentrations are investigated and a fourth reference sample with zero g/l particles is also represented.

In figure 10 the raw data from an immobilization experiments is presented. Three different particle concentrations are investigated and one reference sample is also measured where the particle concentration is zero. In these and all the other immobilization kinetics experiments a single particle solution is made and used. Compared to figure 9 the difference between the particle samples and the reference sample is smaller but still very much measurable. The smaller difference is due to the fact that smaller particle concentrations are studied in the experiments fin figure 10. This is result is expected. If more particles are added, the particles immobilize faster and in larger amounts.

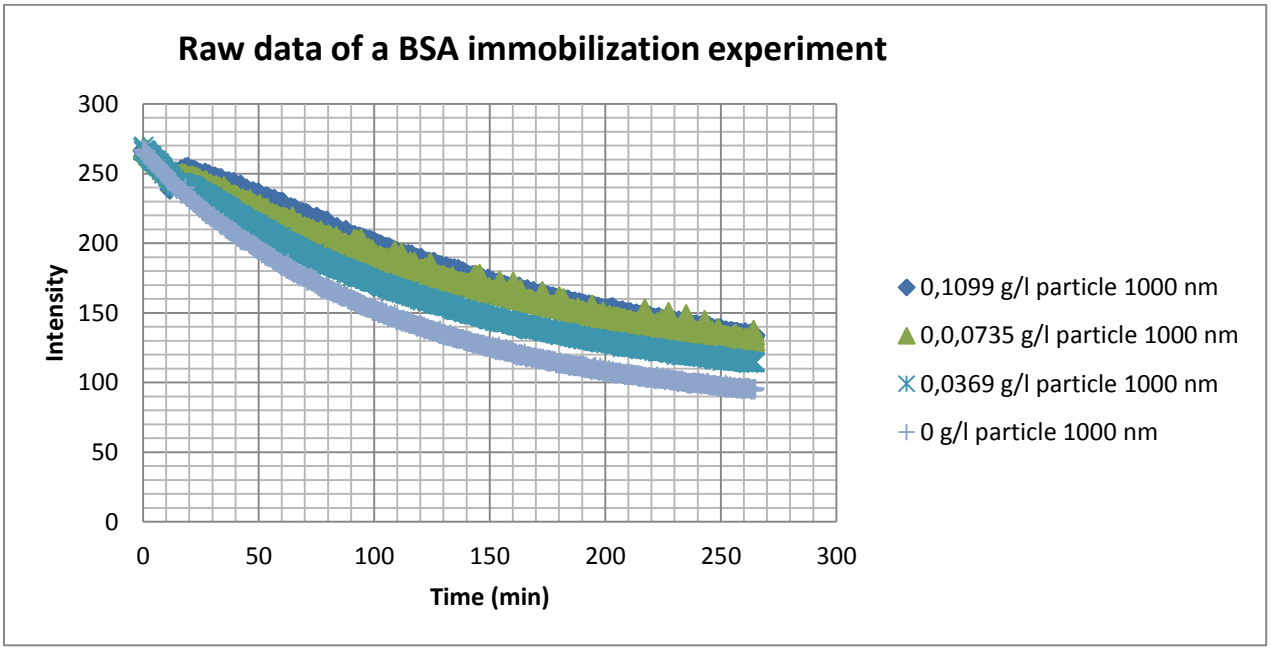

Figure 10. Raw data curves from a BSA immobilization experiment. Three different particle concentrations are investigated and a fourth reference sample with zero g/l particles is also represented.

The immobilization kinetics is quantified with a value called t_1. This value is a half-time for the immobilization kinetics for each specific particle concentration. This quantification is further described in section 2.7 and in equation 15.

In table 4 a summary from the reproducibility experiment in figure 9 is seen, where the reproducibility is quite good, however sample b differs a bit from the other t_1 values.

Table 4. Results from a reproducibility experiment. The reproducibility experiment in figure 9 is presented, in these experiments particles has been weighed three times to the same weight and diluted to the same concentration. The results are the t_1 values. The particle and BSA concentrations are also presented.

[Particle] in cuvette (g/l)	**[BSA] in cuvette (g/l)**	**[BSA] in cuvette (mol/l)**	**t_1 (min)**
0.1995 (a)	0.002984	4.49E-08	24.753 (a)
0.1995 (b)	0.002984	4.49E-08	26.664 (b)
0.1995 (c)	0.002984	4.49E-08	24.625 (c)

In figure 11 a graph of the immobilization kinetics are represented for sample (a) in figure 9 with a particle concentration of 0,1995g/l, in figure 12 the immobilization kinetics are represented for the sample with a particle concentration of 0,1099g/l in figure 10.

The black squares are each corrected data point and the green curve is the residual between the experimental data point and the red exponential curve. The blue lines represent a 95% confidence interval. The data are fitted to the following equation for exponential association:

$$y = y_0 + A_1(1 - e^{\frac{-x}{t_1}}) \quad (15)$$

as described in section 2.7. The interesting value in this study is as mentioned earlier the t_1 value from equation 15 that is a quantification of the immobilization kinetics. The t_1 value represents a half time for the immobilization process. All average t_1 values with experimental setup are presented in tables 5-7.

The equation for the model (eq.15) fits the data better towards the end of the process in both the cases presented (figure 11-12). The green residual curve doesn't behave completely randomly at the start of the immobilization process and this suggests that the model is not perfectly correct for the immobilization process for BSA.

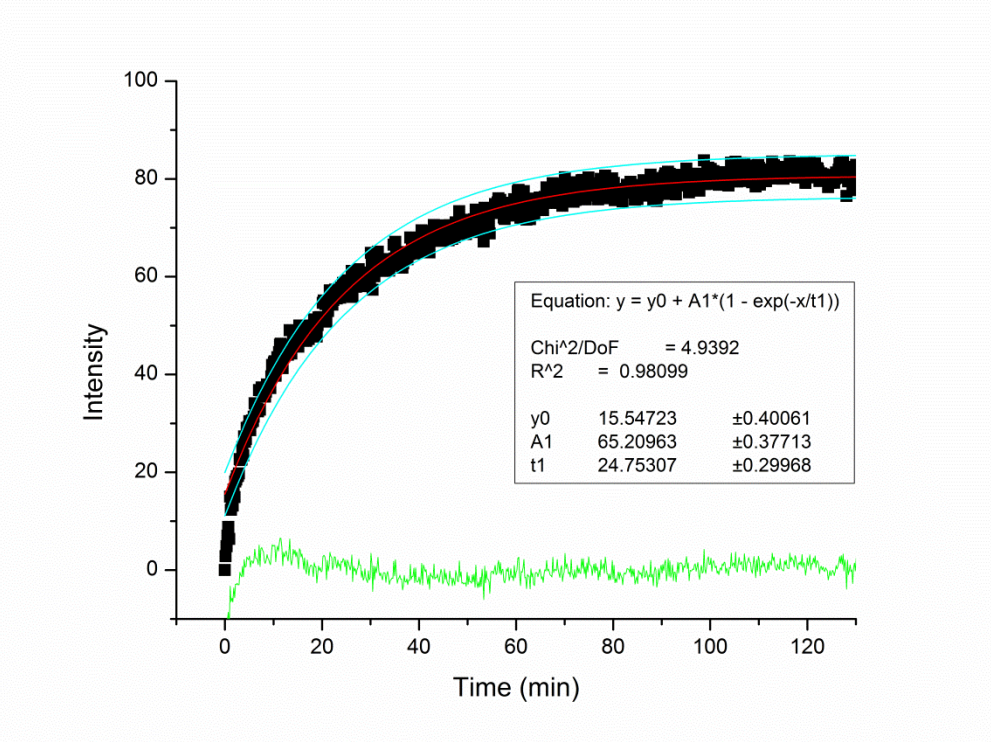

Figure 11. Intensity curve for BSA immobilization kinetics from the reproducibility experiments presented in figure 9. It is sample (a) and the particle concentration is 0,1995 g/l. The black squares are each corrected data point and the green curve is the residual between the experimental data point and the red exponential curve. The blue lines represent a 95% confidence interval. The model equation and the R squared value are also presented in the figure.

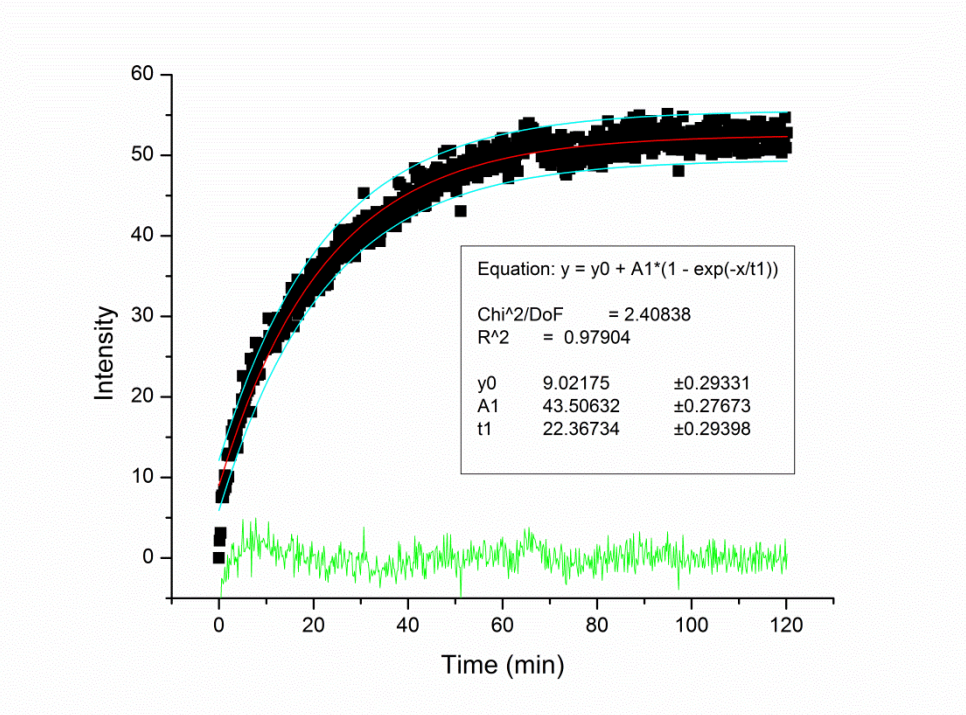

Figure 12. Intensity curve for BSA immobilization kinetics from the BSA immobilization experiment presented in figure 10. The particle concentration is 0,1099g/l. The black squares are each corrected data point and the green curve is the residual between the experimental data point and the red exponential curve. The blue lines represent a 95% confidence interval. The model equation and the R squared value are also presented in the figure.

In figures 13 and 14 raw data from immobilization experiments with lipase are seen. These data are taken from two separate occasions where different amount of particles are added to

the cuvettes. Comparing to the raw data graphs for BSA the difference between the particle samples and the reference sample is much more profound. Lipase immobilize faster and in larger amount over the same time span compared to BSA.

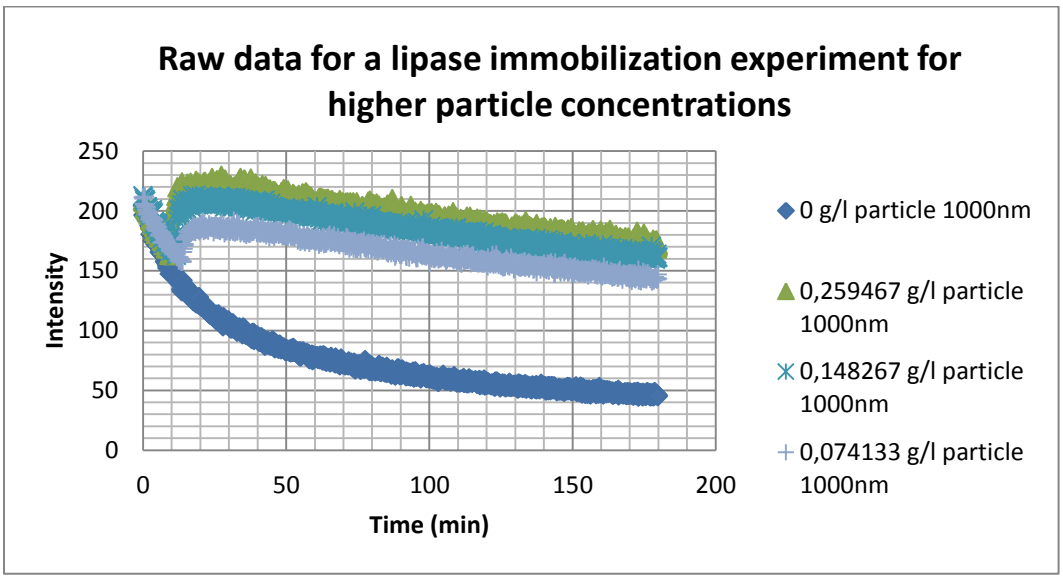

Figure 13. Raw data curves for a lipase immobilization experiments for higher particle concentrations. Three different particle concentrations are investigated and a fourth reference sample with zero g/l particles is also represented.

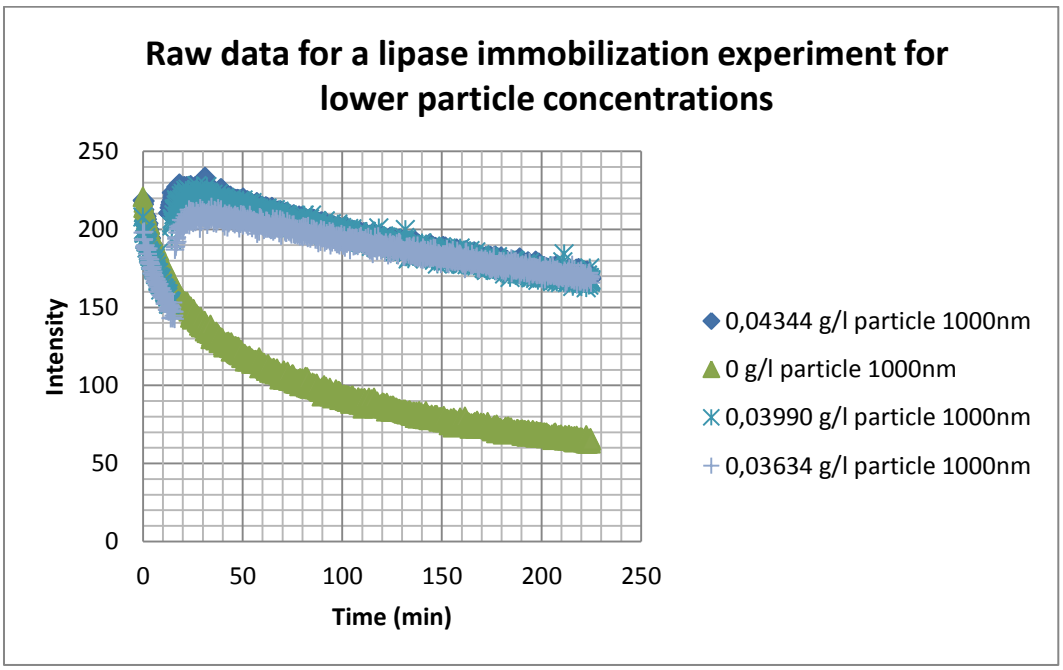

Figure 14. Raw data curves for a lipase immobilization experiment for lower particle concentrations. Three different particle concentrations are investigated and a fourth reference sample with zero g/l particles is also represented.

As with BSA in figures 15 and 16 graphs of the immobilization kinetics for Lipase are represented. The black squares are each corrected data point and the green curve is the residual. The blue lines represent a 95% confidence interval. Also here the green residual curve shows a non-random behavior at the start of the immobilization process. The corrected data point generally fits the model equation (eq.15) better for BSA (figure 11-12) than for lipase (figure 15-16).

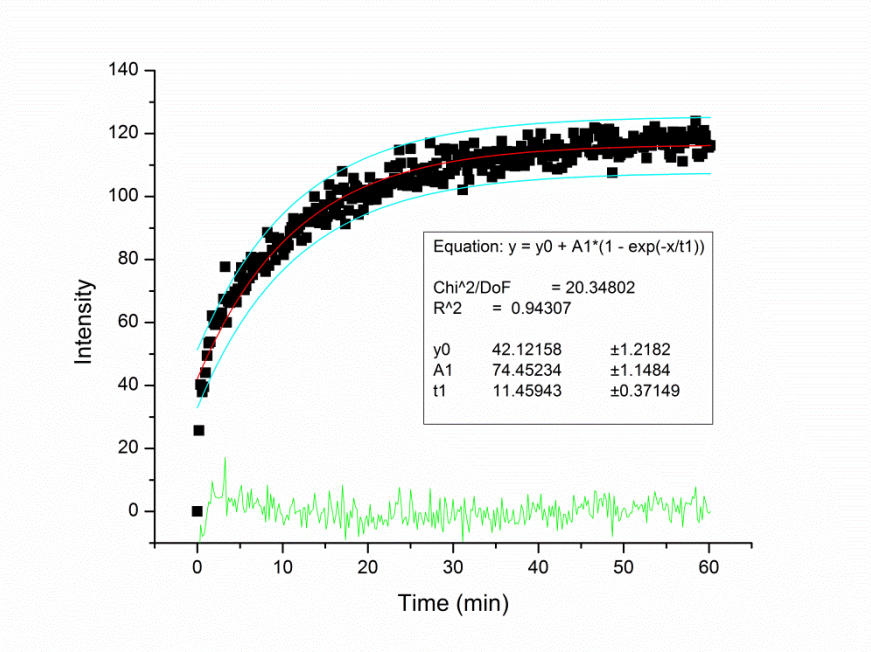

Figure 15. Intensity curve for lipase immobilization kinetics for the experiment presented in figure 13. The particle concentration is 0,259g/l. The black squares are each corrected data point. The green curve is the residual between the data point and the exponential model curve. The blue lines represent a 95% confidence interval. The model equation and the R squared value are also presented in the figure.

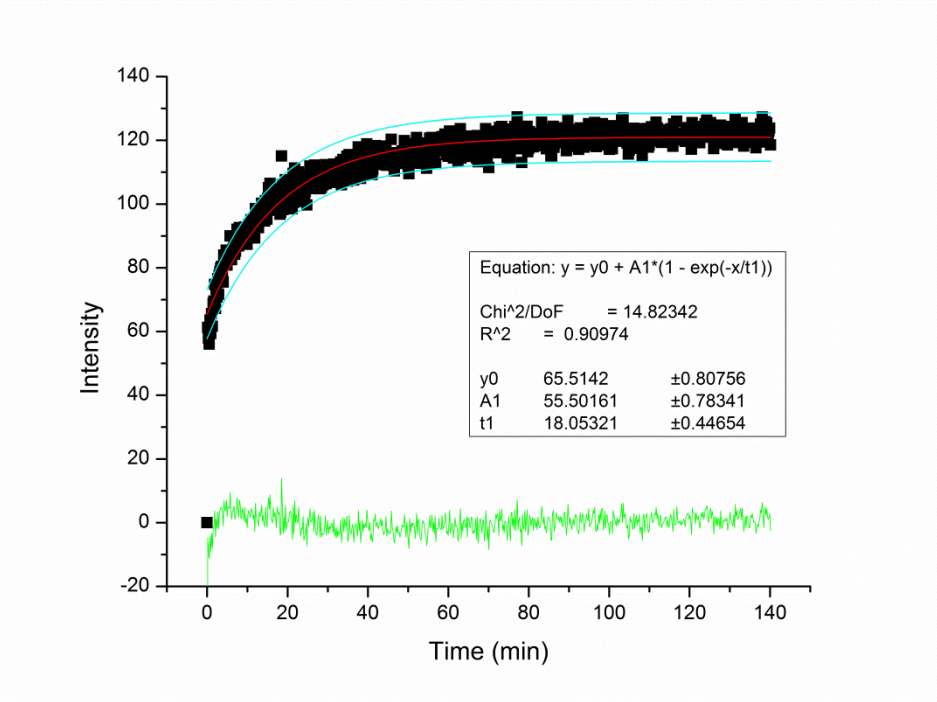

Figure 16. Intensity curve for Lipase immobilization kinetics for the experiment presented in figure 14. The particle concentration is 0,0434g/l. The black squares are each corrected data point. The green curve is the residual between the data point and the exponential model curve. The blue lines represent a 95% confidence interval. The model equation and the R squared value are also presented in the figure.

In table 5-7 are the average t_1 values presented together with particle and protein concentrations for each case. The average t_1 value is an average of all t_1 values with the same

experimental conditions. It is the concentration in the cuvette where the process takes place in the spectrophotometer that is presented. The t_1 average value represents how the fast the process immobilization process occurs for each specific particle concentration.

In table 5 a summary of the BSA immobilization experiments is presented including particle and protein concentrations and average t_1 values. The average t_1 value decreases with increasing particle concentrations.

Table 5. Summary of BSA immobilization experiments. The particle concentration, BSA concentration (constant) and t_1 average values are presented. The t_1 average value decreases with increasing particle concentration.

[Particle] in cuvette (g/l)	[BSA] in cuvette (g/l)	[BSA] in cuvette (mol/l)	t_1 average (min)
0.0185	0.002984	4.49E-08	70.528
0.0369	0.002984	4.49E-08	41.605
0.0624	0.002984	4.49E-08	50.630
0.0735	0.002984	4.49E-08	47.767
0.0917	0.002984	4.49E-08	44.607
0.1099	0.002984	4.49E-08	33.142
0.1239	0.002984	4.49E-08	29.685
0.1459	0.002984	4.49E-08	42.550
0.1817	0.002984	4.49E-08	24.192
0.1995	0.002984	4.49E-08	25.348
0.2143	0.002984	4.49E-08	25.568
0.2172	0.002984	4.49E-08	23.853
0.2524	0.002984	4.49E-08	31.249
0.2873	0.002984	4.49E-08	18.112
0.3220	0.002984	4.49E-08	24.468
0.3564	0.002984	4.49E-08	28.019

In table 6 the results for the lipase immobilization process are presented. These are for higher particle concentrations (the same particle concentration span as for the BSA experiments). The t_1 average value decrease with increasing particle concentrations and compared to BSA the process occurs faster for lipase.

Table 6. Summary of lipase immobilization experiments. Results for higher particle concentrations. The particle concentration, lipase concentrations (constant) and t_1 average values are presented. The t_1 average value decreases with increasing particle concentration.

[Particle] in cuvette (g/l)	[Lipase] in cuvette (g/l)	[Lipase] in cuvette (mol/l)	t_1 average (min)
0.0185	0.00133	4.51E-08	22.592
0.0369	0.00133	4.51E-08	23.110
0.0735	0.00133	4.51E-08	15.156
0.1099	0.00133	4.51E-08	19.589
0.1459	0.00133	4.51E-08	13.746
0.1817	0.00133	4.51E-08	22.133
0.1995	0.00133	4.51E-08	11.564
0.2172	0.00133	4.51E-08	19.960
0.2524	0.00133	4.51E-08	11.459
0.2873	0.00133	4.51E-08	18.840
0.3220	0.00133	4.51E-08	16.399
0.3564	0.00133	4.51E-08	9.028

In table 7 the results of immobilization kinetics for low particle concentration are presented with lipase as the adsorbate. Also here the immobilization occurs faster with higher particle concentration.

Table 7. Summary of lipase immobilization experiments. Results for lower particle concentrations. The particle concentration, lipase concentrations (constant) and t_1 average values are presented. The t_1 average value decreases with increasing particle concentration.

[Particle] in cuvette (g/l)	[Lipase] in cuvette (g/l)	[Lipase] in cuvette (mol/l)	t_1 average (min)
0.00185	0.00133	4.51E-08	26.026
0.00370	0.00133	4.51E-08	65.880
0.00554	0.00133	4.51E-08	42.708
0.00738	0.00133	4.51E-08	52.215
0.01105	0.00133	4.51E-08	44.055
0.01471	0.00133	4.51E-08	29.619
0.01835	0.00133	4.51E-08	26.833
0.02198	0.00133	4.51E-08	27.996
0.02559	0.00133	4.51E-08	18.831
0.02919	0.00133	4.51E-08	15.685
0.03277	0.00133	4.51E-08	18.836
0.03634	0.00133	4.51E-08	25.943
0.03990	0.00133	4.51E-08	17.801
0.04344	0.00133	4.51E-08	18.053
0.04697	0.00133	4.51E-08	15.692
0.05048	0.00133	4.51E-08	14.136
0.05398	0.00133	4.51E-08	13.565
0.05747	0.00133	4.51E-08	14.871
0.06094	0.00133	4.51E-08	14.362
0.06440	0.00133	4.51E-08	12.784

In figures 17-18 the trends of the average t_1 values presented in table 5-6 are seen. Starting with BSA (figure 17) a decrease in t_1 value is seen with an increase of the particle concentration until a particle concentration of approximately 0.17g/l where the curve levels out. This behavior suggests that the immobilization doesn't occur faster above particle concentrations of 0.17g/l.

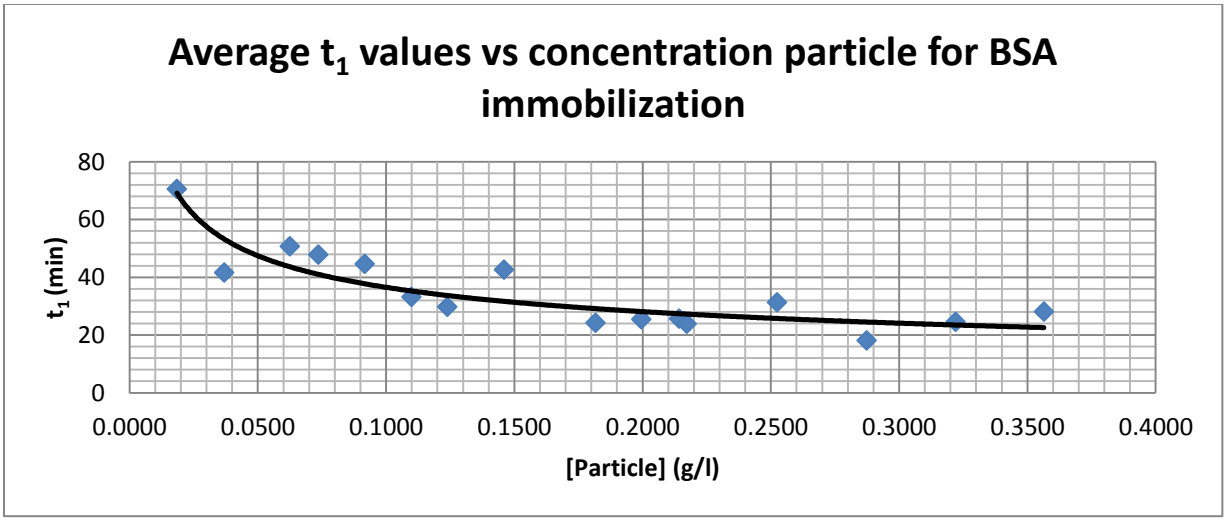

Figure 17. Trend of average t_1 values for the immobilization of BSA. With increasing particle concentrations the curve decreases and levels out. A black curve is added into the figure to help the eye see the trend.

Looking at lipase the immobilization occurs faster at the same particle concentrations as for the BSA experiments (figure 18). A week trend is seen where the average t_1 values decrease

with increasing particle concentrations. There is a lot of spreading of the data points as well. It is however clear that lipase immobilizes faster than BSA.

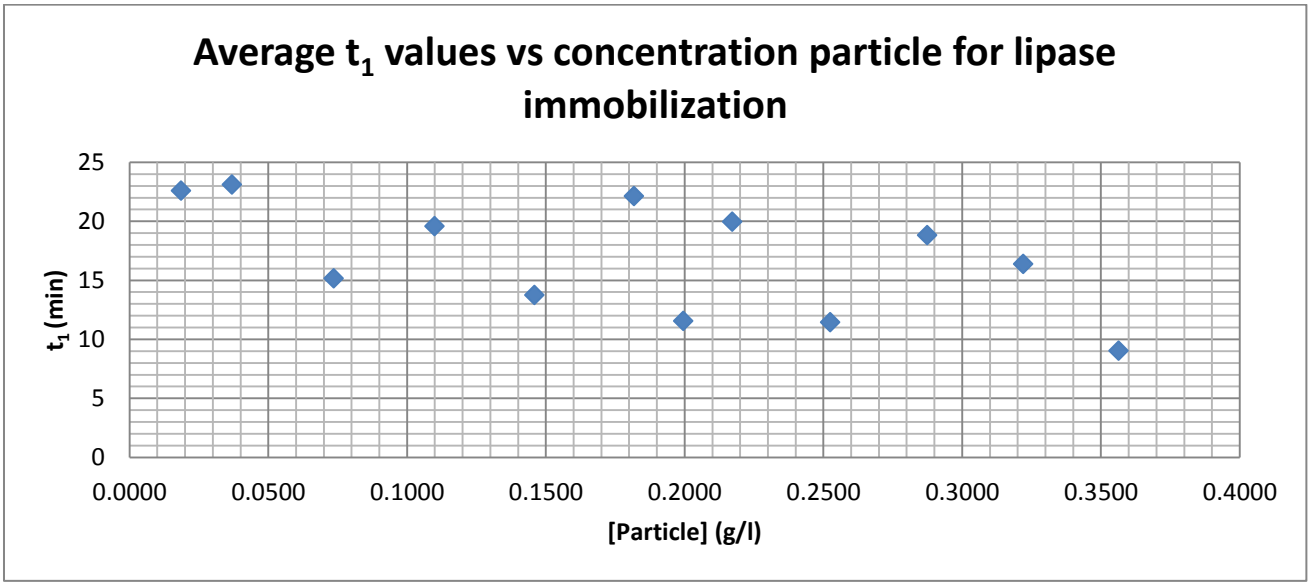

Figure 18. Trend of average t_1 values for the immobilization of lipase. With increasing particle concentrations the trend of the curve is ambiguous. It is slightly decreasing but it is clear that lipase immobilize faster than BSA.

To better study the immobilization kinetics of lipase lower particle concentrations were used so that the procedure is slowed down and the relevant t_1 values are studied. In figure 19 a similar trend for lipase immobilization is seen as for BSA but at lower particle concentrations. Lipase immobilization is much faster than for BSA and the trend curve levels out when a particle concentration of approximately 0.025g/l is reached.

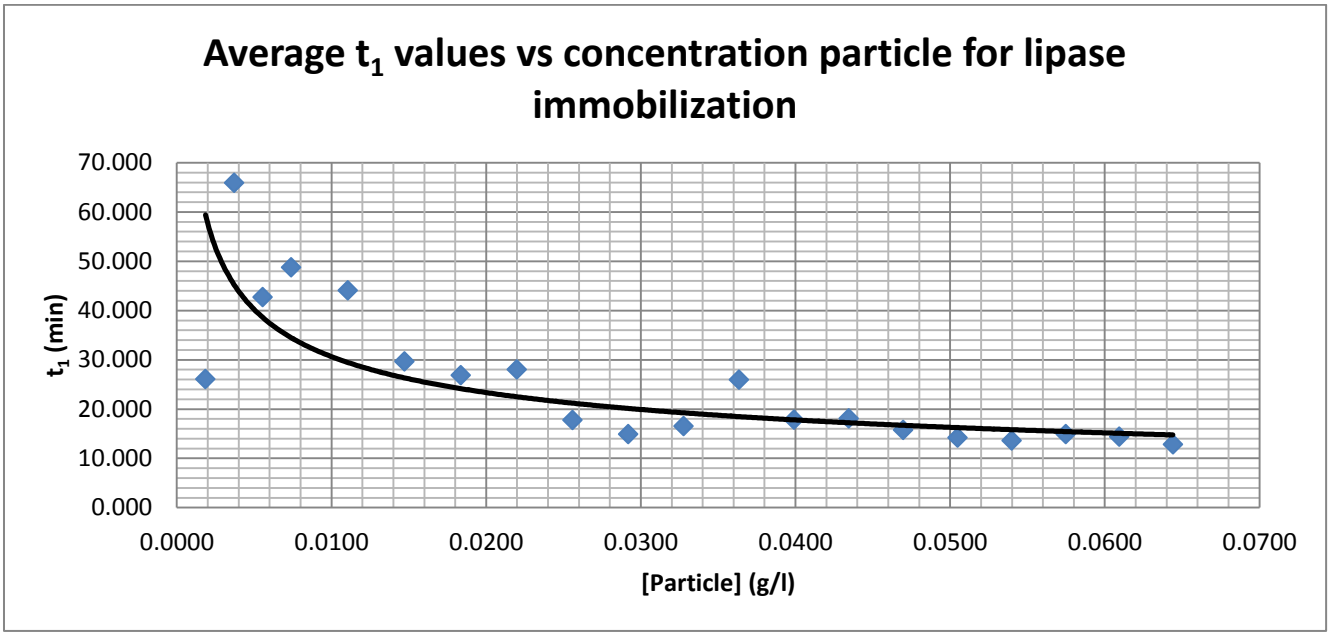

Figure 19. Trend of average t_1 values for the immobilization of lipase. With increasing particle concentrations a decreasing trend is clearly seen. The decrease of the t_1 average values continues until a particle concentration of 0.025g/l where the curve starts to level out. A black curve is added into the figure to help the eye see the trend.

In figures 20-21 the assumed kinetic model for the immobilization (first order with respect to both protein and particle concentration) is partially confirmed. Each average t_1 value (see table 5 and 7) is inversed and this generates the particle concentration specific rate constant

k_n. These k_n values are plotted against each particle concentration. From these graphs (in figure 20 and 21) a trend line is determined out of which the k(T) value can be extracted as the slope. This k(T) value is the temperature specific rate constant general for all particle concentrations (see section 2.7). The k(T) value for BSA is 0.0826 $(g/l)^{-1}min^{-1}$ and for lipase it is 0.8803$(g/l)^{-1}min^{-1}$. The fitting of the curve is better for lipase than for BSA. Neither of the straight lines goes through the origin.

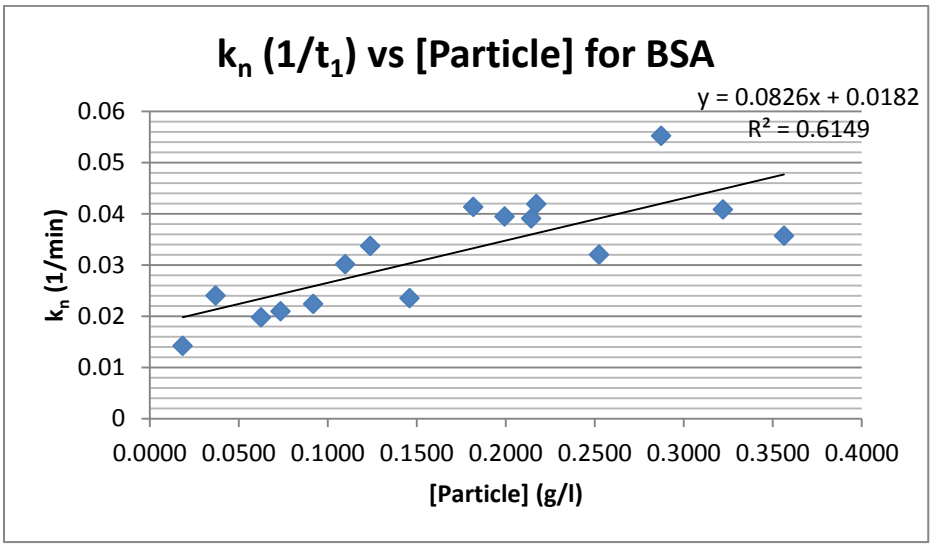

Figure 20. The k_n values (the inverse of the average t_1 values) plotted against the particle concentration. A straight line is fitted to the data points and the k(T) value can be extracted as the slope. The k(T) vale is the temperature specific rate constant with the unit $(g/l)^{-1}min^{-1}$. For BSA the k(T) value is 0.0826 $(g/l)^{-1}min^{-1}$.

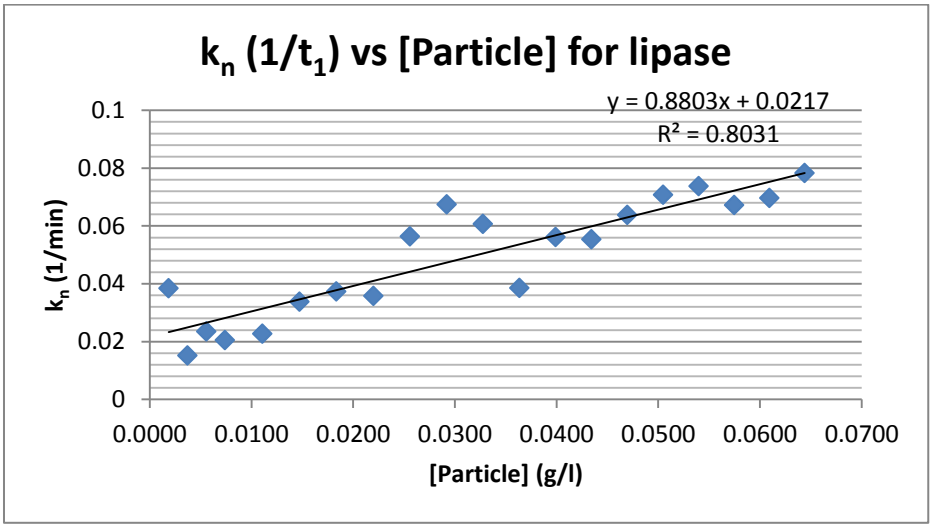

Figure 21. The k_n values (the inverse of the average t_1 values) plotted against the particle concentration. A straight line is fitted to the data points and the k(T) value can be extracted as the slope. The k(T) vale is the temperature specific rate constant with the unit $(g/l)^{-1}min^{-1}$. For lipase the k(T) value is 0.8803 $(g/l)^{-1}min^{-1}$.

4.2. Immobilized Lipase activity assays for studying availability

In the availability experiments lipase activity is investigated by measuring absorbance of a product called 4-nitrophenol. The substrate 4-nitrophenyl acetate and the method itself are described in sections 3.1.5 and 3.2.4. The concentrations of particle and proteins are presented in section 3.2.4. Neither BSA nor the particles showed any hydrolytic activity.

The results from these activity assays are presented in figures 22-25. The x-axis in these figures represents a ratio. When the particle concentration is varied and the protein

concentration is kept constant, respective concentration (g/l) is divided and the particle to protein ratio is presented. In the other case the protein concentration is varied and the particle concentration is kept constant and this is represented by a protein to particle ratio on the x-axis. The activity of the sample is basically the amount substrate hydrolyzed to product by lipase. The specific activity is defined as µmol product per gram protein used in the immobilization and per minute and this is the relevant quantification of the activity observed.

It is important to again mention that the ratios are calculated from the particle and protein concentrations used when the immobilization process is started (see section 3.2.4.). This means that it is not the amount of protein immobilized that is compared to the specific activity; it is the amount of protein present at the start of the immobilization.

In figure 22 an experiment where the particle concentration is varied is presented. It is a clear linear dependence between the specific activity and the particle concentration. As the particle concentration increases the specific lipase activity increases linearly at constant protein concentration.

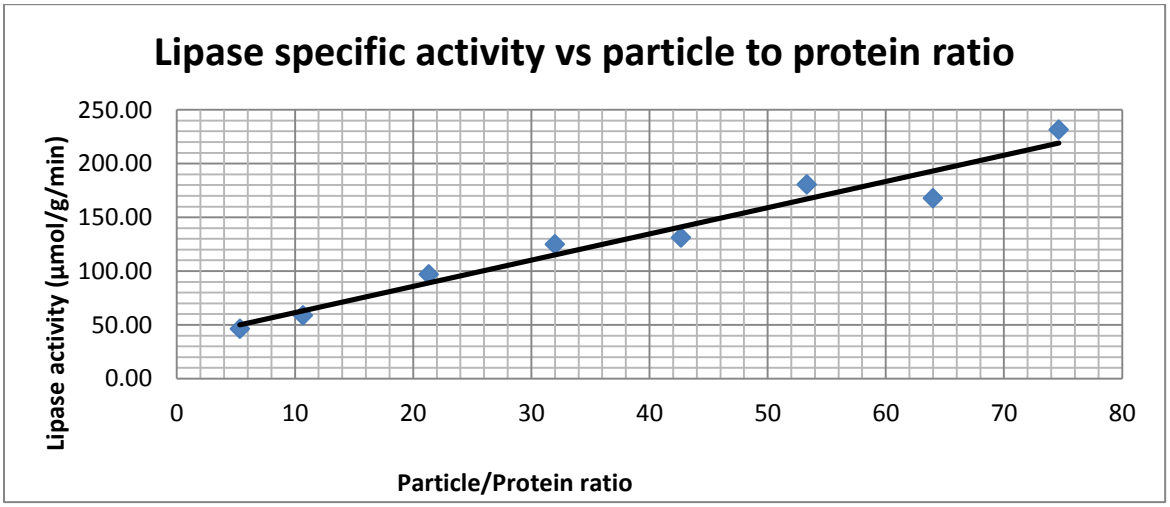

Figure 22. Specific activity of lipase is plotted against the particle to protein ratio. The protein concentration is constant and with increasing particle concentration the specific activity increases linearly. A black trend line is added as a guide for the eye.

The same type of experiments was performed for particle to protein ratios over 90. In figure 23 these results are presented. The specific activity levels out when the particle to protein ration reaches approximately 96. This is seen clearly in figure 23. For particle to protein ratios of 96 and above the specific activity is stable around 600µmol/g/min.

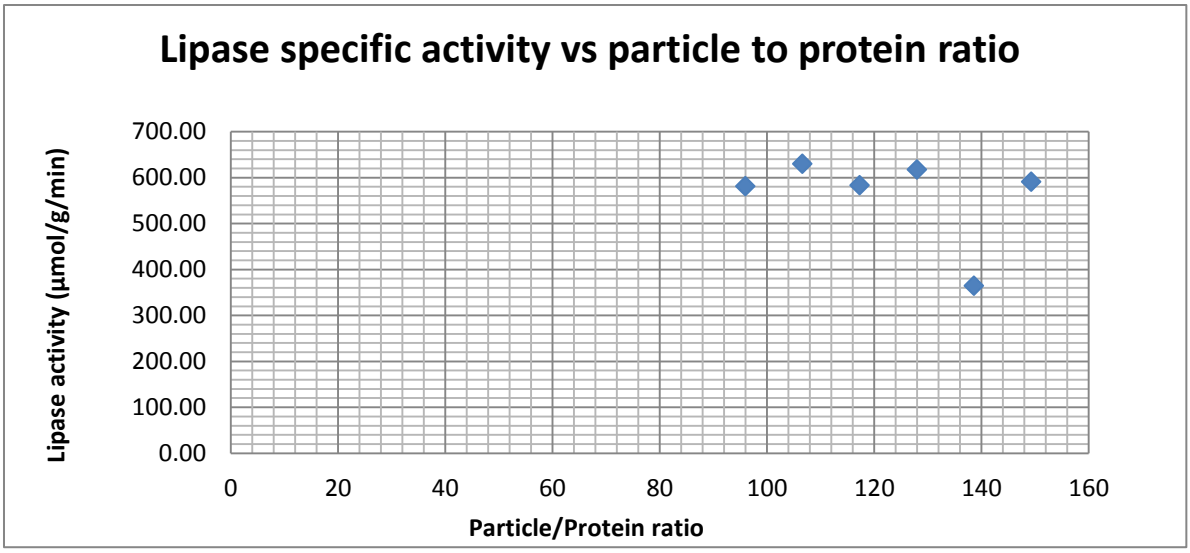

Figure 23. Specific activity of lipase is plotted against the particle to protein ratio. The results are for particle to protein ratios higher than 90. The protein concentration is constant and with increasing particle concentration the specific activity is constant.

When the protein concentration was varied and the particle concentration was kept constant, starting from a particle to protein ratio of 96 or a protein to particle concentration of 0.01, two shapes of curves are seen (figure 24 and 25). The specific activity both decrease and levels out (figure 24) or it increase and levels out (figure 25). It is important to mention that the same type of lipase is used but the lipases are from different batches. The specific activity is much higher in figure 24 because a fresher lipase solution was used.

Looking at figure 24 where the specific activity for free lipase is also presented as the red data point the specific activity decreases and levels out. The difference between the free lipase (0.00934g/l) and the corresponding point for immobilized lipase (the blue data point at a ratio of 0.01) is explained by the fact that not all lipase is immobilized. The particle and protein concentration for this experiment is presented in table 2 in section 3.2.4.

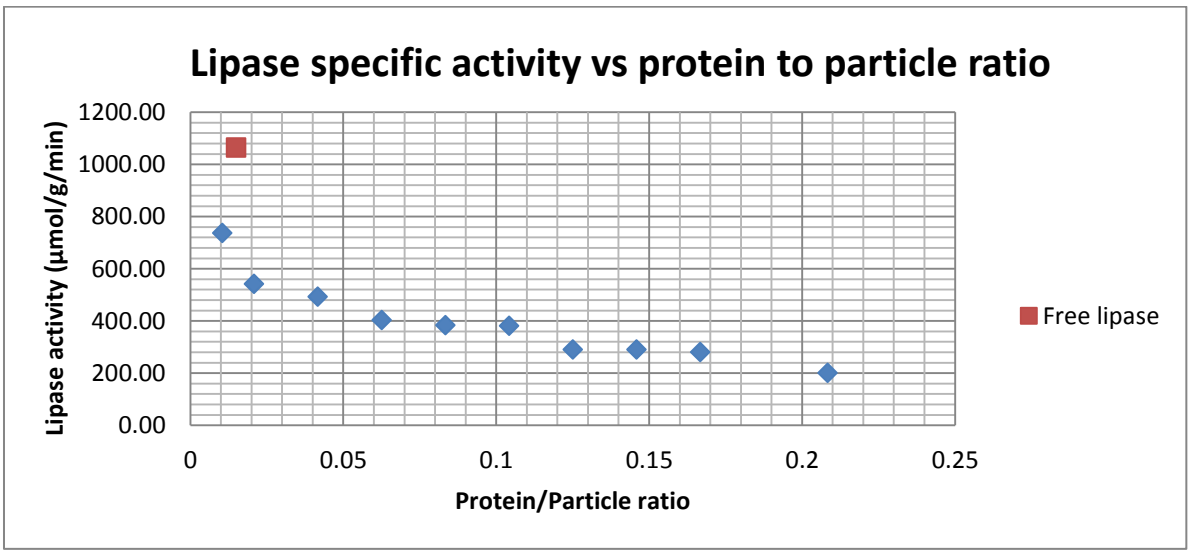

Figure 24. Specific activity of lipase is plotted against the protein to particle ratio. The blue data points represent specific activity of immobilized lipase where the particle concentration is kept constant and the protein concentration is varied. The activity decreases and starts to level out at higher protein to particle ratios. The decreasing trend is clearly seen. The red data point represents the specific activity for free lipase (0.00934g/l). It is higher than for the corresponding blue data point for immobilized lipase.

In figure 25 the shape of the specific activity curve is different even though exactly same experimental procedure is conducted. The only difference between the experiment in figure 24 and in 25 is that approximately two times lower lipase concentrations and particle concentration is used in the experiments presented in figure 25. The ratios are however the same in both cases. The concentrations for the experiment in figure 25 are not presented in section 3.2.4. The specific activity increases and levels out at a protein to particle ratio of 0.07. In figures 26 and 27 the concentration product (pNP) is plotted against the protein to particle ratio. Figure 26 is corresponding the results in figure 24 and figure 27 correspond the results in figure 25.

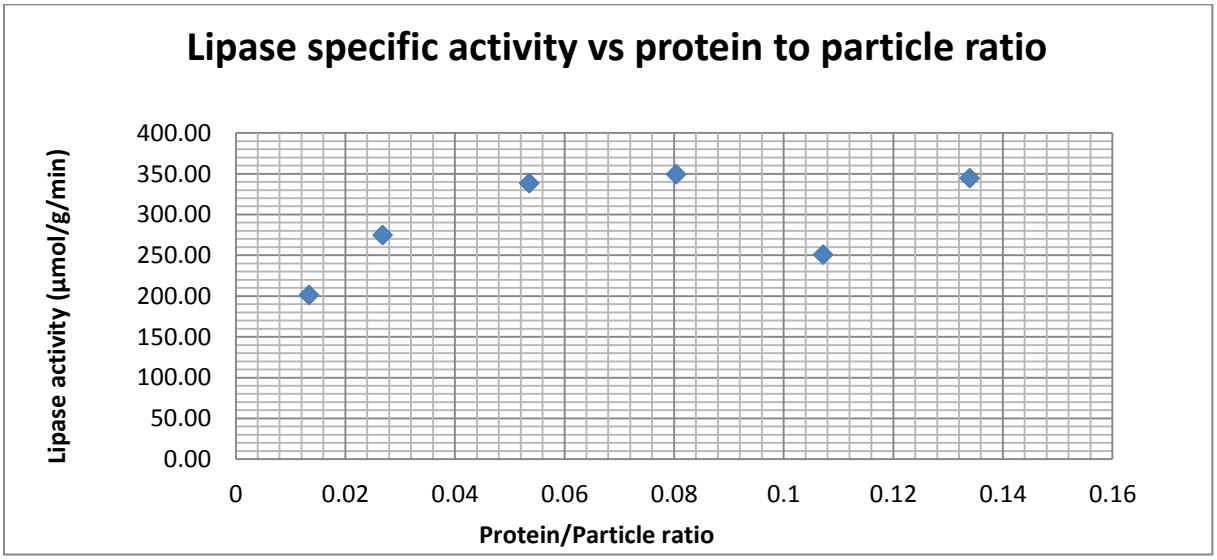

Figure 25. Specific activity of lipase is plotted against the protein to particle ratio. The blue data points represent specific activity of immobilized lipase where the particle concentration is kept constant and the protein concentration is varied. The activity increases and eventually levels out at a protein to particle ratio of 0.07. In this experiment the particle and protein concentrations are approximately half of these used in the experiment presented in figure 24. The ratios are the same.

In figure 26 concentration product increases with increasing protein concentration until a protein to particle ratio of 0.12. After this point the curve levels out.

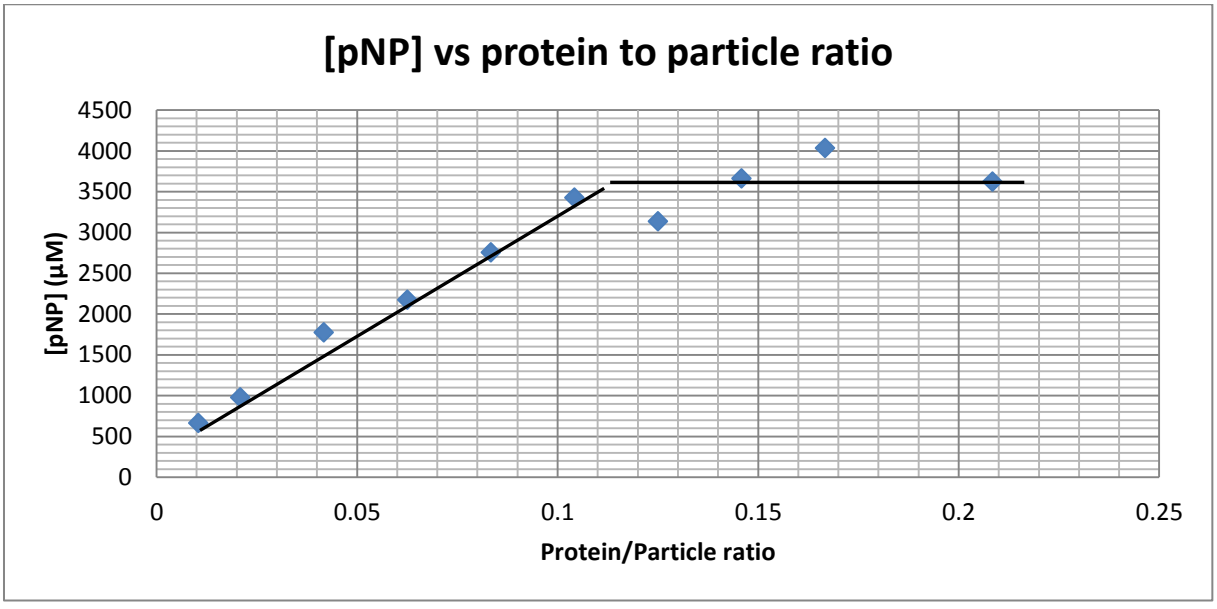

Figure 26. The concentration product (pNP) is plotted against the protein to particle ratio. These results correspond to the results in figure 24. The curve is linear until a protein to particle ratio of approximately 0.12. Above this ratio the curve levels out. Two solid lines has been added as an guide for the eye to see the trend.

The results in figure 27 are similar to those seen in figure 26, the amount of product produced by lipase increases with increasing protein concentrations. A linear increase is seen and if the protein concentrations where to be increased further the curve would probably level out as in the previous case.

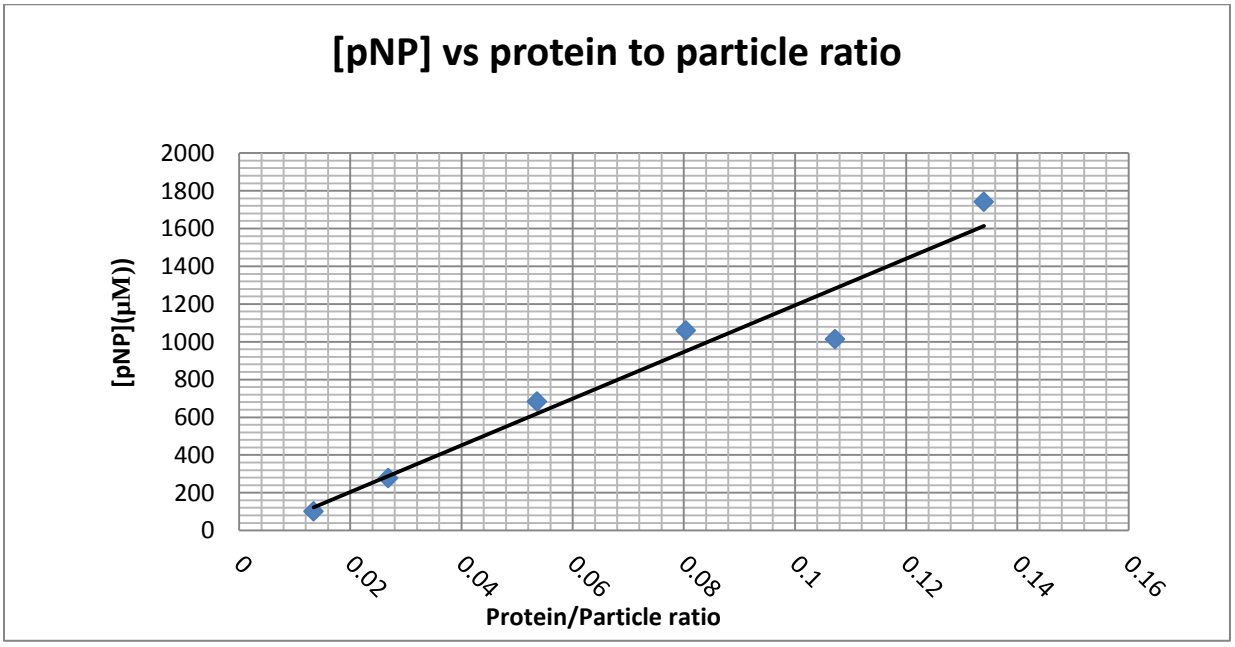

Figure 27. The concentration product (pNP) is plotted against the protein to particle ratio. These results correspond to the results in figure 25. The concentration product increases with increasing protein to particle ratio. The increase is linear. A solid line has been added as an guide for the eye to see the trend.

4.2.1. Co-immobilization of Lipase and BSA

A third way of studying the availability of lipase to the substrate was tested briefly. This method is described at the end of section 3.2.4. The method basically amounts to using both lipase and BSA at the same time. Three types of immobilizations where conducted. In the first type lipase is immobilized for 24 hour, after washing of the particles BSA is added and let to immobilize over 24 hours. In the second type of experiment BSA is instead the first protein to be immobilized for 24 hours followed by 24 hours of immobilization of lipase. Finally both lipase and BSA are added at the same time to a particle solution and let to immobilize. The results are seen in figure 28 and 29.

Looking at figure 28 first the highest specific activity is seen for the samples where lipase and BSA where let to immobilize at the same time (green). The second highest specific activity is seen for the samples where only lipase was immobilized. Letting lipase immobilize for 24 hours and then afterwards letting BSA immobilize for 24 hours (red) gives slightly higher specific activity than the experiment where the BSA was first immobilized (blue). These results should be compared to the reference sample which is the specific activity for immobilized lipase (purple).

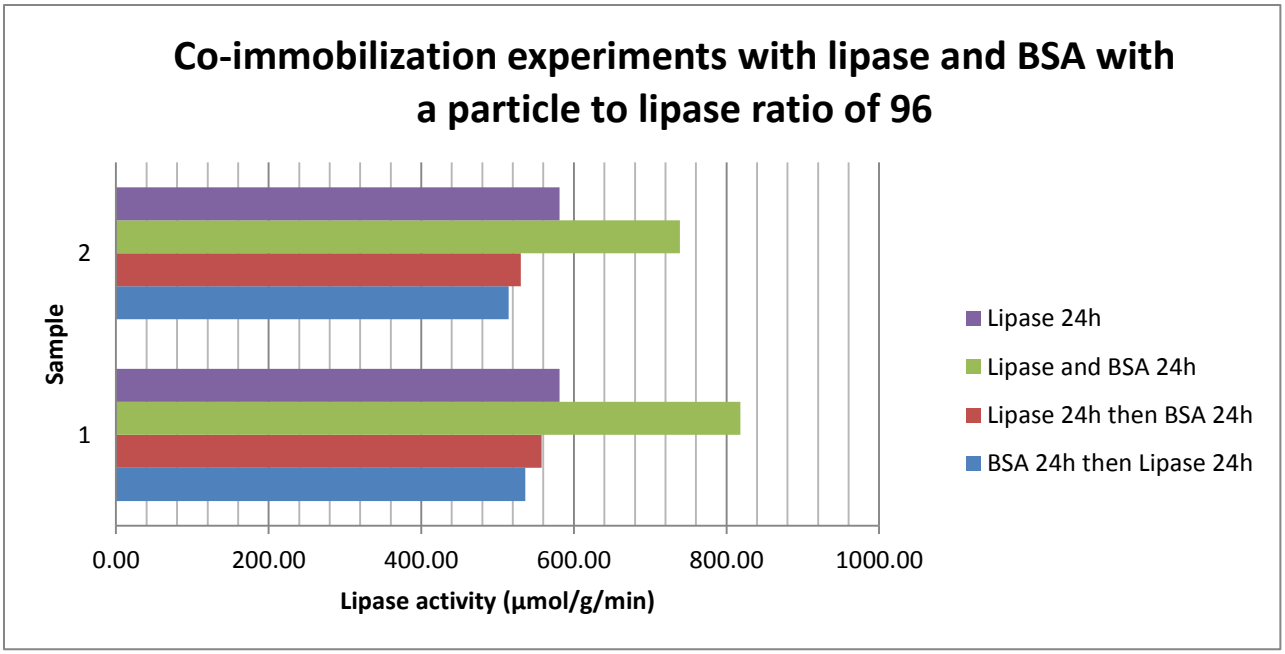

Figure 28. Specific activities for lipase in co-immobilization experiments with lipase and BSA. The purple staple is the specific activity for immobilized lipase. The green staple is the specific activity for the samples where lipase and BSA were immobilized at the same time. The red staple is the specific activity for the experiment where lipase was first immobilized followed by the immobilization of BSA. The blue staple is the specific activity for the experiment where BSA was first immobilized followed by the immobilization of lipase. The highest activity is seen for the green staple and the lowest activity is seen for the blue staple. The particle to lipase ratio in all sample is 96.

The results in figure 28 was interesting, especially the result where the lipase and BSA where immobilized at the same time (green staple).

This type of experiments was repeated and the results is seen in figure 29 (red staple). However this time the sample with just lipase immobilized (green staple) showed higher specific activity. The highest specific activity is seen for the free lipase with a concentration of 0.00934g/l. This is not strange since all lipase is not immobilized in the other cases.

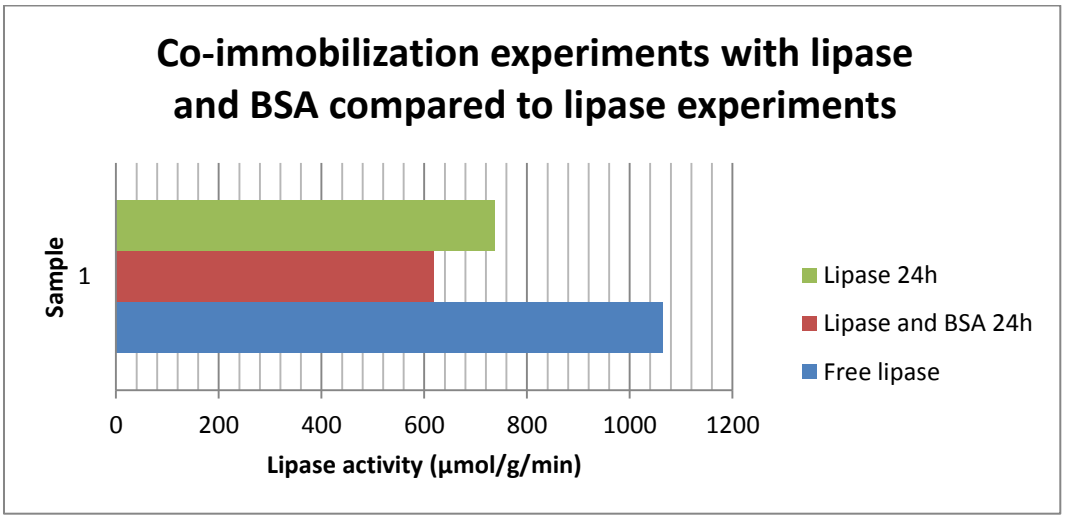

Figure 29. Specific activities for lipase in co-immobilization experiments with lipase and BSA. The green staple is the specific activity for immobilized lipase. The red staple is the specific activity for the sample where lipase and BSA were immobilized at the same time. The specific activity of free lipase is represented by the blue staple.

5. DISCUSSION

In section 5.1 and 5.2 a discussion around the results in section 4.1 and 4.2 is presented. The discussion is an attempt explaining the results.

5.1. Investigation of immobilization kinetics

Comparing BSA and lipase both have negatively net charge in the pH range used in the experiments. Since the MPS surface also is negatively charged repulsion is expected between protein and pore wall. This means that strong hydrogen bonds and van der Waals interactions are the driving force for the proteins in questions to be drawn to particles instead of the cuvette surfaces. These interactions are due to uncharged silanol groups on the MPS surface. One effect of these conditions is however that the electrostatic repulsion between the protein and MPS pore wall could lead to that the protein diffuses further into the particle enabling denser packing of proteins.

At the short time spans of 3-4 hours over which the experiments are carried out no immobilization equilibrium is assumed to be reached. This assumption is based on earlier results where the immobilization kinetics was studied with absorbance (Gustafsson et al 2011). For both lipase and BSA the immobilization occurs faster for higher particle concentrations (see figures 17-19). This is not strange because if the amount of binding sites is increased the protein in question will easier find and bind into to a particular binding site. The higher particle concentration the more binding sites the faster both BSA and lipase immobilize because there are more likely to find a binding site.

The main property that is different between BSA and lipase is the size. BSA is approximately twice the size of lipase in terms of hydrodynamic radius. Both proteins have the same globular shape and thus the diffusion constant as a consequence of the size difference will be larger for lipase. This is the main explanation to the faster immobilization of lipase onto the particles. This is clearly seen when comparing figure 17 (BSA immobilization) and figure 18-19 (lipase immobilization).

Looking at the temperature specific rate constant k(T) it is approximately 10 times higher for lipase (0.8803 $(g/l)^{-1}min^{-1}$) than for BSA (0.0826 $(g/l)^{-1}min^{-1}$). These temperature specific rate constants are based on the assumption that the kinetic model for the immobilization is first order with regard to the protein and particle concentration (see section 2.7). They are extracted as the slope for the straight line fitted to the data points in figure 20 (BSA) and figure 21 (Lipase). In both cases the straight line should go through the origin but this is not the case. One explanation could be that the assumption of first order kinetics is wrong and the model is more complex. Another explanation is that the experimental procedure is not perfect and this gives uncertain and wrong data points, the particle solution preparation could have an effect as an example since the particles aggregate and settle easily. A third explanation could be that the model fitted to the data points in each experiment is too simple or wrong (see equation 15 section 2.7). Looking at the green residual curve in figures 11-12 (BSA) and figures 15-16 (lipase) it doesn't show a random behavior at the beginning of the process and this suggests that the chosen model (eq.15) doesn't explain the immobilization correctly at the start of the process. Generally the model equation (eq.15) fits the data better for BSA and its slower immobilization process. Anyhow the model is probably too simple in both cases and it doesn't describe the whole process properly. A fourth explanation could be that the wrong concentration span is investigated. The experiments should maybe be conducted at lower particle concentrations because that could be the relevant concentration span. The process would be slower at lower particle concentration and therefore easier to study. This is especially relevant for lipase (see figure 15-16) because the increase of intensity is very large

and fast at the beginning of the immobilization process and this results in bad fitting of the model (eq.15) to the corrected data points. Lower particle concentrations however mean lower intensity increase upon immobilization and this means that the process would probably not be detectable with the method used. It is however clear that a straight line appears when the k_n values are plotted against the particle concentration and the immobilization kinetics has been partially described and explained which was the purpose of the report.

An alternative explanation for the faster immobilization for lipase could be the bulkiness and size of BSA. These factors makes BSA more likely to be stuck on the pore wall surfaces and therefore not allowing or slowing down other BSA molecules to get into the pores. Since lipase is smaller and negatively charged they diffuse further into the particles. This means that an immobilized lipase molecule will not hinder other lipase molecules in solution.

5.2. Immobilized Lipase activity assays for studying availability

An important aspect of the availability experiments is what that is actually observed? Is it really the amount immobilized proteins? Since the specific activity is written as per gram enzyme and not per gram immobilized enzyme an important question is how much protein that actually is immobilized? This could be answered by measuring how much enzyme is left in the supernatant after centrifugation of an immobilization reaction mixture. This has been difficult to do since small protein concentrations are used. Based on previously presented results by others (Gustafsson et al 2011) but also looking at the kinetic study is safe to assume that after 24 hours of immobilization at 25°C immobilization equilibrium should have been reached. The only question remaining is how much of the proteins that have been immobilized.

When looking at the lipase activity versus particle to protein ratio the activity increases linearly to a ratio of about 74 (see figure 22). After this point the curve reaches a plateau (see figure 23). An explanation to this behavior is that every lipase immobilized is as available for the substrate as its neighboring lipase. For higher particle concentrations a plateau is reached and therefore there is no reason to have higher particle concentrations then the concentration at the point in which the plateau is reached. Another different explanation for the plateau in figure 23 is that at particle concentrations over the point at which the plateau is reached all lipase that can be immobilize is immobilized and neighboring lipase molecules doesn't disturb the immobilized lipase and therefore the specific activity is constant. Looking at the linear part of the curve (see figure 22), an explanation for this shape is that the higher particle concentration more lipase is immobilized. It has already been shown that higher particle concentrations results in faster immobilization. Another explanation could be that at lower particle concentrations, the environment inside the particle could be too crowded. The enzymes simply hinder neighboring enzymes to hydrolyze pNPA. To know which of these explanation that is correct the free lipase concentration in the supernatant has to be determined.

In the availability experiments in section 4.2 it was assumed that after 24 hours of immobilization equilibrium was reached, this is maybe not the case. A way to test when the equilibrium is reached is to have longer immobilization times over 48-72 hours and compare the specific lipase activities for different immobilization times. This would be interesting because the assumption of 24 hours could be wrong. If this assumption is wrong the linear part of the curve in figure 22 could be explained by how much faster the lipase has immobilized onto particles rather than the more interesting aspect in this study where the increasing specific lipase activity is explained by how much lipase is immobilized and how available each lipase is to the substrate.

When the particle concentration was kept constant and the protein concentration was varied two types of exponentially shaped curves is seen; the curve presented in figure 24 where the specific lipase activity decreases and levels out and the curve in figure 25 where the specific lipase activity increases until a plateau is reached. The only difference between these experiments is that approximately two times lower protein and particle concentrations were used for the experiment presented in figure 25. The protein to particle ratios is however the same in both experiments. The behavior seen in figure 24 can be explained by the fact that it is specific activity that is analyzed. Since the specific activity is per gram protein at the start of the immobilization and not per gram immobilized protein, with increasing protein concentrations the specific activity is expected to decrease. This decrease of specific activity in figure 24 could also be explained by crowding inside the particles. The more protein that are immobilized the more product is produced until a plateau is reached as seen in figure 26. In figure 26 the concentration product is plotted against the protein to particle ratio. When the plateau in figure 26 is reached at the protein to particle ratio of 0.12 the environment could be too crowded and thus not as much substrate is hydrolyzed per gram lipase. This trend in figure 26 is however weak, it is not clear whether it is a plateau or not.

Looking at the behavior seen in figure 25 more lipase results in higher activity until a plateau are reached. One explanation to this plateau is that no more protein can be immobilized to that specific particle concentration (where the plateau is reached). This explanation means that the results don't tell whether the environment inside the particles is too crowded with lipase or not and whether the substrate is as available or not as available to every lipase immobilized. The other explanation is that as the specific activity increases until a point is reached at which the environment is too crowded and the lipases are in the way of each other. This point is at which the plateau is reached. Regardless of explanation the behavior in figure 25 can be looked like an adsorption isotherm for lipase that represents the amount lipase bound inside the particles. These are two different possible explanations for the behavior seen in the results and it remains to explain why the results in figure 24 and 25 do not look the same. To know which explanation that is correct the amount of immobilized lipase has to be determined.

The amount of lipase that actually has been immobilized is important to know because this figure would make it possible to explain the results better. Higher particle and protein concentrations results in more immobilized lipase but the question is whether this increase in amount of immobilized lipase is or is not proportional to the particle or protein concentration increase. Determining the amount of immobilized lipase in each experiment and correlating this to the specific activity makes it possible to explain the results. The economical aspect about how much protein and particle that should be used for as high specific activity as possible has been briefly investigated and the results gives an indication on what particle and protein concentrations that are more economical feasible. For results from these types of experiments to be relevant in future industrial applications of immobilized enzymes, as mentioned before, the amount of immobilized lipase has to be determined.

5.2.1. Co-immobilization of Lipase and BSA

The experiment where both lipase and BSA was used at the same time was performed to see whether the larger BSA molecules hindered or disturbed lipase when it hydrolyses pNPA to pNP (see figure 8 in section 3.2.4). Starting with the results seen in figure 28, adding lipase and BSA at the same time (green staple) gave the highest specific activity. This result is hard to explain especially since the experiments where lipase was immobilized (purple figure 28) showed lower specific activity. One explanation could be that BSA drags lipase with it somehow so that more lipase is immobilized. Besides lipase own immobilization BSA, with its bulkiness, drags additional lipase molecules with it into the particles by interacting with the

lipase molecules (even though both are negatively charged). More lipase is basically immobilized at the same particle concentration with BSA than without. This result also indicates that lack of space inside the particles isn't a relevant issue when BSA is co-immobilized with lipase. Another explanation could be that better packing occurs inside the particles with both lipase and BSA; lipase maybe adopts a more active shape or state in this environment. It also seems that the lipase is as available to the substrate as it was when the BSA wasn't present. It is here again clear that it is not a question of lack of space for lipase inside the particle when BSA is immobilized at the same time, because in that case the specific activity for lipase (purple) should have been higher than for lipase and BSA co-immobilization (green) and this difference should have been profound since a lower concentration of lipase would have been present inside the particles. The two different behaviors can however also be put together to explain the result of co-immobilization. Lipase together with BSA gives a higher specific activity but at the same time there would be a lower concentration lipase inside the particles (if BSA was to take up place so that less lipase could immobilize) and these effects of the co-immobilization could counteract each other. In the end however the winning side would be the increase in specific activity.

In the experiment when the lipase is first immobilized and followed by immobilization of BSA (see red staple figure 28) the specific activity was slightly higher than the experiment that was conducted in the opposite way (blue staple). Maybe the BSA acts as a lock or a door that shuts in the immobilized lipase. The lipase leakage during the course (1 hour) of the lipase activity assay might be minimized with BSA acting like a shut door. Filling the particles with BSA first maybe doesn't leave as much room to fill with lipase (see blue staple figure 28), this lack of space explanation is however not solid and it is contradicted by the results where lipase and BSA were immobilized at the same time (green staple). However comparing the red and blue staple the difference isn't large and not that many conclusions can be drawn. One fact that however strengthens the credibility of the results is that both sample 1 and 2 shows the same trends. Further the result for the experiment where lipase is immobilized first followed by the immobilization of BSA (red staple) indicate that lipase molecules imbedded deep into the particles also hydrolyzes the substrate. This can be assumed because BSA due to its size will not let too many lipase molecules pass through and diffuse out to the edge of the pores (the edge facing the bulk) and hydrolyze the substrate there. The large difference between immobilizing lipase and BSA one at the time (red and blue staple figure 28) and at the same time (green staple) could be due to the increase in specific activity when lipase is packed in between the BSA molecules. Even though it can be assumed that lipase would mix and diffuse in between the BSA molecules if they are immobilized one at the time they would not mix as much or as good if they were to be immobilized at the same time.

The experiment where lipase and BSA was let to immobilize at the same time was repeated and the result is seen in figure 29 (red staple). This time around the specific activity for immobilized lipase was higher (green staple figure 29). The difference between immobilizing only lipase (green staple) and lipase and BSA at the same time (red staple) is not so large. This result could contradict the explanation that BSA makes lipase adopt a more active state. BSA might disturb the lipase. However mixing lipase and BSA at the same time is clearly better than adding them and immobilizing them one at the time.

6. CONCLUSIONS

The conclusions are divided into two sections, one for the immobilization kinetics results in section 4.1 and one for the immobilized lipase assays from section 4.2.

6.1. Investigation of immobilization kinetics

One conclusion is that it was possible to study the immobilization with the new method described in section 3.2.3. As the stained proteins go into the particles a clear increase of fluoresced light intensity is observed and this behavior is possible to study over time. However after 3-4 hours the difference between the sample and the reference decrease due to sedimentation of the particles. This is not a major problem since the interesting part at the beginning of the immobilization process can be studied as shown in the results (see section 4.1).

This method has worked for two types of proteins, both one larger protein (BSA) and for the smaller enzyme lipase. It was not possible to stain lipase as much as BSA because of two reasons. Firstly lipase is smaller; secondly lipase has more than three times less possible binding sites for epicocconone if the possible binding sites are calculated from the amino acid sequence. By altering the settings on the fluorescence spectrophotometer this staining issue could be overcome and the immobilization could also be studied for lipase.

The results from the immobilization kinetics study were logical and expected. Both proteins have the same globular shape. Looking at their charge both have negative net charge since the isoelectric point for both proteins is well below 6 which is the pH at which the immobilization is conducted. Since the main difference between lipase and BSA is the size the diffusion constant will be higher for lipase as a consequence of the smaller hydrodynamic radius.

The t_1 value decreased with increasing particle concentrations and this was the case for both lipase and BSA. This result is also logical since with higher particle concentrations more possible binding sites are available for the protein in question. This means that the protein will find a binding site faster and this result in a lower t_1 value. Also the temperature specific rate constant k(T) was approximately 10 times higher for lipase which is a consequence of the faster immobilization for lipase.

6.2. Immobilized Lipase activity assays for studying availability

The main idea to study the availability by conducting activity assays is to see when there is a limit on how much lipase that can be immobilized and packed into the particles. The hypothesis is that at this limit the environment for the lipase inside the particles would be too crowded. The lipase would have less activity because the substrate will have a more difficult way to the enzyme and because neighboring lipases could disturb the hydrolysis of the substrate.

The results from these experiments were hard to interpret. When the particle concentration was varied and the lipase concentration was kept constant the specific activity increased and leveled out above particle to protein concentrations above 96. When the particle concentration was kept constant and the protein concentration varied two different shapes of curves was seen. The specific activity either decreased and leveled out or increased and reached a plateau. Too crowded environment inside the particle could be the explanation for all of these results.

However it is clear that not all lipase is immobilized. Earlier experiments (Gustafsson et al 2012) show that about a third of the amount of lipase is immobilized. The amount lipase not immobilized would be an interesting figure to know, but since low protein concentrations where used in these availability experiments it was difficult to analyze the left over lipase

concentration in the supernatant. There were some brief attempts using epicocconone to stain protein and measure fluorescence. These attempts were not successful and the results from these experiments are not presented in this report.

Finally the immobilized lipase activity study gave an idea on how much particle and protein that should be used to get as high as possible specific activity but further studies around these particle and protein concentrations need to be conducted. This is of importance for the economical aspect of using immobilized enzymes industrially.

6.2.1. Co-immobilization of Lipase and BSA

At the end of the project a few experiments with both BSA and lipase where performed. Immobilizing BSA together with lipase had effect on the specific activities. Immobilizing lipase first and BSA afterwards gave slightly higher specific activity than conducting the experiment the other way around. Immobilizing lipase and BSA at the same time gave interesting high specific activity. The specific activity didn't decrease as it was assumed. BSA doesn't seem to disturb lipase when they are co-immobilized. All these effect should be further studied by varying the concentration of both the proteins used and the particles.

7. FUTURE PERSPECTIVES

Looking at future perspective there are aspects in short term but also in the long term for using immobilized enzymes to catalyze reactions for a greener chemical industry.

Starting with short term aspects it would be interesting to perform the activity assays so that the amount of free lipase can be determined and this will indirectly give an idea of how much lipase that has immobilized. One of the future perspectives of this research is there for experiments where the same systematic analysis of particle to protein ratio compared to specific activity is conducted at higher protein concentrations so the protein concentration of free proteins left in the supernatant could be measured. To understand the progress better the supernatant (containing free lipase) after the centrifugation after the immobilization could be analyzed to measure protein concentration either with fluorescence of epicocconone or with absorbance of the lipase. The fluorescence method has a lower detection limit whilst measuring the concentration at 280nm with absorbance spectroscopy is more sensitive if high enough lipase concentrations are present in the supernatant. The fluorescence method is more suitable for larger proteins as BSA since more epicocconone can bind in. Because lipase is a smaller protein with less binding sites (amine groups) for epicocconone the sensitivity is not too good. A way to overcome these sensitivity problems is thus, to use higher concentrations of lipase and particles to increase the detection while at the same time having the same protein to lipase ratios in the experiments.

The experiment where BSA and lipase are used at the same time showed some interesting results. Mixing enzymes and immobilizing them in different orders is an aspect in this area of research to further investigate. Additional proteins than lipase and BSA can also be used. Other enzymes and proteins can be used to enhance activity, shut in enzymes (like in the case of BSA). Varying the concentration of the particles and especially the proteins used could also give interesting results.

Talking generally, other experimental conditions could be varied in both types of experiments, both the immobilization kinetic investigation and the availability experiments. On example could be the effect of temperature which is always interesting when studying kinetics but also for enzyme activity. Effect of pH can also be further studied especially in the immobilization kinetics experiments.

Finally looking at long term, conducting experiments with chains of enzyme catalyzed reaction would be relevant. Shorter metabolic reaction pathways catalyzed by a couple of enzymes could be studied. The enzymes could be immobilized in separate particles or into same particles. Immobilizing them into the same particles would give an environment similar to the one inside a dense cell. The different enzymes in the same metabolic pathway would be close to each other and this could enhance the total reaction rate. The enzymes could be immobilized in different order or at the same time. The various reaction steps could be performed in a reaction tube each with one substrate at the time or in one single reaction tube with only the primary substrate added at the start. Different enzymes not part of the same metabolic pathway can also be immobilized together. Adding respective enzyme substrate and measuring activity could say something about how the environment inside the particle is effected by the co-immobilization.

As with all research the possibilities are infinite but the latter aspect of using several enzymes in a metabolic pathway is a relevant aspect that could be of special interest for the industry.

8. ACKNOWLEDGMENTS

I would like to thank my supervisor Nils Carlsson for his help and guidance throughout this project. The discussions with my supervisor have been very good, educational and have helped me understand how research is conducted. I would also like to thank Björn Åkerman for the opportunity to let me conduct my master thesis at the division of Chemistry and biochemistry.

9. REFERENCES

Atkins, P. de Paula, J. Friedman, R. (2009). *Quanta, Matter, and Change: A molecular approach to physical chemistry*. Oxford New York: Oxford University Press. Chapter 10 and 11.

Banerjee, U C. Chisti, Y. Sharma, R. (2001) Production, purification, characterization, and applications of lipases. *Biotechnology Advances*, vol. 19, no 8, pp. 627-662.

Bell, P. Karuso, P. (2003) Epicocconone, A Novel Fluorescent Compound from the Fungus *Epicoccum nigrum*. *Journal of the American Chemical Society*, vol. 125, no 31, pp. 9304-9305.

Carlsson, N. (2011) *Implications of light scattering and secondary structure in protein concentration determination*. Gothenburg: Chalmers University of technology. (Licentiate Thesis within the Department of Chemistry and Biotechnology).

Ge, S. Kojio, K. Takahara, A. Kajiyama, T. (1998) Bovine serum albumin adsorption onto immobilized organotrichlorosilane surface: influence of the phase separation on protein adsorption patterns. *Journal of Biomaterials Science. Polymer Edition*, vol. 9, no 2, pp. 131-150.

Gerngross, T U. (2004) Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. *Nature biotechnology*, vol. 22, no 11, pp. 1409-1414.

Gustafsson, H. (2012) *Enzyme immobilization in mesoporous silica*. Gothenburg: Chalmers university of technology. (Licentiate Thesis within the Department of Chemistry and Biotechnology).

Gustafsson, H. Thörn, C. Holmberg, K. (2011) A comparison of lipase and trypsin encapsulated in mesoporous materials with varying pore sizes and pH conditions. *Colloids and Surfaces B: Biointerfeces*, vol. 87, no 2, pp. 464-471.

Harris, D. (2010). *Quantitative Chemical Analysis*. 8th ed. New York New York: W. H. Freeman. Chapter 17.

Joseph R. Lakowicz. (2006). *Principles of Fluorescence Spectroscopy*. 3rd ed. New York New York: Springer. Chapter 1-3.

Karuso, P. Coghlan, D. Mackintosh, J. (2005) Mechanism of Reversible Fluorescent Staining of Protein with Epicocconone. *Organic letters*, vol. 7, no 1, pp. 2401-2404.

Linko, Y-Y. Jääskeläinen, S. Wu, X,Y. (1996) An investigation of crude lipases for hydrolysis, esterification, and transesterification. *Enzyme and Microbial Technology*, vol. 19, no 3, pp. 226-231.

Liu, G. Li, Y. Liu, Y. Shangguan, D. Shen, B. Yu, X. Zhang, H. Zhang, W. Zhao, R. (2004) Quartz crystal biosensor for real-time kinetic analysis of interaction between human TNF-α and monoclonal antibodies. *Sensor* & *Actuators: B. Chemical*, vol. 99, no 2, pp. 416-424.

Mackintosh, J. Veal, D. Karuso, P. (2005) Fluoroprofile, a fluorescence-based assay for rapid and sensitive quantitation of proteins in solution. *Proteomics*, vol. 5, no 18, pp. 4673-4677.

Malmsten, M. (1994) Protein Adsorption at Phospholipid Surfaces. *Journal of Colloid And Interface Science*, vol. 172, no 1, pp. 106-115.

Mathews, C. van Holde, K E. Ahern, K (2000). *Biochemistry*. 3rd ed. San Francisco: Addison Wesley Longman. Chapter 15.

McDonald, K. Huang, T. (2012) Bioreactor systems for *in vitro* production of foreign proteins using plant cell cultures. *Biotechnology Advances*, vol. 30, no 2, pp. 398-409.

Murphy, C. (2012) The microbial cell factory. *Organic & biomolecular chemistry*, vol. 1, no 1, pp. 1949-1957.

Peters, T. (1975). Putman FW. ed. *The Plasma Proteins*. Academic Press. pp. 133–181.

Skagerlind, P. Bergenståhl, B. Hult, K. Jansson, M. (1995) Binding of *Rhizomucor miehei* lipase to emulsion interfaces and its interference with surfactants. *Colloids and Surfaces B: Biointerfaces*, vol. 4, no 3, pp. 129-135.

Tamiya, E. Endo, T. Morita, Y. Nagatani, N. Takamura, Y. Yamamura, S. (2005) Localized surface plasmon resonance based optical biosensor using surface modified nanoparticle layer for label-free monitoring of antigen-antibody reaction. *Science and Technology of Advanced Materials*, vol. 6, no 5, pp.491-500.

Tamiya, E. Endo, T. Hiepa, H M. Kerman, K. Kim, D-K. Nagatani, N. Nakano, K. Yonezawa, Y. (2006) Multiple Label-Free Detection of Antigen-Antibody Reaction Using Localized Surface Plasmon Resonance-Based Core-Shell Structured Nanoparticle Layer Nanochip. *Analytical chemistry*, vol. 78, no 18, pp. 6465-6475.

Thörn, C. Gustafsson, H. Olsson, L. (2011) Immobilization of feruloyl esterases in mesoporous materials leads to improved transesterification yield. *Journal of Molecular Catalysis B: Enzymatic*, vol. 72, no 1, pp. 57-64.

Vulfson EN. Industrial applications of lipases. In: Woolley P, Peterson SB, editors. *Lipases—their structure,biochemistry and applications*. Cambridge: Cambridge Univ. Press, 1994. pp. 271–88.

Wright, A.K. Thompson, M.R. (1975) Hydrodynamic structure of Bovine Serum Albumin determined by transient electric birefringence. *Biophysical Journal*, vol. 15, no 2, pp. 137-141.

Wurm, Florian M. (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature biotechnology*, vol. 22, no 11, pp.1393-1398.

Yohannesa, G. Wiedmera, S. Elomaab, M. Jussila, M. Aseyevb, V. Riekkolaa, M-J. (2010) Thermal aggregation of bovine serum albumin studied by asymmetrical flow field-flow fractionation. *Analytica Chimica Acta*, vol. 675, no 2, pp. 191-198.