Dynamics of Enzymes at Interfaces
- lipase adsorption and mobility on solid surfaces

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Doctoral Thesis
Stockholm, Sweden 2007
Andreas Sonesson. *Dynamics of enzymes at interfaces – lipase adsorption and mobility on solid surfaces.*

Akademisk avhandling som med tillstånd av Kungliga Tekniska Högskolan framlägges till offentlig granskning för avläggande av teknologie doktorsexamen måndagen den 23 april 2007 kl. 10.00 i sal FB53, AlbaNova, KTH.

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Front: The motion of a single Quantum Dot-lipase molecule on a triglyceride substrate surface.
ABSTRACT

This thesis aimed to give more insight in the dynamics of enzymes at interfaces. The adsorption and mobility of adsorbed proteins can e.g. give a better understanding of structure-function properties of interfacially active enzymes. Studied enzyme was the lipase from *Thermomyces lanuginosus* (TLL).

Adsorption of TLL to surfaces of different hydrophobicity was studied by Dual Polarization Interferometry (DPI), Surface Plasmon Resonance (SPR) and ellipsometry. It was found that TLL had highest affinity and adsorbed to largest adsorbed amount on a hydrophobic, C18 terminated surface. Moreover, activity studies of adsorbed TLL suggested that a larger fraction of the lipases were orientated with the active site facing the surface on hydrophobic surfaces.

Mobility of adsorbed enzymes was studied by means of Fluorescence Recovery After Photobleaching (FRAP) with Confocal Laser Scanning Microscopy (CLSM). CLSM was also used as a tool to image the role of TLL in the detergency of lipids from single cotton fibers. The TLL surface mobility was measured on model surfaces of different hydrophobicity. The rate of TLL surface diffusion was strongly dependent on the surface density of lipase, which was explained by sterical hindrance and intermolecular repulsion. The diffusion was both lowest and decreased as a function of time after adsorption on the most hydrophobic surface. This was thought to be due to a larger fraction of adsorbed TLL oriented with the active site towards the hydrophobic surface and that this fraction increased as a function of time.

The presence of surfactants affected the TLL mobility on hydrophobic surfaces. The diffusion increased more than tenfold when TLL was coadsorbed with C_{12}E_{6}/LAS above the critical micellar concentration (cmc) of the surfactant. This was thought to be due to a surfactant induced desorption-rebinding mechanism of TLL. Total Internal Reflection Fluorescence Correlation Spectroscopy (TIR-FCS) supported this theory and was implemented as a technique to quantify kinetic processes of protein-surfactant interactions at surfaces.

The surface mobility of TLL was higher on a trimyristin substrate surface compared to the model hydrophobic surface. Single particle tracing of lipases could be performed by conjugation of TLL to Quantum Dots (QDs). The microscopic behavior of QD-lipases on trimyristin suggested that the enzyme operated in two different modes on the surface, which gave the trajectories of single lipase molecules a “bead on a string” appearance.
LIST OF PAPERS

The thesis is based on the following papers:

I. Imaging the detergency of single cotton fibers with confocal microscopy – the effect of surfactants and lipases
   Andreas W. Sonesson, Thomas H. Callisen, Ulla M. Elofsson, and Hjalmar Brismar
   Submitted for publication

II. A comparison between Dual Polarization Interferometry (DPI) and Surface Plasmon Resonance (SPR) for protein adsorption studies
    Andreas W. Sonesson, Thomas H. Callisen, Hjalmar Brismar, and Ulla M. Elofsson
    Colloids and Surfaces B: Biointerfaces, 2007, 54, 236-240

III. Adsorption and activity of Thermomyces lanuginosus lipase on hydrophobic and hydrophilic surfaces measured with dual polarization interferometry (DPI) and confocal microscopy
     Andreas W. Sonesson, Thomas H. Callisen, Hjalmar Brismar, and Ulla M. Elofsson
     Submitted for publication

IV. Lipase surface diffusion studied by Fluorescence Recovery After Photobleaching
    Andreas W. Sonesson, Thomas H. Callisen, Hjalmar Brismar, and Ulla M. Elofsson
    Langmuir 2005, 21, 11949-11956

V. Adsorption and mobility of a lipase at a hydrophobic surface in the presence of surfactants
   Andreas W. Sonesson, Ulla M. Elofsson, Hjalmar Brismar, and Thomas H. Callisen
   Langmuir 2006, 22, 5810-5817

VI. Protein-surfactant interactions at hydrophobic interfaces studied with Total Internal Reflection Fluorescence Correlation Spectroscopy (TIR-FCS)
    Andreas W. Sonesson, Hans Blom, Kai Hassler, Ulla M. Elofsson, Thomas H. Callisen, Jerker Widengren and Hjalmar Brismar
    Submitted for publication

VII. Mobility of Thermomyces lanuginosus lipase on a trimyristin substrate surface
     Andreas W. Sonesson, Hjalmar Brismar, Thomas H. Callisen, and Ulla M. Elofsson
     Langmuir, 2007, 23, 2706-2713

VIII. Tracking single lipase molecules on a trimyristin substrate surface using Quantum Dots
      Andreas W. Sonesson, Ulla M. Elofsson, Thomas H. Callisen, and Hjalmar Brismar
      Submitted for publication

The author has done all of the experimental work and writing of Papers I-V and VII-VIII. In Paper VI, the experimental work was done together with Dr H. Blom and writing together with Dr H. Blom and Dr K. Hassler.
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1. Introduction

Proteins and enzymes that are present or function at interfaces are of great importance in nature. Membrane-water interfaces are a main constituent of cells; 1 cm³ tissue consists of roughly 30 m² of phospholipid bilayer structure. Moreover, about 50 % of all proteins that are synthesized become membrane-associated and many important functions and metabolic pathways governed by proteins take place at the interface, such as the photosynthesis, post-translational modifications of proteins and lipid metabolism. This work focused on one type of interfacial enzymes called lipases, which are present in almost all type of organisms and catalyze the digestion of lipids in complex environments. As an example, pancreatic lipases must work on the interface of emulsion particles, consisting of a core of dietary di- and triglycerides covered with e.g. polar lipids and bile salts. Almost all lipases are activated by the interface but the interfacial catalysis mechanisms are far from fully understood. Interfacial turnover by lipases cannot be described by conventional Michaelis-Menten kinetics, due to complex equilibria between enzyme, substrate, products and difficulties in defining the substrate concentration in the interface etc. A well-studied lipase is secreted phospholipase A₂ (PLA₂) that performs the initial digestion of phospholipid compounds. The PLA₂ surface dynamics has been analyzed in terms of two modes of interactions between the enzyme and the surface, which in the PLA₂ case is phospholipids vesicles: hopping, where the enzyme is able to leave the interface between catalytic cycles or scooting, where the enzyme is trapped at the interface after adsorption. Thus, in the scooting mode, substrate turnover only occurs at the vesicle interface to which the lipase binds initially, whereas in hopping mode, all vesicles will be hydrolyzed to roughly the same extent during the reaction.

This project aimed to gain more insight in the interfacial catalysis by lipases. The lipases studied were variants of the lipase from the fungus Thermomyces lanuginosus (TLL), an enzyme with pronounced interfacial activation. Studied properties of this enzyme were the dynamics at different interfaces, in terms of adsorption and surface mobility, the latter being a complex subject that has received very little attention in the literature. TLL has important technical applications since it is an important component in detergent formulas, where it is added to aid in the removal of triglyceride stains from textile surfaces. Enzymes with fine-tuned performance allow washing at lower temperatures, provide fabric care and reduce washing time and water consumption. In the detergency process, the enzyme needs to function optimally in a complex-, and for the lipase, unfriendly environment, e.g. together with proteolytic enzymes (proteases) and at high surfactant concentrations. Thus, more detailed insight in structure-function properties of
lipases and how mobility of the enzyme is correlated with activity could aid in the development of new lipase variants that work optimally in different environments and on different surfaces.

The project had three major objectives

- study adsorption and mobility of TLL on hydrophilic and hydrophobic model surfaces and see the effect of surface crowding and time after adsorption on the lateral mobility of the enzyme.
- study adsorption and mobility of TLL when coadsorbed with surfactants. Proteins and surfactants are known to interact both in solution and at interfaces, but little is know how it affects the surface mobility of the protein.
- study adsorption and mobility of TLL on triglyceride substrate surfaces and relate activity to mobility of the enzyme.

Except for the techniques used to measure adsorption to surfaces (Dual Polarization Interferometry, Surface Plasmon Resonance and Ellipsometry) a major part of the studies was performed with Confocal Laser Scanning Microscopy (CLSM), a method commonly used in cell biology and biophysics. Thus, a minor objective of the project were to implement this tool to address problems that arise in surface chemistry. This mainly involved fluorescence recovery after photobleaching (FRAP) of proteins on surfaces, which was used to quantify the long-range ensemble diffusion coefficient of the studied protein. Moreover, confocal microscopy was used for single molecule tracking of lipases on substrate surfaces using Quantum Dots (QDs) and for imaging the role of lipases in the detergency of single cotton fibers soiled with different lipids. Total Internal Reflection Fluorescence Correlation Spectroscopy (TIR-FCS), a technique with single molecule sensitivity, was implemented as a tool to quantify kinetics of protein-surfactant competition for binding sites on surfaces.
2. Background

2.1 Lipases

Lipases are enzymes classified as glycerol ester hydrolases (E.C 3.1.1.3) and can be found in the human blood plasma, pancreas, as well as in different bacteria and moulds. The primary substrate for glyceride lipases is triglycerides, the main constituents of vegetable oil and animal fat. Lipases catalyze the hydrolysis of triglycerides and partial glycerides to free fatty acids and glycerol. There is a high degree of sequence homology between lipases from different species and these enzymes are crucial for lipid digestion, e.g. hydrolyzing dietary triglycerides\(^7\),\(^8\), but also involved in lipid mediated cell-signalling\(^9\). Lipases in the gastric and pancreatic juices function at the surface of lipid emulsions, and regulating the activity of these enzymes can help to control obesity and aid in the treatment of cystic fibrosis\(^7\). There is a broad field of technical applications of lipases, extending from the food industry to fats and oil production, but they are most widely used as a technical enzyme in modern detergency\(^10\).

A majority of lipases display so-called interfacial activation, leading to kinetics that cannot be explained by the usual Michaelis-Menten theories. Interfacial activation relates to the dramatic increase in catalytic turnover of lipases at a substrate interface or when the critical micellar concentration of a water soluble substrate is exceeded\(^10\),\(^11\). This is mainly explained by a conformational rearrangement in the protein structure that takes place at the interface\(^12\). With a few exceptions, an amphiphilic lid of various size (5-23 amino acids) covers the active site in aqueous solution, making it inaccessible to substrates. Upon association with the surface, the lid domain rolls over and exposes the hydrophobic active site region. Therefore, lipases are found in two different structural conformations; the closed form when the lid covers the active site and the open form, when the active site is accessible to substrates (Fig. 1A and B). Organic solvents may also facilitate a transfer from the closed to the open form, due to lowering of the dielectric constant\(^12\). The active site of lipases consists of a catalytic triad (Ser, Asp, His), similar to that of serine proteases\(^13\). Due to the three-dimensional alignment of the active site, the serine residue is activated by hydrogen bonding to histidin. Activated serine then performs a nucleophilic attack on the carbonyl bond of the substrate, which initiates the catalytic cycle (Fig. 1C).
Figure 1. Three-dimensional structure of the closed (A) and open (B) form of Thermomyces lanuginosus lipase. (C) Alignment of oleic acid in the active site. Images generated from the coordinates in the pdb files 1tib and 1gt6 available in the Protein Data Bank (http://www.rcsb.org/pdb/home/).
2.2 Proteins at solid surfaces

2.2.1 Protein adsorption

In almost all systems where a surface is exposed to an aqueous solution containing proteins, a spontaneous accumulation of proteins at the interface occurs, regardless of the specific properties of the surface or the protein. Protein adsorption alters the characteristics of the surface and often induces structural rearrangements in the protein tertiary structure. Therefore, protein adsorption is relevant in a variety of fields, such as the food industry and in medical science. Specific examples are blood coagulation and complement activation on implant materials\textsuperscript{14}, interfacial catalysis by enzymes, ion-exchange chromatography and biosensor technology\textsuperscript{15,16}

Protein adsorption from aqueous solutions to solid surfaces is the net result of several combined interactions in which the protein, the surface and the aqueous medium are involved. Proteins will adsorb to the surface if the change due to adsorption in Gibbs free energy of the system ($\Delta_{\text{ads}} G$) < 0 (see Eq. 1)\textsuperscript{16}

\[
\Delta_{\text{ads}} G = \Delta_{\text{ads}} H - T\Delta_{\text{ads}} S < 0
\]  

(1)

$H$ is the enthalpy of the system, $S$ is the entropy and $T$ the temperature. When proteins adsorb at interfaces, the enthalpy changes are usually very small, and hence, the increase in entropy is the factor dominating the adsorption process. There are three main driving forces for protein adsorption\textsuperscript{15,16}:

i) **hydrophobic interactions**

Dehydration of hydrophobic regions of both protein and surface contributes significantly to an increase in entropy and thus to the adsorption driving force. In an aqueous environment, globular protein tend to be folded with their hydrophobic regions buried in the interior, but the protein surface will always exhibit both hydrophilic and hydrophobic patches. The entropy penalty for hydrating non-polar patches is high, and adsorption of the protein to an interface is therefore one mechanism to increase the entropy of the system.
ii)  

*electrostatic interactions*

Interactions between charges on the surface and charged groups or dipoles on the protein affect the adsorption. In solution, most interfaces carry electrical charge due to covalently bound surface groups or specific adsorption of low molecular weight ions. Adsorption of charged proteins will therefore result in electric attraction or repulsion. However, since charged proteins have been found to adsorb on surfaces of similar charge, hydrophobic dehydration might dominate over electrostatic forces. The maximum adsorption of proteins on surfaces is pH-dependent, i.e. dependent on the charge of the protein. The maximum adsorbed amount is often found at a pH around the isoelectric point (pI) of the protein, when the net charge of the protein is zero. At this pH, lateral repulsion will be minimal, allowing a maximum packing density of the adsorbed protein layer.

iii)  

*conformational changes in protein structure*

Interactions with a surface might induce changes the three-dimensional structure of a protein. In aqueous solution, hydrogen bond formation between peptide units in the polypeptide chain and dehydration of hydrophobic amino acid residues are the most important factor that determines the protein structure and stability. Hence, the forces that favor protein folding outweigh the large loss in conformational entropy that occurs upon folding. However, when adsorbing to an interface, parts of the protein structure might interact with the surface, e.g. by forming hydrogen bonds with a hydrophilic surface. In this way, proteins can increase the conformational entropy without exposing hydrophobic residues to solution.

Protein adsorption is strongly irreversible, i.e. diluting the proteins in bulk solution does usually not lead to any desorption from the surface, which would be expected of a system in equilibrium. Adsorbed proteins bind to the surface with many segments, and a large driving force would be required for all to desorb simultaneously, something that bulk-dilution rarely leads to. However, other types of proteins or other surface active molecules might replace adsorbed proteins. In competitive adsorption of different proteins, the surface will first be covered by the protein with the highest rate of arrival, i.e. dependent on the concentration and diffusion rate, which is proportional to the hydrodynamic size of the protein. In general, this means that if the concentrations are similar, the protein of lowest molecular weight will initially adsorb. This protein may later be replaced, and in the end the protein of highest molecular weight will most likely dominate the surface.\textsuperscript{15,17}
The most common way to present data of protein adsorption is with an adsorption isotherm. In
the isotherm, the adsorbed amount of protein at constant temperature is plotted as a function of
concentration of protein in the bulk solution. Protein adsorption isotherms usually reach plateau
values, i.e. when a maximum packing density on the surface has been reached. The affinity of the
protein to the surface is reflected in the initial slope of the isotherm.16

### 2.2.2 Mobility of adsorbed proteins

The structure of a protein adsorbed at a solid-liquid interface might relax and alter the properties
of the protein layer with time after adsorption18,19. For example, it has been shown that the small
enzyme lysozyme reorient on a solid surface as the adsorbed protein layer exceeds a critical
surface coverage20. This suggests that proteins that remain longer on a surface might obtain
several different interfacial structures and orientations21. Other studies has shown that irreversible
adsorbed polymers22 and proteins23 indeed can be mobile and migrate on solid surfaces. For
proteins, the first observation was reported in the early 1980’s when it was found that adsorbed
bovine serum albumin (BSA) could migrate on a coverslip glass surface without re-entering the
liquid bulk phase24. Further work with different proteins on a variety of surfaces has since been
reported, and is reviewed elsewhere23. The lateral diffusion is thought to enable efficient packing
of an adsorbed protein layer and thereby facilitate a high surface concentration.

Diffusion results from the randomized movement of particles due to their thermal energy. On a
microscopic level, collision between particles and surrounding medium result in a Brownian
motion of each particle and the mean velocity is dependent on both the absolute temperature and
size of the particle. For an entire population of moving particles, this will lead to a net flow of
particles from regions of high concentration to low concentration. All diffusion processes are
described by the diffusion equation, i.e. Fick’s second law of diffusion, which in one dimension
reads:25.

\[
\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \tag{2}
\]

The diffusion equation describes the space-time evolution of a diffusive species with
concentration \(c(x,t)\) (for one dimension). \(D\) is the diffusion coefficient and is dependent on both
the particles and the medium through which particles diffuse. Assuming a spherical diffusing
particle, $D$ can be related to radius ($r$), temperature ($T$) and viscosity ($\eta$) of the medium through which diffusion occurs using the Stokes-Einstein relation (Eq. 3):

$$D = \frac{kT}{6\pi r\eta}$$  \hspace{1cm} (3)

Microscopic models of a one-dimensional walk can estimate the time it takes for the particles to diffuse a given distance (Eq. 4). If $t_{1/2}$ is the time it takes for 50% of the particles to travel a distance of at least $x_{1/2}$, then

$$t_{1/2} = \frac{x_{1/2}^2}{D}$$  \hspace{1cm} (4)

$D$ is thus used to compare different diffusion processes. For example, oxygen diffusing in air has a $D$ in the order of $10^{-1}$ cm$^2$/s and a protein in water about $10^{-6}$ cm$^2$/s. Proteins diffusing at an interface, confined in two dimensions, have lower diffusion rates. Protein diffusion on solid surfaces have been found to be a very slow process, with some systems partially, and in some cases, even totally immobile. For mobile systems, $D$ is reported in the range $10^{-11} - 10^{-8}$ cm$^2$/s which is some order of magnitude slower than protein diffusion in a lipid bilayer, a process more to consider as a diffusion in a two-dimensional liquid with $D = 10^{-7} - 10^{-8}$ cm$^2$/s. It has been found that protein properties, e.g. conformational flexibility and molecular weight, combined with surface properties, e.g. hydrophobicity and charge, affect the protein mobility on solid surfaces but the exact mechanism is not well explained. Adsorbed proteins are often tightly bound to the surface, and diffusion requires many physisorbed segments of the protein to move. Thermal fluctuations of the polypeptide chain might give the protein a crawling motion with segments of the protein constantly attached to the surface during the migration.

Theoretical models of two-dimensional diffusion indicate that the diffusion is strongly dependent on surface density of particles. These models suggest that as the density of particles increases, the probability of finding a vacant position nearby decreases. Moreover, a system with distance-dependent interparticle forces leads to a decrease in diffusion rate as the surface density of particles increase. Brownian dynamics simulations of protein mobility on solid surfaces do also support a lower relative diffusion rate as the surface area fraction covered by protein increases. Therefore, in order to compare different proteins at different surfaces, the ideal method is to measure the mobility of single protein molecules or to do repeated measurements on surfaces.
with different densities of proteins, enabling an extrapolation to zero surface density ($D_0$). Unhindered diffusion can only occur when there are no particle interactions, i.e. the diffusion coefficient at infinite surface dilution $D_0$ is only dependent on protein-surface interactions.

An important factor in surface diffusion is the immobile fraction of the adsorbed protein population. The surface diffusion coefficient describes the dynamics of the adsorbed protein while the immobile fraction might be an indication of the distribution of adsorbed orientations. No reported system of proteins on surfaces has displayed a 100% mobile fraction,$^{23}$ and the immobile fraction is thought to arise from different orientations on the surface or a clustering of proteins, rendering them immobile.$^{23, 27}$

### 2.3 Protein-surfactant interactions

#### 2.3.1 Interactions in solution and at interfaces

Surfactants (surface-active agents) are molecules with a tendency to adsorb at interfaces. The driving force for adsorption is to reduce the interfacial tension. Surfactants are amphiphilic and are categorized into ionics or nonionics, depending on the nature of the hydrophilic part. An important characteristic is the critical micellar concentration (cmc), the concentration at which unimers start to aggregate into micelles in solution. Micelle formation reduces the hydrocarbon-water contacts of the alkyl chain of the surfactant, thereby reducing the free energy of the system.$^{37}$

Surfactants and proteins interact with each other, both in solution and at interfaces. Protein-surfactant interactions have broad significance e.g. in lipid-protein interactions in biological membranes and in technical applications such as food chemistry, detergency formulations, gel electrophoresis and enzyme catalysis in microemulsions.$^{38}$

In solution, low concentrations of ionic surfactants complex with most proteins, which can be shown indirectly e.g. by surface tension or viscosity measurements.$^{39}$ Aggregation with surfactants might affect protein conformational stability$^{37, 40}$ and the hydrophobicity of the protein surface$^{41, 42}$. Higher surfactant concentrations might lead to partial or complete unfolding of the tertiary protein structure$^{40, 43}$. Destabilization is thought to arise from electrostatic repulsion between charged surfactants that have bound to the protein and penetration of the hydrophobic parts of surfactants into the protein structure, thereby destroying stabilizing hydrophobic interactions
between amino acid residues. Different types of structures are proposed for the protein-surfactant complex, but the most common is the “bead on a string” model, in which micelle-like aggregates are formed along the polypeptide chain. Unlike ionic surfactants, nonionic surfactants usually bind weakly to proteins. The explanation for this is the low cmc of nonionic surfactants, making micelle formation more favorable than binding to the protein.

Surfactants can influence the surface activity of proteins and enzymes in several ways. By simply accumulating at interfaces, surfactants might affect properties such as charge and hydrophobicity of the surface and facilitate wetting of oily substrates. Under competitive adsorption to an interface, protein-surfactant interactions can either lead to complete hindrance of adsorption, reduced protein adsorption or in some cases, the protein-surfactant complex becomes more surface active. The latter could be due to a more surface active surfactant-protein complex compared to the bare protein or a more efficient packing of the protein at the interface, due to charge neutralization that reduces lateral repulsion.

Sequential adsorption of protein and surfactant tend to displace a preformed protein layer. Studies with different surfactants and protein systems suggest that a certain surfactant threshold concentration is required for protein removal. Protein displacement is proposed to occur by two mechanisms; replacement of the protein at the surface by the surfactant or surfactants binding to the protein and thereby solubilizing it. For example, removal of β-lactoglobulin by surfactants at both solid and air-water interfaces have been found to be initialized at heterogeneities or defects in the protein layer, where surfactants can adsorb and expand, thereby compressing the protein network until it desorbs from the surface.

### 2.3.2 Detergency mechanisms

One important technical application where surfactant and protein interactions are important is the detergency of fabric. Detergency is a complex problem because the textile substrate might be yarns spun from different fibers, e.g. polyester-cotton mixtures, and some part of the fabric might be treated with dye. Moreover, the soil might originate from different sources, e.g. protein stains from blood and lipid stains from chocolate. Therefore, detergent formulations consist of several individual components; surfactants and enzymes that actively remove the soil but also additives such as hardness sequestering agents (complexing agents, builders), bleach and different polymers. Hence, enzymes must be stable and active in a potentially unfriendly environment.
Three processes are considered as the major mechanisms for removal of oily soils from a surface (Fig. 2):

- Roll up, i.e. lifting of the entire soil particle due to wetting of the surface by surfactants, which reduces the surface-water interfacial tension. The contact angle between soil and surface gradually increase as the soil is rolled up. Roll-up is more often achieved when the contact angle of the oil to the surface is $>90^\circ$, e.g. on polar textiles such as cotton.

- Emulsification (necking) of the soil due to a reduction in interfacial tension between the oil and the surfactant solution. The contact angle between soil and surface remains constant during the necking process. Emulsification mechanisms are usually independent of the surface.

- Solubilization of the soil into e.g. a microemulsion in solution. This mechanism is also independent of the type of underlying surface.

Roll up has been seen as the predominant mechanism in oily soil removal, whereas the others are considered predominant under special conditions, e.g. at low temperatures.

![Figure 2. Three different mechanisms for oily soil removal. (A) Roll-up (B) Emulsification (necking) (C) Solubilization.](image)

Surfactants are the principal active and single most important component of all detergent formulations. The most important function is to lower surface tensions and a combination of anionic and nonionic surfactants are commonly used. Anionic surfactants have fast kinetics in establishing equilibrium interfacial tensions and are preferred for removing particulate soil and suspending the soils in the solution by electrostatic stabilization. Nonionic surfactants, on the other hand, have slower kinetics but achieve very low absolute interfacial tensions. They are most efficient at removing oily soil by solubilization and emulsification. Hence, by using a combination of the two, both fast kinetics and low interfacial tensions are achieved. Common anionic surfactants used in detergent formulations are primary alcohol sulphates (PAS) or linear...
alkylbenzene sulfonates (LAS). Nonionics are often alcohol ethoxylates, $C_nE_m^{37,53}$. Requirements that need to be fulfilled for an efficient detergency mechanism is a surfactant concentration above the cmc and a temperature at the phase inversion temperature (PIT) of the oil-water-surfactant mixture. This will lead to a three-phase system with a microemulsion in equilibrium with excess oil and water, which results in extremly low interfacial tension of the microemulsion towards both oil and water and thus maximum solubilization of the soil$^{37,53}$.

Enzymes were first introduced in detergent formulations in the 1980’s. This was because enzymes efficiently remove some types of soil that can not be solubilized by surfactants, such as triglycerides and proteinaceous stains. Due to enzyme activity, these stains are transformed into smaller fragments that are more easily solubilized. Moreover, from an environmentally point of view, enzymes have contributed to lower water consumption, washing at lower temperatures and providing care of the fabric. The major classes of enzymes used are proteases to remove protein stains, amylases for removal of starch, cellulases to maintain quality of the cotton and lipases for removal of triglyceride stains. The presence of nonionic and anionic surfactants may affect the activity of detergency enzymes. As an example, lipases are suggested to be mostly inactive during the first wash cycle although still able to adsorb on the soil surface. During subsequent cycles, when the water content of the fabric and surfactant concentrations are reduced, lipases are more activate and can hydrolyze the substrate$^{5,54,55}$.
3. Materials

3.1 Lipases, surfactants and buffers

The lipases studied were from the fungus *Thermomyces* (formerly *Humicola* *lanuginosus*) (TLL). TLL is a lipase with pronounced interfacial activation and was the first lipase used in a detergent formulation, called Lipolase®.

Two different variants of TLL were used, denoted in this text as TLL-α and TLL-β, and were provided by Novozymes A/S, Bagsvaerd, Denmark. The difference between the two variants was that TLL-β was less charged than TLL-α at the studied pH (pH 9). TLL-α was also modified to allow a well-defined labeling of a fluorophore. This was done with either AlexaFluor 488 or fluorescein isothiocyanate (FITC) purchased from Molecular Probes (Leiden, The Netherlands). Labeling procedures were performed according to the protocols given by the manufacturer (www.probes.com) with the exception that TLL-α was left to react with the probe overnight in +4 °C. All labeling procedures led to a fluorophore/lipase ratio < 1. In Paper VIII, TLL was labeled with PEG-coated CdSe-ZnS Quantum Dots (QDs), using the protocol suggested by the manufacturer (www.evidenttech.com). The QDs had a hydrodynamic diameter of ~25nm and were functionalized so they could react with amine- or carboxyl terminal groups on the protein.

The activity of the two lipase variants was measured towards a fatty acid ester (Paper VII) both before and after Alexa 488 labeling (see Table 1).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLL-α</td>
<td>0.49</td>
</tr>
<tr>
<td>TLL-α-Alexa488</td>
<td>0.26</td>
</tr>
<tr>
<td>TLL-β</td>
<td>1.00</td>
</tr>
<tr>
<td>TLL-β-Alexa488</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Hence, TLL-β was most active in its native form, but lost all activity after fluorophore labeling. TLL-α was studied in Papers II, V, VII, VIII and TLL-β in Papers I, III, IV, VI, VII.
Surfactants used were the nonionic C₁₂E₆ (hexaethylene glycol mono n-dodecylether) and the anionic LAS (linear alkylbenzene sulphonate) at a molar ratio of C₁₂E₆/LAS (1:2 mol%). The surfactants were used as a model system for the main fraction of surfactants in common household detergent formulations.

The majority of lipase experiments were performed in glycine pH 9.0 buffer (10 mM NaCl, 0.05 mM EDTA, 50 mM glycine, 1mM NaN₃), with exception of Paper III, where also glycine pH 7 buffer was used and in Paper I, where 1 mM CaCl₂ was added to the buffer.

### 3.2 Surfaces

Silica wafers were modified so that both model hydrophilic- and hydrophobic surfaces were obtained, as well as surfaces of intermediate hydrophobicity. All wafers were initially cleaned for 5 min in 80°C 5:1:1 (v/v/v) H₂O:NH₃:H₂O₂, thoroughly rinsed in Milli-Q and then cleaned for 5 min in 80°C 5:1:1 (v/v/v) H₂O:HCl:H₂O₂, and finally rinsed with Milli-Q and ethanol. Hydrophilic silica surfaces had a contact angle with water of about 20°.

The procedure for creating surfaces of intermediate hydrophobicity with desirable homogeneity has been described elsewhere. Cleaned silica were immersed in an unstirred solution of 0.1%(v/v) dichlorodimethylsilane (DDS) in p-xylene for 20 min. The surfaces were then cleaned in xylene and ethanol. Silica treated in this way resulted in a contact angle of about 60°.

Hydrophobic C₁₈-terminated silica was prepared by immersing cleaned wafers in an unstirred solution of 0.5%(v/v) octadecyltrichlorosilane (OTS) in toluene for 24 hours. The wafers were then rinsed in chloroform and put in a chloroform bath for 2 min. Finally, the wafers were rinsed with ethanol and water. This procedure led to surfaces with contact angle about 105°. Coverslip glasses (Menzel- Gläser, Braunschweig, Germany) were cleaned and C₁₈- modified with the similar protocol as used for the silica wafers and had a contact angle of about 101°.

The hydrophobic C₁₈- surface used in the SPR instrument was a commercial HPA chip from Biacore made by covalent fixation of a self-assembled monolayer of alkanethiolates on a gold surface, cf. [www.biacore.com](http://www.biacore.com). The C₁₈-and unmodified sensor surfaces used in DPI was purchased from Farfield Sensors Ltd (Crewe, UK).
Substrate surfaces of trimyristin were created by a protocol described by Engström and Bäckström. Poly(vinyl chloride) (PVC) surfaces were spin-coated with trimyristin using a PWM32 photo resist spinner (Headway Research, Inc., Garland, Texas). A 50 µl drop of 15% (w/w) trimyristin in toluene was placed in the center of the surface. The surface was rotated at a speed of 4000 rpm for 4 min, with an acceleration of 100 rpm/s. This led to a thin trimyristin film on the PVC surface, with a contact angle with water of about 121° and a root mean square (rms) roughness parameter of 0.3 µm.
4. Experimental techniques

4.1 Dual Polarization Interferometry (DPI)

The sensor chip in Dual Polarization Interferometry (DPI) is a sandwich of two horizontally stacked waveguides of silicon dioxide doped with silicon nitride. An insulation layer separates the reference waveguide from the upper, sensing waveguide, which is the only one in contact with the analyte solution. The short edge of the sensor chip is illuminated with a laser beam that splits and propagates by total internal reflection through both waveguides. At the output of the chip, the two beams are allowed to diverge and form an interference pattern that is detected with a CCD camera in the far-field (Fig. 3A). Molecules at the surface of the sensing waveguide will interact with the evanescent field of the laser and thus cause a phase shift, which is detected as a spatial change of the interference pattern (Fig. 3B). Two polarization states of the laser are used that allow the detection of two independent phase shift responses. Absolute values of the thickness and refractive index of the adsorbed film are obtained by relating the spatial shift to an adsorbed single homogenous equivalent layer in terms of thickness and refractive index. This is done by the software that solves the electromagnetic equations of the system for both states of polarization. More detailed descriptions about the instrumentation and theories behind the method can be found elsewhere.\textsuperscript{58-61} Thus, for each time point, the refractive index and thickness are obtained, which can be used to calculate adsorbed mass using e.g. the de Feijter formula (Eq. 5):\textsuperscript{62}

\[
\Gamma = d_f \frac{n_f - n_{\text{buffer}}}{dn/dc} \quad (5)
\]

\(\Gamma\) = adsorbed mass (mg/m\(^2\)), \(d_f\) = film thickness (nm), \(n_f\) = refractive index of the film, \(n_{\text{buffer}}\) = refractive index of the buffer, \(dn/dc\) = refractive index increment with concentration. For proteins, a \(dn/dc\) of 0.18 ml/g is often used.

The adsorbed mass can also be calculated using the formula derived by the Cuypers et al.\textsuperscript{63} (Eq. 6 and Eq. 7).

\[
\Gamma = \frac{0.3d_f f(n_f)(n_f - n_{\text{buffer}})}{A - M} - \frac{(n_{\text{buffer}} - 1)}{(n_{\text{buffer}} + 2)} \quad (6)
\]
where

\[ f(n_f) = \frac{n_f + n_{\text{buffer}}}{(n_f^2 + 2)(n_{\text{buffer}}^2 + 2)} \]  

(7)

For globular proteins, normal values of the partial specific volume \( \langle \sigma \rangle \) and the ratio between the molar weight and the molar refractivity \( \langle M/A \rangle \) are 0.75 and 4.1, respectively.\(^{63,64}\)

![Figure 3](image.jpg)

**Figure 3.** (A) The DPI principle. The interference pattern of the two light waves is detected in the far-field. (B) Molecules within the evanescent field extending from the upper sensor surface will cause a spatial shift of the fringe pattern, detected by the CCD camera. Figure generated from information given by Farfield Sensors Ltd. (Crewe, UK).

An AnaLight Bio200® DPI instrument from Farfield Sensors Ltd (Crewe, UK) was used in this work. Lipase adsorption was measured on unmodified or C18-modified sensor waveguides purchased from the manufacturer. The length of the chamber was 15 mm and the volume was 2 µl. For the hydrophobic surfaces, the data was interpreted with the main assumption that proteins were adsorbing as a separate layer on top of the C18 layer.

### 4.2 Surface Plasmon Resonance (SPR)

A surface plasmon is a surface-bound charge density wave propagating along a metal surface. Excitation of a surface plasmon is a phenomenon sensitive to the optical properties of the medium adjacent to the metal surface\(^{65,66}\). In an SPR-based device, a laser is reflected on the backside of a sensor gold surface, and the reflected light intensity can be sampled continuously as a function of the angle of incidence \( \Theta \). A resonance coupling of a surface plasmon will result in a minimum in reflected light intensity at a certain angle of incidence \( \Theta_m \), which is the measured parameter (Fig. 4A). The plasmon excitation (or resonance) is a function of the angle of the incident light and the effective refractive index of the layer adsorbed on the gold surface. A small
shift in resonance angle is proportional to the change in refractive index on the surface. As the analyte adsorb on the sensor surface, the refractive index changes, which causes a shift in resonance angle (Fig. 4B). Thus, by varying the incident light until an intensity drop is observed, the resonance angle $\Theta_m$ is continuously measured and plotted in a sensorgram (Fig. 4C).

Adsorption was measured with a Biacore 3000 instrument (Biacore, Uppsala, Sweden). The Biacore expresses the shift in $\Theta_m$ as resonance units (RU). The RU contains a calibration constant that converts shifts in $\Theta_m$ to adsorbed amount (mg/m$^2$). It has been found that 1 RU corresponds to 1 μg/m$^2$ for a variety of proteins, carbohydrates and other carbon-rich molecules. $\Theta_m$ was measured on a spot of 0.26 mm$^2$.

### Figure 4.

(A) The SPR configuration used to detect protein adsorption in Biacore. At a certain angle of incidence, $\Theta_m$, surface plasmon resonance gives rise to a drop in reflected light intensity. (B) As analyte adsorb on the sensor surface, the resonance angle shift (e.g. $\Theta_m1 \rightarrow \Theta_m2$). (C) By continuously measure the shift in $\Theta_m$, a sensorgram is obtained.

#### 4.3 Ellipsometry

Ellipsometry is an optical method used to characterize surfaces, thin films and multilayers. The technique monitors the change in state of polarization of a monochromatic plane wave upon reflection on a substrate. The change in polarization depends on the optical properties of the surface, the surface layer and the ambient. The experimentally obtained parameters are the ellipsometric angles $\Delta$ and $\Psi$, determined by a procedure called null-ellipsometry. The light passes a rotatable polarizor and compensator before being reflected on the surface and a rotatable analyzer.
before being detected (Fig. 5). The polarizer and analyzer are rotated until a minimum in detected light is found, and the so-called nulling angles of the polarizer and analyzer correspond to $\Delta$ and $\Psi$, respectively. If the optical properties of the substrate and the ambient are known, the thickness and refractive index of the surface layer can be determined numerically by the changes in $\Delta$ and $\Psi$ upon adsorption to the surface. The refractive index and thickness can in turn be used calculate adsorbed mass by similar means as in DPI (Eq. 5-7).

![Ellipsometric setup](image)

**Figure 5.** The ellipsometric setup allowed measuring adsorption in situ under continuous stirring.

The ellipsometric setup in this work allowed in situ measurements of protein adsorption and has been further described elsewhere. Measurements were performed using a Rudolph thin film ellipsometer, type 436 (Rudolph Research, Farfield NJ). A surface was mounted in a cuvette of about 3 ml, and buffer and sample could be injected while $\Delta$ and $\Psi$ was calculated under continuous stirring (Fig. 5).

### 4.4 Confocal Laser Scanning Microscopy (CLSM)

#### 4.4.1 Principle

Confocal laser scanning microscopy (CLSM) allows imaging of a fluorescent sample with high resolution. Fluorescence is the result of an excitation-relaxation process that occurs in certain molecules, generally polyaromatic hydrocarbons or heterocycles, called fluorophores (Fig. 6C). The excitation and emission wavelength is dependent on the molecular structure of the fluorophore. Instead of illuminating a broad, extended region of the sample, which is done in a common wide-field fluorescence microscope, only a single point of the sample is illuminated at a time. A focused laser beam is used to create the illuminated spot, which beam waist is determined by diffraction effects in the optics and is in the order of 0.5 µm. Reflected or fluorescent light
from that illuminated spot is collected by the objective, separated by a dichroic mirror so that the emitted fluorescence can be focused onto the detector, which is typically a photomultiplier tube (PMT). The microscope becomes confocal when a small aperture in front of the detector, a pinhole in the range of 10-100 µM, effectively blocks light from all out-of-focus parts of the sample (Fig. 6A). Hence, since the pinhole is conjugate to the focal point of the lens, it is a confocal pinhole. The principle of a confocal microscope is seen in Fig. 6A and B. The dichroic beam splitter and emission filter must match the excitation and emission spectra of the fluorophore (Fig. 6B and C). Rotating mirrors that scan the laser spot in x-, y-, and z-direction over the specimen are used to create an image (a micrograph). The beam scanning setup enables a higher temporal resolution compared to stage scanning microscopes. Due to the depth-discriminating properties of the instrument, optical sectioning and 3D-reconstructing of a sample can be performed.

**Figure 6.** (A) Principles of a confocal microscope. The pinhole aperture in front of the detector effectively blocks reflections from out-of-focus sections of the specimen. (B) Filter settings to match the absorption and emission spectra of fluorescein; NFT = dichroic mirror, LP = long pass filter. (C) Molecular structure and absorption/emission spectra for fluorescein.

The confocal microscope is diffraction limited, i.e. the size of the focused laser spot is only limited by diffraction by the lenses in the microscope. The resolution is dependent on the
wavelength of the light and the numerical aperture (N.A.) of the focusing objective. The lateral Rayleigh resolution of a fluorescent sample, assuming the excitation and emission wavelengths are equal, reads:

\[ D = \frac{0.45 \lambda}{N.A.} \]  

(8)

When imaging an infinitely thin layer, the confocal microscope produces an axial intensity function with a full-width-half-maximum (FWHM) that can be approximated with Eq. 9:

\[ FWHM = \frac{\lambda}{(N.A.)^2} \]  

(9)

The depth resolution, or the optical slice thickness, is defined as the FWHM. Thus, using a wavelength \( \lambda = 500 \text{ nm} \) and an objective with N.A. = 1.4, the theoretically best resolution is \( \sim 0.18 \mu\text{m} \) laterally and \( \sim 0.39 \mu\text{m} \) in depth.

To image proteins on surfaces, a Zeiss LSM Pascal or Meta scanner was used with either an upright or inverted Axiovert 100M microscope, with a 40 \( \times \) 1.3 oil objective and the 488 nm line of an Ar-laser. A flow cell designed for a 13 \( \times \) 18 mm surface was constructed, with a 100 \( \mu\text{m} \) thick silicon rubber and a coverslip glass that limited the volume to about 15 \( \mu\text{l} \). A flow system, consisting of a syringe pump in conjunction with a 6-port injection valve, was used so buffer and sample could be pumped or injected to the flow cell.

4.4.2 Fluorescence Recovery After Photobleaching (FRAP)

A common method to quantify protein diffusion processes is Fluorescence Recovery After Photobleaching (FRAP). The technique was developed in the 1970’s for studying protein diffusion in lipid monolayers and bilayers and is now widely used in pharmaceutical research and cell biology. The procedure requires that the species of interest has an intrinsic fluorescent moiety or is labeled with a fluorophore. For most proteins, labeling is required. A defined region of the fluorescent sample is photo-bleached, which is an irreversible destruction of fluorescent dye molecules. Photobleaching can be caused by several different processes, all thought to originate from intersystem crossing of the excited dye from the singlet to the triplet state. Since the lifetime in the triplet state is longer than the singlet state, the fluorophore can
undergo reactions that lead to semioxidized or semireduced radical forms and thus lose the delocalized electrons needed for excitation\(^{80, 81}\). Photobleaching can be performed e.g. by illuminating the sample with a high-intensity laser. If the molecules are mobile, the intensity of the bleached region will recover due to mixing of bleached and unbleached molecules, i.e. unbleached molecules will be transported into the bleached region and vice versa (Fig. 7). The fluorescence recovery is sampled and fitted to the solution of the diffusion equation with the specific boundary- and initial conditions of the bleached region. The data obtained usually provide information of both the diffusion coefficient \(D\) as well as mobile fraction \(f\) of the diffusive population\(^{78, 82}\). FRAP averages the movement of many molecules and is thus an ensemble average measurement of the fluorescent species of interest.

The FRAP data analysis is dependent on the size and shape of the bleached region and the geometry of the medium through which diffusion occurs. A different approach is used if diffusion is measured on a two-dimensional surface compared to e.g. diffusion along the surface of dendrite in a nerve cell\(^83\). Since the solution of the diffusion equation can lead to very complex mathematical expressions, a recommended strategy is to reduce the 2D diffusion process into a 1D-diffusion, e.g. by bleaching a disc geometry and analyze the intensity recovery as a function of radius\(^82\). A common bleaching procedure when studying protein diffusion on solid surfaces has been to use two coherent laser beams to superimpose a fringe pattern on the adsorbed protein layer, so called fluorescence recovery after pattern photobleaching (FRAPP)\(^{84, 85}\). FRAPP can be used in combination with total internal reflection fluorescence (TIRF)\(^{27, 86}\) so that only molecules near the surface contribute to the detected fluorescence. The FRAPP- and the TIRF-FRAPP measure and average the diffusion over a 100 \(\mu\)m to 1 mm distance of the surface. Other reported strategies have been to use Confocal Laser Scanning Microscopy\(^{30, 31}\) or a CCD camera\(^26\) to form spots on the surface. This has been done by e.g. electronically zoom during bleaching, so that only a small rectangular regions were illuminated\(^28\). For enzymes diffusing on substrate surfaces, another approach has been used to quantify enzyme diffusion. The substrate surface was then fluorescently labeled so that the monitored fluorescence decrease due to enzymatic activity indirectly could be related to enzyme mobility\(^29\).

In this work two different FRAP procedures were used to measure protein lateral mobility on solid surfaces; spot bleaching and bleaching of a large rectangle (Fig. 7). For the spot bleaching, the theoretical solution for the fractional fluorescence recovery profile \(f(t)\), has been determined e.g. by Axelrod \textit{et al.}\(^82\) (Eq. 10):
**Figure 7.** Two different FRAP procedures (A) Spot bleaching of lipase diffusion on trimyristin where the average intensity of the bleached circle is plotted in a recovery profile (Paper VII). (B) Bleaching of a large rectangular area makes it possible to analyze the intensity profile $I(x,t)$ as one-dimensional diffusion. Example from lipase diffusion on silica (Paper IV).

$$f(t) = 1 - \frac{\tau_D}{t} \exp\left(-2\frac{\tau_D}{t}\right) \left[ I_0(2\frac{\tau_D}{t}) + I_2(2\frac{\tau_D}{t}) \right]$$

$$- 2 \sum_{k=0}^{\infty} \frac{(-1)^k(2k+2)!(k+1)!}{(k)!^2(k+2)!^2} \left[ \frac{\tau_D}{t} \right]^{k+2}$$

(10)

where $I_0$ and $I_2$ are modified Bessel functions and $\tau_D$ is the characteristic diffusion time. The diffusion coefficient $D$ can be calculated using the relationship in Eq. 11:
\[ D = \gamma_D \left( \frac{\omega^2}{4 \tau_{1/2}} \right) \]  

where \( \omega \) is the radius of the bleached circle, \( \tau_{1/2} \) is the time when the recovery profile had reached half its maximum (Fig. 7A) and \( \gamma_D \), a constant defined as \( \tau_{1/2} / \tau_D \). For a circular beam, \( \gamma_D = 0.88 \). From the intensity profile (Fig. 7A) the mobile fraction \( f \) could be determined as the saturated plateau, \( F_S \), in the recovery process.

When using a confocal microscope with scanning capabilities limited to line scans, another approach was required. A large rectangular area was bleached and the intensity profile \( I(x,t) \) of the middle section could be averaged and analyzed as a one dimensional diffusion relaxation of a step function (Fig 7B). \( I(x,t) \) is the sum of the contributions from the mobile fraction \( f \) and the immobile fraction \( (1-f) \) (Eq. 12):

\[ I(x,t) = f \times F(x,t) + (1-f) \times H(x) \]  

\( F(x,t) \) is the solution of the diffusion equation for a one dimensional diffusion from a step function\(^{25} \) and \( H(x) \) is the initial step function, which leads to the following expression of \( I(x,t) \) (Eq. 13):

\[ I(x,t) = f \times I_0 \int_{-\infty}^{x/\sqrt{2Dt}} \frac{1}{\sqrt{2\pi}} e^{-y^2/2} dy + (1-f) \times I_0 H(x) \]  

Normalizing to the intensity of the unbleached area, \( I_0 \), and differentiating gives (Eq. 14):

\[ \frac{\partial I(x,t)}{\partial x} = f \times \frac{e^{-x^2/4Dt}}{\sqrt{4\piDt}} + (1-f) \times \partial(x) \]  

A median filter operator could remove the contribution from the immobile fraction and hence, the maximum slope between the two areas simplified to an expression from which \( D \) can be estimated (Eq. 15):

\[ \left| \frac{\partial I(x,t)}{\partial x} \right|_{x=0} = \frac{f}{\sqrt{4\pi Dt}} \]
However, this approach relied on the assumption of a slow diffusion process on the surface, i.e. that the bleached area was sufficiently large so that the analyzed intensity profile could be treated as a one-dimensional step function. To estimate the mobile fraction $f$, a separate bleaching of a much smaller rectangle was required. In that recovery process, only the plateau value of the intensity was considered and used to estimate $f$.

4.4.3 Single molecule tracking using Quantum Dots (QDs)

Quantum Dots (QDs) are inorganic semiconductors in the nanometer size that can absorb photons and fluoresce at sharp wavelengths. The excitation and emission wavelengths are dependent on both the size and composition of the QD. QDs typically consist of a CdSe or CdTe core with a ZnS shell and can be functionalized in order to be more biocompatible. They are superior to traditional organic dyes due to their high extinction coefficients and photostability, making it possible to image single molecules in a fluorescence microscope without bleaching. QDs can be conjugated to proteins, which have successfully allowed imaging and tracking of single receptors in cell membranes.

To analyze the movements of a QD-conjugated particle, the detected trajectory of a single particle motion is used. The spreading tendency of the particle is reflected in the mean square displacement (MSD), which can be calculated from the collected images/trajectories applying the following relation (Eq. 16):

$$MSD(n\Delta t) = \frac{1}{N-n} \sum_{i=1}^{N-n} \left[ (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2 \right]$$

where $x_i$ and $y_i$ are the particle coordinates on frame $i$, $N$ is the total number of steps in the trajectory, $\Delta t$ is the time between frames and $n\Delta t$ is the time interval over which the MSD is calculated. The diffusion coefficient can then be estimated by fitting the first points in the MSD function versus time using the relation (Eq. 17):

$$MSD = 4Dt$$

In this work, PEG coated CdSe-ZnS Quantum Dots (Fig. 8A) were conjugated to lipases. Single QD-lipase particles could be imaged and tracked using the confocal microscope (Fig. 8B). Trajectories were calculated using the Volocity 4.0.1 software.
4.5 Total Internal Reflection Fluorescence Correlation Spectroscopy (TIR-FCS)

Fluorescence correlation spectroscopy (FCS) is a technique that analyses the fluorescence intensity fluctuations from within a focused laser spot, which requires samples of low concentration (nM). Measurements are performed in a non-scanning confocal setup with the excited and detection volume limited to about one femtoliter by the focused laser-spot and a pinhole. The technique has single-molecule sensitivity and the length and variation of the emitted fluorescence carries information about dynamic events of the investigated sample or system. The autocorrelation of the emitted fluorescence intensity provides information of e.g. kinetics and number of individuals participating in a specific dynamic process. Studied processes have been e.g. diffusion, flow, binding of biomolecules, rotation, photo-physics, bleaching and conformational changes\textsuperscript{92-95}.

However, since the axial size of the focused laser spot is in the magnitude of 1 - 2 µm, it is appropriate to use total internal reflection (TIR) excitation in order for the technique to be surface sensitive\textsuperscript{96, 97}. Total internal reflection can occur when light hits the boundary between mediums of different refractive index. If the light travels through the medium of high refractive index and strikes the interface at an angle above a certain critical angle, all light will be reflected. The critical angle \( \theta_c \) can be deduced from Snell’s law:

\[
\theta_c = \arcsin\left(\frac{n_2}{n_1}\right)
\]  \hspace{1cm} (18)
where \( n_1 \) is the refractive index of the dense medium and \( n_2 \) is the refractive index of the less dense medium.

**Figure 9.** Principle of TIR-FCS. A drop of highly diluted fluorescent analyte is placed on the glass surface. The evanescent field of the totally internally reflected laser excites the dye and the fluctuations of the emitted fluorescence is detected and autocorrelated. An example of the autocorrelation function of FITC on C18-glass is shown to the right, where three different regions (singlet-triplet transition, free diffusion and surface interaction) at different correlation times are visible.

However, although total internal reflection occurs, an evanescent field of less than 100 nm will protrude into the medium of low refractive index (Fig. 9). In TIR-FCS, the boundary between a glass surface and water (or buffer) is used to create the evanescent field that excites molecules in proximity to the interface (Fig 9). For the experiments in this work, an objective based TIR-FCS setup, described by Hassler et al. 98, was used and is schematically shown in Fig. 9. A drop of fluorescent sample was placed on top of the glass surface and the autocorrelation of the fluorescence in the evanescent field could be detected. An example of a normalized autocorrelation function (ACF) for the fluorescent dye FITC on C18-modified glass is shown in the right of Fig. 9. Three distinct regions in the ACF are visible; a fast region \( (\tau < 5 \mu s) \) that corresponds to the singlet to triplet transition of the dye (i.e. a fast on-off flickering when passing the excited volume), a middle region that reflects the free diffusion through the evanescent field and a third region at long correlation times that reflects the surface interactions. Fitting the autocorrelation functions to the model for reversible or competitive adsorption/desorption kinetics makes it possible to quantify e.g. interaction rates at the surface. A detailed description of the theoretical models behind TIR-FCS is found in Paper VII.
5. Summary of key results and discussion

5.1 The effect of lipases in the detergency process

With the aim of demonstrating the role of lipases during detergency and in the complex environment where it must function, a confocal microscope was used to image the effect of both surfactants and lipases (Paper I). A nonionic:anionic surfactant composition of 300 µM C₁₂E₆/LAS (1:2 mol%) was chosen to mimic the detergents used in a common household detergent formulation. This composition was used throughout the whole project. The detergency mechanism of lipids from single cotton fibers could be imaged by soaking the fiber in olive oil, lard or tricaprin (tri-C₁₀₃). The lipids were then labeled with the fluorescent dye NileRed, that partitions into lipid structures. After mounting the fibers in a flow-cell to the CLSM, detergent compositions could be injected and the detergency imaged and quantified as the decrease in NileRed intensity along the fiber. When using only the surfactant composition, different detergency mechanisms (roll up, emulsification and solubilization) were visualized in the image series depending on the type of lipid studied (Fig. 2-4 in Paper I). The effect of adding lipases to a detergent formulation is most apparent at temperatures just below the melting point of the fat/oil⁹⁹. At higher temperatures, when the lipid is fluid, surfactants can remove most of the soil, which is why lipases are particularly important when washing at low temperatures. The effect of lipases in detergency was demonstrated when studying detergency of lard, which is produced from the fat portions of the pig. Lard is a fat (i.e. solid at room temperature) and has about equal amounts of saturated fatty acids (~ 50%) and unsaturated fatty acids (40 – 45%)¹⁰⁰. The intensity of a lard-soiled fiber as a function of time in the detergency process is shown in Fig. 10 A. At t = 0, the fiber was exposed to the 300 µM C₁₂E₆/LAS solution, which led to a 50 % decrease of the fat content. Most of the lard was emulsified and rolled up into emulsion droplets along the fiber, which seemed to stick to the surface of the fiber, visualized in the images in Fig. 10 B. Since lard is a mix of saturated and unsaturated lipids, micron-sized necking/emulsification process was probably induced in the unsaturated, more fluid fraction. However, this process was limited in effectiveness by the more solid-like saturated fraction. A subsequent treatment of 300 µM C₁₂E₆/LAS + 100 nM TLL-β resulted in a further reduction of the lard content, as seen by the decrease in signal between 1500 and 1600 s after the start of the detergency (Fig. 10 A). The lipase effect was also visible in the images. Some of the preformed emulsion particles that still stuck to the fiber during the surfactant injection could be removed from the surface when lipase was added to the system (Fig. 10 B). Hence, lipases could reduce the lard content of the fiber by hydrolyzing triglycerides into more water-soluble products. It is of general interest to further
understand the mechanisms behind the enzyme activity and what factors affect it. To further characterize the mechanisms involved in the lipase activity at a lipid-water interface, the different steps, i.e. adsorption, activity, mobility and coadsorption in the presence of surfactants, were studied in depth and the results are presented in the following Sections.

Figure 10. (A) A plot of NileRed intensity, which is proportional to the lard content, vs. time in the detergency of lard from three different fibers. The detergency consisted of a surfactant injection and a subsequent surfactant + TLL-β injection. The start of the two injections is indicated by the arrows. (B) Frames from the image series of detergency of lard from a single cotton fiber. The detergency protocol was identical to (A); the first injection was with only surfactants (upper row) and the second with surfactants + TLL-β (bottom row). During the first injection, the formation of emulsification particles is indicated by the white arrows. During the second injection, the white arrows indicate a region where on the fiber where the intensity decreased and an emulsion particle leaving the surface due to lipase activity. Scale bar is 50 µm.
5.2 Lipase adsorption to surfaces of different hydrophobicity

In order to establish a general understanding of how surface properties affected adsorption of the lipase variants, adsorption of TLL was measured on both hydrophobic and hydrophilic surfaces. In Fig. 11, the DPI results from adsorption to C18 modified sensor surfaces and unmodified (hydrophilic) sensor surfaces are displayed. For TLL-\( \alpha \), the adsorption was measured for 6 min + rinsing with buffer for 4 min (Paper II), whereas for TLL-\( \beta \), the adsorption was measured for 20 min + 10 min rinsing (Paper III). However, a high affinity of TLL towards C18 surfaces was confirmed by ellipsometer measurements, where no TLL desorption upon rinsing was found even at short adsorption times (Paper IV). Thus, due to the high affinity of TLL to the hydrophobic C18 surface, the difference in adsorption times could be assumed to be insignificant and the results for the two variants to be comparable. It was evident from the adsorption isotherms, as measured after the rinsing phase, that TLL-\( \beta \) reached a higher saturated adsorbed amount compared to TLL-\( \alpha \) on C18 surfaces, 1.90 vs. 1.35 mg/m\(^2\) (Fig. 11 A, top). Moreover, TLL-\( \beta \) might have had a higher affinity for the hydrophobic surface since the initial slope of the isotherm appeared to be higher. The difference in the maximum adsorbed amounts between the two lipase variants could be related to the net charge of the proteins. At pH 9, TLL-\( \beta \) carries less negative charge than TLL-\( \alpha \), which allows a more densely packed protein layer on the surface due to less lateral repulsion. From the plot of the thickness \( d \) of the protein layer as a function of adsorbed amount \( \Gamma \) on C18 surfaces (Fig. 11 A, middle), it was found that the adsorbed TLL-\( \beta \) layer had a higher thickness \( d \) around 3.5 nm at \( \Gamma > 1.5 \text{ mg/m}^2 \) compared to TLL-\( \alpha \) \( d \) between 1.5 - 2.0 nm at \( \Gamma > 1.0 \text{ mg/m}^2 \). At lower adsorbed amounts, the thickness of both variants decreased. This might be explained by partial unfolding if the surface is sparsely covered combined with limitations in the DPI technique, as discussed in Papers II and III. Moreover, the values of the refractive index (RI) was lower for TLL-\( \beta \) compared to TLL-\( \alpha \), and decreased almost linearly with adsorbed amount (Fig. 11 A, bottom). Hence, taking into account the average radius in solution of about 2.3 nm\(^{101}\), these results indicated that the TLL-\( \beta \) structure did not undergo any major unfolding on the C18 surface and that this enzyme adsorbed with a similar structure density independent of the adsorbed amounts. These observations are consistent with earlier reported FTIR data on TLL adsorption on C18-surfaces\(^{102}\), which has suggested that very few conformational changes occurred upon adsorption. TLL-\( \alpha \), on the other hand, seemed to have a more flexible structure that partially unfolded on the C18 surfaces, since a lower thickness was found for this enzyme (Fig. 11 A, middle). This might have contributed to the lower maximum adsorbed amounts measured, i.e. that an adsorbed TLL-\( \alpha \) molecule occupied a larger surface area than an adsorbed TLL-\( \beta \) molecule. The higher RI values (Fig. 11 A, bottom) also
suggested a more compact layer, consistent with a more collapsed structure on the surface compared to TLL-β.

Figure 11. (A) Adsorption of TLL-α and TLL-β to hydrophobic (C18-modified) sensor surfaces. Adsorbed amounts are plotted vs. bulk concentration of lipase (top), whereas thickness (middle) and refractive index (bottom) is plotted vs. adsorbed amount. (B) Adsorption of TLL-β to hydrophilic sensor surfaces. Adsorbed amounts are plotted vs. bulk concentration of lipase (top), whereas thickness (middle) and refractive index (bottom) is plotted vs. adsorbed amount.
Both SPR and ellipsometry were also used to study the TLL adsorption. An identical adsorption isotherm on C18 surfaces was found in SPR as with DPI (Paper II) for TLL-α, but ellipsometry resulted in higher maximum adsorbed amounts for both lipase variants; 2.1 mg/m² at a TLL-β bulk concentration of 0.1 – 3.0 µM (Paper IV) and 1.75 mg/m² at TLL-α bulk concentration 1.0 µM (Paper V). The discrepancy could be due to the different adsorption conditions. In DPI and SPR, small flow cells (µl) were used whereas in ellipsometer the adsorption is measured in a cuvette (3 ml) under continuous stirring. Other reasons could be different surface properties of the silanized C18 silica used in the ellipsometer and the purchased C18 sensor surfaces used in DPI and SPR, e.g. packing density of acyl-chains and defects or impurities in the surface layer, that might affect the contact angle. Nevertheless, ellipsometry gave similar qualitative results, i.e. a higher adsorbed amount for TLL-β compared to TLL-α. This was also observed qualitatively in the confocal microscope when fluorescently labeled TLL-β and TLL-α was let to adsorb on spin-coated trimyristin substrate surfaces (Paper VII). TLL-α was found to adsorbed to a maximum adsorb amount on trimyristin corresponding to 65 % of the TLL-β maximum adsorbed amount, which is equivalent to was what found by DPI for the lipase variants on the C18 model surface (Fig. 11 A, top).

TLL-β adsorption was also studied on unmodified hydrophilic DPI sensor surfaces (Fig. 11 B). Compared to the hydrophobic surface, the maximum adsorbed amount was lower, only around 1.50 mg/m², and the initial slope of the adsorption isotherm was also found to be lower (Fig. 11 B, top), which suggested a lower affinity to the hydrophilic surface. The same observation has been made with several other proteins64, 103-105 and are supported by theoretical and experimental kinetic data, which have suggested that the conversion rate to irreversibly adsorbed protein states increase with surface hydrophobicity15, 104.

The thickness of adsorbed TLL-β was found to be similar on the hydrophilic surface compared to the C18 surface (Fig. 11 B, middle), with d = 3.5 nm at \( \Gamma > 1.0 \) mg/m², and the RI seemed to follow the same trend as on the hydrophobic surface at \( \Gamma > 1.0 \) mg/m² (Fig. 11 B, bottom). This would be a further confirmation of the robustness of the TLL-β structure. However, at low adsorbed amounts, the refractive index was more scattered and a few high values were found compared to the C18 surface, which might indicate that some partial unfolding of TLL-β had occurred that led to a more compact structure on the surface. However, it is interesting to note that although TLL-β had the same trend in thickness and RI on both the hydrophobic and hydrophilic surface, the maximum adsorbed amounts differed. This suggests that a hydrophobic
surface might result in a higher packing density. Due to the hydrophobicity of the active site, there is a strong affinity for TLL to be adsorbed with the active site facing downwards on hydrophobic surfaces. Therefore, one might expect to find a larger population of TLL oriented with the active site outwards at a hydrophilic surface compared to at a hydrophobic\textsuperscript{106}.

To further analyze the orientation of adsorbed lipases, the activity of adsorbed TLL-\(\beta\) towards a substrate in solution was measured (Paper III). If TLL was adsorbed with the active site facing the surface, one might expect a lower activity towards a substrate in solution and the opposite if the active site was facing the bulk. Thus, activity measurements could serve as an indirect way of measuring the orientation on the surface. This was studied with confocal microscopy and 5-carboxyfluorescein diacetate (5-CFDA), that upon TLL hydrolysis turned into a fluorescent product. By monitoring the intensity increase as a function of time, the relative mean activity of TLL-\(\beta\) adsorbed to the surface was measured as a function of time after adsorption on C18-modified silica and hydrophilic silica. The results are displayed in Fig. 12. Although the data was quite scattered, it was confirmed that the relative activity was lower on the hydrophobic silica (Fig. 12 A) compared to the hydrophilic (Fig. 12 B). Hence, a larger population of adsorbed TLL-\(\beta\) molecules was thought to be oriented with the active site facing the surface on the C18 surface, which has been proposed previously\textsuperscript{106}. Moreover, it was found that the activity seemed to decrease as a function of time, from \(\Delta I/\Delta t \approx 1.5\ \text{min}^{-1}\) to \(\approx 0.5\ \text{min}^{-1}\) on the C18 surface and from \(\Delta I/\Delta t \approx 2.5\ \text{min}^{-1}\) to \(\approx 1.0\ \text{min}^{-1}\) on the hydrophilic silica. This might indicate that the structure of TLL-\(\beta\) changed as a function of time after adsorption. Two different bulk concentrations, based on the adsorption isotherms in Fig. 11 of TLL-\(\beta\) was used to give rise to surfaces of high and low adsorbed amounts. Interestingly, the overall activity was more or less independent of the adsorbed amount, an observation that could origin from the CFDA concentration used, which might not have been optimized for the TLL surface concentrations used. Hence, substrate concentration might have limited the overall activity at high adsorbed amounts of lipase. The surface mobility of TLL might also contribute to the high activity found at low adsorbed amount. This will be discussed further in the following chapter. More mobile species on the surface might lead to longer exposure time of the active site towards solution leading to a higher overall activity detected. These results support the theory that no dramatic unfolding occurred upon adsorption, since adsorbed lipases were still enzymatically active independent of surface and adsorbed amount.
For further studies of the mobility of adsorbed lipases, the enzymes had to be labeled with a fluorescent probe (Alexa488). To see whether the attachment of the probe affected the affinity of the surface, samples of the same total lipase concentration, 300 nM but with different fractions of the labeled population were adsorbed under no-flow conditions to a C18 surface in the confocal microscope (Paper IV and V). When the fraction of labeled lipase decreased, the intensity decreased linearly down to the origin of coordinates, i.e. the value found with only an unlabeled population. This was observed for both TLL-α and TLL-β and suggested that unlabeled and labeled TLL had similar affinity under no-flow and at the studied concentration. However, SPR analysis revealed that labeled TLL-α adsorbed to lower amounts compared to unlabeled at bulk concentrations < 900 nm under flow conditions (data not shown), i.e. that the labeling did have an effect on the adsorption properties of the lipase. The labeling probably led to a more unstable structure that more easily underwent conformational changes upon adsorption, which in turns would lead to a lower adsorbed amount compared to the unlabeled. The labeling had also a negative effect on the activity of both TLL-α and TLL-β (Table 1, p. 13), with TLL-β completely inactive after labeling.
5.3 Lipase mobility on surfaces of different hydrophobicity

It is well known that the catalytic activity of TLL is strongly modulated by adsorption at interfaces and subsequent conformational changes in the lid region of the enzyme\(^4,10,12\). To what extent the activity of TLL relates to the mobility of the enzyme is not well understood. Therefore, to see what factors affected the lateral mobility of adsorbed lipases, the surface diffusion properties was systematically measured on surfaces of different hydrophobicity (Paper IV, V and VII). The studied surfaces were model surfaces of hydrophilic silica and silica methylated with dichlorodimethylsilane (DDS) or octadecyltrichlorosilane (OTS (C18)) and a substrate surface of trimyristin, schematically shown in Fig. 13. The surface diffusion properties of TLL were analyzed with FRAP using rectangular bleaching (Fig. 7B) on the model surfaces and with spot bleaching (Fig. 7A) on the substrate surface. Ellipsometry data of TLL adsorption on the C18 modified silica surfaces were used to calibrate fluorescence intensity to relative surface density of lipase \(\phi/\phi_{\text{max}}\), which enabled measurements of the diffusion coefficient at different \(\phi/\phi_{\text{max}}\) on the model surfaces (Paper IV and V). For the trimyristin surface, no ellipsometer data was available. Relative adsorption isotherms on the substrate surface could however be established by measuring the surface mean fluorescence intensity after adsorption with the fluorescently labeled lipase variants. The surface intensity that corresponded to the plateau value in the TLL-\(\beta\) isotherm was considered as \(\phi_{\text{max}}\), so that all surface intensities could be normalized to get \(\phi/\phi_{\text{max}}\) for each FRAP experiment. FRAP was performed after rinsing the surface with buffer, to ensure that the recovery was due to lateral mobility and not exchange processes with lipases in bulk. The average lateral diffusion coefficient \(D\) was calculated and averaged in two time intervals after adsorption, 0-90 min and 90-180 min, respectively, denoted \(D_{t=0-90}\) and \(D_{t=90-180}\).

![Figure 13](image-url)

**Figure 13.** The model surfaces (silica or silanized silica) and the substrate surface of trimyristin had different chemistry and degree of hydrophobicity (\(\sigma = \text{contact angle with water}\)).
Figure 14. The lateral diffusion of the two lipase variants on the different surfaces. The diffusion coefficient $D$ is plotted on a log scale vs. the relative surface density of lipase, $\theta/\theta_{\text{max}}$. The lateral diffusion was averaged in two time intervals after adsorption, 0-90 min (•) and 90-180 min (△), respectively. Exponential functions were fitted to the data so that the diffusion at infinite surface dilution (zero surface density), $D_0$, could be extrapolated. The fits were forced to pass through $D = 10^{-12}$ cm$^2$/s as $\theta/\theta_{\text{max}} \to \infty$, which was a diffusion rate considered as immobile in this work (Paper IV and V).
The FRAP results on the different model surfaces are displayed as $D_{t=0-90}$ and $D_{t=90-180}$ vs. $\theta/\theta_{\text{max}}$ in Fig. 14 A-F. On all surfaces, two distinct features were seen. First of all, the lateral diffusion process was slow, with calculated diffusion coefficients in the order of $10^{-11}$ cm$^2$/s. For comparison, a globular protein of TLL-size has a $D$ of $10^{-6}$ cm$^2$/s in free solution and proteins in biological membranes have a $D$ of $10^{-8}$ - $10^{-7}$ cm$^2$/s. Moreover, the surface diffusion of the lipases was one order of magnitude lower than reported for BSA on a variety of surfaces.

Second of all, there was a distinct dependence of $D$ on $\theta/\theta_{\text{max}}$ on all types of surfaces, with an approximate tenfold decrease in diffusion rate when $\theta/\theta_{\text{max}}$ increased from 0.1 to 0.7 on the hydrophilic silica and the DDS surface (Fig. 14 A and B). Similar trends were seen on trimyristin substrate surface and the C18 surface for both TLL-β and TLL-α (Fig. 14 C to F), but less pronounced on the C18 surface. The strong dependence on surface density is probably explained by sterical hindrance and intermolecular repulsion at high adsorbed amounts, which has been predicted theoretically and by Brownian dynamics studies.

The hydrophobicity of the surface had a noticeable effect on surface mobility of adsorbed lipase. In order to compare the different surfaces, $D$ was extrapolated to infinite surface dilution, $D_0$, using an exponential fit as suggested by theoretical work on surface mobility. All estimated $D_0$ values of studied diffusion processes measured with FRAP in this work are displayed in Table 2 (page 44). It was found that $D_{0,t=0-90}$ and $D_{0,t=90-180}$ was about three times higher on the hydrophilic Si-surface and DDS surface compared with the C18-terminated OTS surface. However, TLL was most mobile on the trimyristin substrate surface; $D_{0,t=0.90}$ was more than 18 times higher for TLL-α and four times higher for TLL-β on the triglyceride surface compared to the hydrophobic C18 surface.

The difference in mobility on the model surfaces might be explained by differences in the affinity for the surfaces. As found in the adsorption experiments, TLL-β had a higher affinity for the hydrophobic surface compared to the hydrophilic surface (Fig. 11). Further, TLL-β is more likely to expose the hydrophobic active site region towards the hydrophobic surface, which was reflected in the lower activity towards a substrate in solution (Fig. 12). The orientation with the lid region facing the surface might minimize the free energy of adsorption and is therefore probably an orientation of low mobility. On hydrophilic surfaces, one might expect to find TLL in a diverse number of orientations, some which are expected to be more mobile than the orientation with the active site facing the surface. Moreover, it was found that the two lipase variants, TLL-α and TLL-β had similar slow diffusion rates on the hydrophobic C18 surface.
14 C and D). Hence, any difference in adsorption affinity on the C18-surface (Fig. 11) had no effect on the surface mobility.

Both active and inactive lipase was more mobile on the substrate surface compared to the hydrophobic model surfaces, although the triglyceride surface was more hydrophobic. This must be due to the properties of the substrate surface. Tentative explanations are that the triglycerides themselves are mobile within the spin-coated film or that a small amount of water is incorporated, making the surface more fluid-like. The effect of the activity on the mobility, i.e. the difference in mobility between the active TLL-α and inactive TLL-β, and other effects seen with an active lipase will be further discussed in Section 5.5 on page 45.

Apart from the wettability of the surface, the lateral mobility was affected by time after adsorption on the two most hydrophobic model surfaces and on the trimyristin substrate surface (Fig. 14 B - F). The apparent lateral diffusion decreased with time, reflected by a lower $D_{0, t=90-180}$ compared to $D_{0, t=0-90}$ on those surfaces (Table 2, p. 44). This might be explained by an orientational redistribution of adsorbed TLL molecules with time so that a larger fraction of lipase molecules become “trapped” in states of low free energy, e.g. oriented with the active site facing the surface. This would also explain why a decrease with time was not found on the hydrophilic-Si surface. For TLL-α, the decrease in mobility with time on the trimyristin surface might also be connected with its activity (Section 5.5). The decrease in activity towards the substrate in solution found on the C18-surface (Fig. 12 A) supported such an explanation, i.e. that more lipase molecules become trapped on a hydrophobic surface with the active site inaccessible for a substrate in solution. However, the activity also decreased on the hydrophilic surface (Fig. 12 B), which suggested long-term structural rearrangements on hydrophilic surfaces that did not affect the mobility but decreased the activity. Another possibility for the decrease in lateral mobility is that the lipase molecules partially unfolded on the surface during the FRAP experiments (0 – 180 min after adsorption). However, since the DPI-experiments indicated that any conformational changes in TLL structure only occurred on the hydrophilic surface where no decrease in diffusion rate was found, this explanation seems less likely. It should also be noted that the decrease in lateral mobility was most pronounced at low $\theta/\theta_{\text{max}}$, which suggested that crowding effects were dominant at high adsorbed amounts of lipase.

While the diffusion coefficient is a measure of the mobility of the enzyme, the mobile fraction might reflect the different adsorption states on the surface. With the rectangular FRAP-model
Summary of Key Results and Discussion

used (Fig. 7 B) combined with the low diffusion rates found on the model surfaces, it was not possible to estimate the mobile fraction with shorter time intervals than an average over the whole 180 min time interval after adsorption. It was found that the mobile fraction on the model surfaces followed the same trend as $D_m$, i.e. that it was highest on the hydrophilic and DDS surface, around 0.60 (Table 2, p. 44), and lower on the OTS surface, around 0.45. On the trimyristin surfaces, the mobile fraction was essentially independent on lipase variant, time after adsorption and $\theta/\theta_{max}$ and fluctuated between 0.4 -0.6 (Paper VII). The nature of the mobile fraction is not at all fully understood. It was thought to be due to one or several of the following explanations:

i) The immobile fraction is a substrate effect, i.e. lipases could after adsorption be trapped in discontinuities in the surface that prevents lateral diffusion.

ii) The immobile fraction reflect lipases adsorbed in an orientation that minimizes the free energy of adsorption, e.g. the orientation with the active site facing the surface, which would render them immobile. In that case, one might have found an increasing immobile fraction with time on the hydrophobic surfaces, which in turn hindered mobile lipases and thus led to an apparent lower diffusion coefficient.

iii) The immobile fraction is a subpopulation of the lipases that is unstable and unfolds or aggregates on the surface. This might be caused by fluorophore labeling at unfavorable sites, since the DPI results with unlabeled TLL showed little conformational changes on hydrophobic surfaces (Fig. 11 A). It was confirmed by activity measurements (Table 1, p. 13) and SPR adsorption studies that the labeling did affect the lipase structure, possibly by making it more unstable (cf. discussion on p. 34).

It is difficult to conclude what mechanism is behind the lipase migration on the model surfaces. One possibility is a desorption/readsorption migration. The lipases would then alternately desorb and readсорb, thereby facilitating a motion across the surface. However, no desorption of TLL was visible upon rinsing the surface with buffer when using DPI (Paper II and III), SPR (Paper II) or ellipsometry (Paper IV), and thus, this mechanism is unlikely to occur unless surfactants are present (see Sections 5.4). Another possibility is that the actual migration is due to segmental movement. A more flexible protein structure would then be more mobile on hard surfaces compared to a rigid structure. Comparing the structure of BSA and TLL, BSA has a low native state stability and is known to undergo changes in secondary and tertiary conformation upon adsorption. This could facilitate a crawling motion on solid surfaces. TLL, on the other hand, is
a quite rigid molecule except for the flexibility of the lid domain. Hence, the lower mobility of TLL compared to BSA recorded in this work is not surprising, taking in account both the structural stability of TLL and the strong interaction with the hydrophobic surface. A rolling migration is also a tentative diffusion mechanism of TLL. However, the highest mobility of adsorbed TLL was found on hydrophilic surfaces with low $\theta/\theta_{\text{max}}$, where the DPI results indicated that conformational changes might have occurred. This would intuitively lead to more contact points with the surface and thus suppress lateral mobility, but in this case the unfolding might have led to a more flexible protein structure that facilitated segmental movement across the surface.

5.4 Lipase surface mobility in the presence of surfactants

It was evident from the surface diffusion experiments that the lateral mobility of a lipase was very low on a hydrophobic surface. To see whether the presence of surfactants modulated the lipase lateral mobility, surface diffusion measurements, equivalent to those described in the previous section, were conducted with lipase coadsorbed with surfactants (Paper V). The nonionic: anionic surfactant mixture, C$_{12}$E$_6$/LAS (1:2 mol%), which was used when studying detergency mechanisms on soiled cotton fibers (Paper I) was used throughout the diffusion experiments. It was found that this system had a critical micellar concentration (cmc) between 100 to 200 µM (Paper V). This rather broad micellar formation region is probably due to the polydispersity of the anionic technical surfactant LAS. Diffusion was measured with TLL-α on the most hydrophobic model surface, the C18 terminated silica surface, to see if the low mobility found without surfactants was affected (Fig. 14 C and D). The lipases were let to coadsorb to the surface with either 25, 150 or 500 µM C$_{12}$E$_6$/LAS, and the surface was rinsed with surfactant solution before conducting FRAP, so that no lipases initially in bulk contributed to the recovery. The lipase lateral diffusion decreased with $\theta/\theta_{\text{max}}$ and with time after adsorption for all surfactant concentrations but it was evident that the presence of surfactants at a concentration at or above cmc strongly enhanced the surface mobility of TLL-α. The results are summarized in Fig. 15. $D_0$ increased more than tenfold, from $0.8 \times 10^{-11}$ cm$^2$/s without surfactants to $13.1 \times 10^{-11}$ cm$^2$/s at surfactant concentration of 500 µM (Fig. 15 and Table 2). The dramatic increase in lipase mobility was also accompanied by an almost two-fold increase in mobile fraction when passing the cmc (Fig. 15 and Table 2).
To gain further insight in the mechanism behind the increase in lipase surface diffusion observed in the presence of surfactants, several other experiments were performed:

- It was found in the confocal microscope that the surface fluorescence intensity from adsorbed TLL-α decreased as a function of time after adsorption when coadsorbed with surfactants. The intensity of the C18-surface, with θ/θ_max of TLL-α between 0.10 and 0.15, is plotted as a function of time and C_{12}E_{6}/LAS concentration in Fig. 16 A. Every 15th minute after adsorption, the flow cell was rinsed with a short pulse of surfactants to assess if lipase displaced from the surface, still in the confocal volume, contributed to the detected signal. This suggested that a large fraction, from 10 % with 25 µM C_{12}E_{6}/LAS to 45 % with 500 µM C_{12}E_{6}/LAS, of the initially adsorbed TLL-α was displaced during the 180 min after adsorption when FRAP was conducted.

- Displacement of TLL-α by surfactants was also observed with SPR (Paper V). 0.9 µM unlabeled TLL-α was coadsorbed with different concentrations of C_{12}E_{6}/LAS to a C18-terminated chip surface and the steady-state surface excess (mg/m^2) was corrected for
bulk contribution and measured before rinsing. By comparison of the surface excess data with and without enzyme, the net contribution of enzyme to the surface excess, i.e. 
\((TLL + C_{12}E_{6}/LAS) - (C_{12}E_{6}/LAS)\), was estimated and plotted in Fig. 16 B. At cmc (150 
\(\mu M\) \(C_{12}E_{6}/LAS\)) or above, no net contribution from TLL was visible, which meant that 
all lipase was displaced from the surface.

![Displacement of TLL-\(\alpha\) (CLSM)](image1)

**Figure 16.** (A) Displacement of TLL-\(\alpha\) measured in CLSM. Time evolution of the surface fluorescence intensity from lipase coadsorbed with different concentrations of \(C_{12}E_{6}/LAS\). \(\theta/\theta_{\text{max}}\) was between 0.1 and 0.15. The intensity values were normalized to the value found without surfactants, so that bleaching of the dye etc. would not contribute to the intensity decrease. (B) Displacement of TLL-\(\alpha\) measured with SPR. The surface excess on a C18 surface before rinsing of \((TLL-\alpha + C_{12}E_{6}/LAS)\) minus the surface excess with only \(C_{12}E_{6}/LAS\) is plotted vs. surfactant concentration.

- The TLL-\(\alpha\) surface mobility at low surface density of lipase \((\theta/\theta_{\text{max}} < 0.20)\) and with 150 
\(\mu M\) \(C_{12}E_{6}/LAS\) was also measured with FRAP under flow conditions (Paper V). The flow 
diluted displaced TLL-\(\alpha\) more effectively from the bulk, reducing the probability of TLL-
\(\alpha\) to rebind to the surface. It was found that the apparent surface diffusion decreased 
when applying a flow, which indicated that desorption-rebinding was likely to have a 
significant contribution to the lateral mobility under no-flow conditions.

- TIR-FCS was used to study on-off kinetics of TLL-\(\beta\) when competing with \(C_{12}E_{6}/LAS\) 
for binding sites on a C18 modified glass surface (Paper VI). 20 nM FITC-labeled TLL-\(\beta\) 
was let to coadsorb with 1 or 10 \(\mu M\) surfactant and the autocorrelation function (ACF) 
was detected immediately after addition of the sample to the surface. It was clearly visible 
in the ACF (Fig. 17) that the term in the ACF representing surface binding of TLL-\(\beta\), \((\tau 
> 0.01 \text{ s})\) decreased with surfactant concentration. The ACFs were fitted to models of
reversible adsorption kinetics, and the deduced surface reaction rate, $R_b$, for TLL-β increased with surfactant concentration. Under the experimental conditions, $R_b$ was equal to the dissociation rate of the lipase and thus, the inverse of $R_b$ reported on the average time the lipase was bound to the surface. Hence, lipase irreversibly bound to the surface without surfactants was shown to bind to the surface for shorter periods of time in the presence of surfactants.

![Figure 17. Autocorrelation functions (ACFs) for TLL-β on C18-modified glass surfaces without and in the presence of 1 or 10 µM C12E6/LAS. The amplitude at $\tau > 0.001$ s reflects the surface interaction of the lipase. The surface interaction rate $R_b$ could be quantified by fitting the ACFs to theoretical models of reversible binding kinetics.](image)

The results obtained from the different techniques indicated that the lipase surface diffusion in the presence of surfactants was boosted due to a desorption-rebinding mechanism. It was evident that the surfactants were able to displace TLL-α from the hydrophobic surface, and that the presence of surfactants decreased the average time the lipase was bound to the surface (Paper V and VI). A proposed model for lipase surface mobility is given in Fig. 18. Without surfactants, lipases migrate across the hydrophobic surface through lateral diffusion without leaving the surface (Fig. 18 A). The docked configuration, with the active site exposed to the surface, is assumed to represent an adsorbed state of very low mobility, which is practically measured as immobile in the FRAP experiment. In the presence of surfactants, a fraction of the adsorbed lipases are displaced from the surface (Fig. 18 B). This fraction probably represents the most mobile species of adsorbed lipases that have bound less tightly to the surface. The redistribution of lipases via desorption-rebinding contributes to an apparent higher diffusion coefficient. However, displaced lipases might also diffuse into bulk, which would further explain why a
decrease in observed mobility with time was observed. Thus, in the presence of surfactants, the decrease in mobility with time is thought to be due to both an orientation-redistribution, similar to what was proposed for diffusion without surfactants, and a dilution of the most mobile species off the surface. Since the proposed “hopping diffusion” was possible to monitor and quantify with FRAP, displaced lipase were thought to readsorb near the desorption site. Otherwise, the fluorescence recovery would not have followed the solution of the diffusion equations (Eq. 12 - 15), which means that the lipase surface mobility in the presence of surfactants could macroscopically be treated as a lateral diffusion process.

Figure 18. A proposed model for lipase surface diffusion without and in the presence of surfactants. (A) The lipase adsorbs to the surface in several different orientations. The orientation with the active site facing the surface is considered to be of very low mobility. Lipases diffusion occurs without leaving the surface. (B) In the presence of surfactants, the lipase lateral mobility is severely enhanced. Lipases diffusing on the surface can be displaced by surfactants but also readsorb to the surface, leading to an apparent higher lateral mobility. However, displaced lipases can also diffuse into bulk solution, which leads to a dilution of the most mobile lipases on the surface.

Table 2. Summary of the FRAP experiments of TLL-α and TLL-β on different surfaces

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Surface</th>
<th>C&lt;sub&gt;12&lt;/sub&gt;E&lt;sub&gt;6&lt;/sub&gt;/LAS (µM)</th>
<th>D&lt;sub&gt;0, t=0-90&lt;/sub&gt; (× 10&lt;sup&gt;-11&lt;/sup&gt; cm&lt;sup&gt;2&lt;/sup&gt;/s)</th>
<th>D&lt;sub&gt;0, t=90-180&lt;/sub&gt; (× 10&lt;sup&gt;-11&lt;/sup&gt; cm&lt;sup&gt;2&lt;/sup&gt;/s)</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLL-β</td>
<td>Si-OTS (C18)</td>
<td>-</td>
<td>0.8</td>
<td>0.5</td>
<td>~ 0.45</td>
</tr>
<tr>
<td>TLL-α</td>
<td>Si-OTS (C18)</td>
<td>-</td>
<td>0.8</td>
<td>0.8</td>
<td>~ 0.40</td>
</tr>
<tr>
<td>TLL-β</td>
<td>Si-DDS</td>
<td>-</td>
<td>2.7</td>
<td>1.1</td>
<td>~ 0.60</td>
</tr>
<tr>
<td>TLL-β</td>
<td>Si</td>
<td>-</td>
<td>2.7</td>
<td>2.0</td>
<td>~ 0.60</td>
</tr>
<tr>
<td>TLL-α</td>
<td>Si-OTS (C18)</td>
<td>25</td>
<td>1.0</td>
<td>1.0</td>
<td>~ 0.40</td>
</tr>
<tr>
<td>TLL-α</td>
<td>Si-OTS (C18)</td>
<td>150</td>
<td>9.3</td>
<td>7.0</td>
<td>~ 0.70</td>
</tr>
<tr>
<td>TLL-α</td>
<td>Si-OTS (C18)</td>
<td>500</td>
<td>13.1</td>
<td>7.9</td>
<td>~ 0.80</td>
</tr>
<tr>
<td>TLL-β</td>
<td>Trimyristin</td>
<td>-</td>
<td>4.1</td>
<td>3.0</td>
<td>0.4-0.6</td>
</tr>
<tr>
<td>TLL-α</td>
<td>Trimyristin</td>
<td>-</td>
<td>17.9</td>
<td>10.4</td>
<td>0.4-0.6</td>
</tr>
</tbody>
</table>
5.5 Effect of activity on lipase surface mobility on a triglyceride substrate surface

The mobility of the two lipase variants differed significantly on spin-coated surfaces of trimyristin, which is a triglyceride (tri C14) and thus a substrate for the lipases (Paper VII). The active TLL-α was found to be more than four times as mobile than the inactive TLL-β. (Fig. 14 E-F and Table 2). On surfaces saturated with lipase, the diffusion of the active and inactive TLL was similar, about $1.0 \times 10^{-11}$ cm$^2$/s (Table 2). This suggested that crowding effects dominated the diffusion rate at high surface density. An explanation why the active lipase, able to hydrolyze the underlying substrate, migrated faster across the surface compared to the inactive variant might be difference in affinity for the trimyristin surface. However, since the lateral mobility was similar on model hydrophobic surfaces, where no distinct difference in affinity was found, the difference in mobility on the trimyristin surface might arise from the activity of TLL-α.

The effect of activity on desorption of adsorbed TLL-α and TLL-β from trimyristin was studied in the confocal microscope (Paper VII). The surface intensity was continuously measured during flow after adsorption of the fluorescently labeled lipase variants. It was found that the active TLL-α desorbed from the surface, i.e. the fluorescence intensity signal decreased as a function of time, whereas the signal from TLL-β remained constant. During no-flow conditions, most desorbed molecules could be expected to rebind to the surface. This was also an evidence that the active TLL-α bound less tightly to the trimyristin surface or that the catalytic cycle included either desorption or transformation to an adsorbed state that detached from the surface upon rinsing. This might have contributed to the higher mobility found for the active lipase.

Furthermore, an active lipase diffusing in the interface region would generate fatty acids upon triglyceride degradation. The negatively charged myristic acid residues may accumulate at the triglyceride surface, since the measurements were performed under no-flow conditions and the solubility in water for myristic acid is extremely low (0.2 ng/μl). Accumulation of fatty acids at the substrate surface may also induce molecular reorganizations of the surface that alters the hydrolysis rate of a lipase.$^{108,109}$ A build-up of negative charge at the interface would increase the electrostatic repulsion with the overall negatively charged lipase. This could affect the mobility in two different ways. One possibility is that the production of fatty acids that become incorporated in the surface region inhibit the mobility of the lipase. Another possibility is that increased electrostatic repulsion has the opposite effect, i.e. increases lateral TLL-mobility. The spot-bleaching (Fig. 7A) allowed detailed analysis on the effect of activity on TLL mobility on trimyristin.
triglycerides. The FRAP method allowed estimation of both $D$ and $f$ on the trimyristin surfaces in intervals of 15 min after adsorption instead of 90 min as with the rectangular bleaching (Fig. 7B). Examples of the time evolution for $D$ on surfaces of low and high $\theta/\theta_{\text{max}}$ for both lipase variants are shown in Fig. 19. For TLL-$\alpha$ at low $\theta/\theta_{\text{max}}$, the diffusion was strongly dependent on the time after adsorption, dropping from $13.7 \times 10^{-11} \text{ cm}^2/\text{s}$ in the interval 0-15 min after adsorption to $4.4 \times 10^{-11} \text{ cm}^2/\text{s}$ in the interval 165-180 min after adsorption (Fig. 19 A). At high $\theta/\theta_{\text{max}}$, the diffusion was almost independent of time after adsorption. With the inactive TLL-$\beta$, only a small decrease was observed at low $\theta/\theta_{\text{max}}$ and no decrease was found with TLL-$\beta$ at high $\theta/\theta_{\text{max}}$ (Fig. 19 B). On the model hydrophobic surfaces, the decrease was thought to arise from orientational redistribution or relaxation of the lipase structure on the surface. On the substrate surface of trimyristin, product inhibition may also have contributed to the decrease in diffusion rate with time seen with the active lipase at low $\theta/\theta_{\text{max}}$, which supports why a significantly decrease was not seen with the inactive lipase variant. However, one might also argue that the generation of fatty acids results in an increase in mobility. Electrostatic repulsion could force the lipase to explore other parts of the surface e.g. by inducing an orientation that bind with low affinity to the surface (see further Section 5.6). This could explain why desorption from the trimyristin surface under flow conditions was found with the active lipase but not with the inactive.

![Figure 19](image_url)

**Figure 19.** Lateral diffusion coefficient averaged in 15 min time intervals for the active TLL-$\alpha$ (A) and the inactive TLL-$\beta$ (B), respectively, on trimyristin substrate surfaces is plotted as a function of time after adsorption. The diffusion on surfaces of low (*) and high (Δ) adsorbed amounts is displayed.
5.6 Lipase diffusion mechanisms on a triglyceride substrate surface

To look further into what mechanisms governed the lipase diffusion on a triglyceride surface, single particle tracking was performed with TLL-α conjugated to Quantum Dots (QDs) on spin-coated trimyristin surfaces (Paper VIII). FRAP measured the macroscopic diffusion behavior of an ensemble of molecules, whereas the use of QDs can yield information of the microscopic diffusion behavior of single lipase molecules in the system. 20 nM QD-lipase samples were used, which resulted in a very low lipase concentration on the surface, i.e. a much lower $\theta/\theta_{\text{max}}$ than what could be achieved during the FRAP measurements ($\theta/\theta_{\text{max}} < 0.1$). Trajectories of single QD-lipase molecules on trimyristin could be collected within 15 min after adsorption using the confocal microscope. Two examples are shown in Fig. 20. The trajectories had a “bead on a string” appearance, with a restricted motion in certain regions of the surface, then migration or transport to another region where the restricted diffusion continued. This gave rise to clusters in the trajectories and suggested that the lipase operated in two different modes on the surface, hereafter referred to as “cluster mode” and “transport mode” The lipase diffusion coefficient could be quantified by calculating the mean square displacement (MSD) of the whole trajectories and apply Eq. 17 (p. 25). This resulted in an average $D = 8.0 \pm 5.0 \times 10^{-10} \text{ cm}^2/\text{s}$, i.e. a bit higher than $D_{0,c=0.95}$ found for TLL-α on the same surface (Table 2).

![Figure 20](image-url)
Trajectories were also recorded when applying an external flow to the system. Two examples are shown in Fig. 21. It was apparent that the lipase movement was affected by the flow; the single molecules were “pushed” in the direction of the flow, i.e. a driven diffusion motion. However, the molecules could at intermittent periods of time undergo random movement similar to the cluster regions found with no-flow. The diffusion coefficient calculated in the cluster mode during flow was similar to what was found with no-flow, with $D = 5.4 \pm 3.7 \times 10^{-10} \, \text{cm}^2/\text{s}$. To study the difference between the cluster and transport mode, the trajectories were analyzed in more detail in terms of the step-length between sampled timepoints. It was found that the step-length was always higher in the transport mode compared to the cluster mode. Moreover, the step-length during cluster mode under flow was equivalent to what was found with no-flow (≈0.5 μm) but increased to several μm in the transport mode.

**Figure 21.** Trajectories of single QD-lipase movements on trimyristin under flow conditions. The flow direction is indicated by the arrows. To the right are the regions of the trajectories magnified where the lipase motion was restricted, i.e. the cluster mode.
Hence, the trajectories measured during flow supported the theory that the lipase operated in two different modes on the surface. The cluster mode seemed unaffected by external influence such as flow, since both $D$ and the step-length were unaffected. This mode is likely associated with enzymatic degradation of the substrate and thus an orientation of the lipase molecule with the hydrophobic active site region facing the surface. The transport mode, when migrating between the cluster regions seen in the trajectories (Fig. 20 and 21), was affected by the applied flow, which resulted in a pronounced increase in step-length. However, the single molecule tracking suggested that no complete detachment of the lipase from the surface occurred during transport mode, since no desorption into bulk solution was visible. This might be explained by the possibility of some segments of the lipase always being in contact with the surface, e.g. a crawling or rotational diffusion process. A tentative explanation for how and why the conversion from cluster mode to transport mode occurs could be generation of negatively charged fatty acids at the interface (in analogy with what was discussed on page 46). This could affect the lipase molecule, which is overall negatively charged, to reorient and operate in the transport mode. In the presence of surfactants, which would solubilize the fatty acids, the cluster modes would then be larger, i.e. continue for longer periods of time. Another explanation could be that the lipase operates in cluster mode until it reaches discontinuities in the trimyristin film, which force the lipase into transport mode. The proposed model is schematically shown in Fig. 22.

**Figure 22.** Proposed model for lipase surface migration on the trimyristin surface. The lipase is thought to operate in two different modes. During cluster mode, the lipase motion is restricted to the cluster regions found in the trajectory and the motion is not affected by external influence such as flow. The lipase is thought to be oriented with the active site facing the surface and hydrolyze the triglyceride. In the transport mode, i.e. between the clusters, the active site is no longer associated with the substrate. This migration is affected by external flow, and might be a crawling- or a rotational diffusion process with some segments always attached to the surface.
Hence, the QD and FRAP experiments show the difference in microscopic and macroscopic diffusion. On the microscopic, single molecular level, the lipase mobility could not be described as a random diffusion process but the behavior of an ensemble of lipase molecules, i.e. the macroscopic diffusion measured with FRAP could be treated as a lateral diffusion process. It should be noted that the proposed model does not include a complete desorption of lipases from the surface while diffusing, which was suggested when analyzing the desorption behavior of the two lipase variants by applying a flow (p. 45). However, a five times higher flow was used in the desorption experiments (Paper VII) compared to the flow in the QD experiments (Paper VIII), which might explain why no desorption of QD-lipase complexes was found. Nevertheless, the surface diffusion of TLL-α on trimyristin seemed to include a state of the enzyme where it was not fully attached to the surface, where it could or could not be desorbed depending on the experimental conditions.
Conclusions

6. Conclusions

- The adsorption of *Thermomyces lanuginosus* lipase (TLL) was affected by the hydrophobicity of the surface. Adsorbed amount and affinity was higher on hydrophobic surface compared to a hydrophilic surface. Moreover, a larger fraction of molecules adsorbed with the active site facing solution on a hydrophilic surface.

- The lateral mobility of TLL was higher on hydrophilic surfaces compared to hydrophobic. On hydrophobic surfaces, the surface diffusion decreased with time after adsorption, explained by more lipases being oriented with the active site facing the surface with time.

- The presence of surfactants clearly modulated the mobility of adsorbed lipases. This is thought to arise from a surfactant induced desorption-rebinding mechanism. Lipases on the surface can be displaced from the surface by surfactants but also readсорb, which lead to a higher apparent lateral diffusion or exploration of the surface. Lipase displacement was confirmed by confocal microscopy and SPR studies and with TIR-FCS, it was established that the time the lipase was bound to the surface decreased with increased surfactant concentration.

- On a triglyceride substrate surface, the lipase motion can be described by two different modes. In the *cluster mode*, the lipase is oriented with the active site facing the surface, leading to enzymatic degradation of the substrate. Lipase motions are then restricted to certain regions of the surface and unaffected by external flow. In the *transport mode*, the lipase migrates across the surface and is bound less tightly to the surface, since this mode was clearly affected by an external flow. This was confirmed by single molecule tracking of lipase molecules conjugated to Quantum Dots.
7. Outlook

This project aimed to give insight in the interfacial activity of lipases and see how different factors affected the lateral mobility of the enzyme. Since this is a rather unexploited area of research, the work performed within this study gave rise to several ideas for future work:

i) It would be very interesting to use triglyceride substrate surfaces in the ellipsometer or construct a triglyceride sensor surface for DPI or SPR. This would both allow quantification of lipase adsorption to a substrate and a measure of subsequent activity by studying the decrease in e.g. film thickness with time. In this work, only qualitative results could be achieved in the confocal microscope. Moreover, if it was possible to coat coverslip glass with thin triglyceride films, lipase-surfactant dynamics at a real substrate could be quantified with TIR-FCS.

ii) The lipase mobility was in this project measured on trimeystein (tri-C\(_{14}\)), whose fatty acid residues have extremely low solubility in water. An interesting approach is therefore to use spin-coated surfaces of tricaprin (tri-C\(_{10}\)), where the capric acid is soluble in water. By performing FRAP with active lipase on tricaprin where the formed fatty acids could leave the interface would give further insight in how the lateral migration was affected by the generation of charge at the interface.

iii) Labeling lipases with Quantum Dots yielded new information of the mechanism behind lipase mobility. It would therefore be of great interest to study lipase-surfactant interactions at the single molecule level on the studied surfaces to see if the desorption-rebinding mechanism is seen. On the triglyceride surfaces, it would be valuable to study if the cluster- or the transport mode was affected by surfactants.

iv) The proposed model for lipase migration on triglycerides, with the enzyme operating in two modes, would be interesting to further validate by modeling. The lipase could be modeled as a molecule with one hydrophobic patch with high affinity for the surface and the rest of the molecule with less affinity. Regeneration of the bead-on-string trajectory would be a great support for the proposed model.
8. Acknowledgements

Many people have supported me during the work in this project, but there are some people that I would like to thank in particular:

Of course, a lot of credit goes to my supervisors, Prof. Hjalmar Brismar, Dr. Ulla Elofsson and Dr. Thomas Callisen. Thank you for excellent support and guiding in the project and for starting up this interesting collaboration! I have really appreciated your interest in teaching me your different areas of expertise.

Novozymes A/S, Denmark is acknowledged for financial support of the project. Special thanks to Erik Gormsen for useful discussions and input to the project. Lene Bjorg Cesar and Leigh Murphy are acknowledged for assistance in the laboratory work.

Thanks to Dr. Hans Blom and Dr. Kai Hassler at Department of Biomolecular Physics, KTH, for a very nice collaboration. I have learned a lot from you and really appreciated our discussions (hope you enjoyed working with “bioslem” and “voodoo science”…). Also thanks to Dr. Katrin Boschkova, YKI, for pep-talks and general support.

Rolf Helg at AlbaNova is acknowledged for excellent work on different flow cells for the confocal microscope and Britt Nyström at YKI for all the help in finding literature. Thanks also to Hasse, Annika and Anne-Marie for helping me in the lab at YKI.

Thanks to my colleagues and friends at YKI for a really nice time. Special thanks to my roommates Tobias, Jessica, Carina, Txell and Brita for nice lunches, innebandy etc. Thanks to the PhD-students at Cell Physics; Jacob, Gustav, Sanna, Erland, Padideh and Victor for making my time at AlbaNova really pleasant.

Last but not least, thanks to Hanna and the rest of my family for love and support!
9. References

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Acknowledgements