Abstract

Enzymes are fascinating nanomachines, which catalyze the reactions essential for life. Studying enzymes is therefore important in a biological and medical context, but the catalytic potential of enzymes also finds use in organic synthesis. This thesis is concerned with the fundamental question whether the catalytic reaction of an enzyme can cause it to show enhanced diffusion. Additionally, this thesis examines if it is possible for enzymes to collectively affect fluid flow, when they are incorporated in functional biohybrid nanostructures.

The diffusive behavior of enzymes is important for their distribution within cells and impacts their ability to reach their substrates. Several reports within the last decade have claimed that enzymes show diffusion enhancements of up to 80 %, when they are catalytically active. Examining these claims is important to achieve an understanding of their biological function. These reports were based on fluorescence correlation spectroscopy (FCS), which measures the fluorescence fluctuations of individual fluorophores (here, labeled enzymes) passing through a small focal volume. FCS is a powerful tool to study diffusion, but several photo- and biophysical processes can interfere with FCS measurements. The work presented in this thesis made theoretical predictions how these effects can lead to misinterpretations specific to FCS experiments of active enzymes. Additionally, these simulations were supported by multi-detector FCS experiments, which showed that the 80 % diffusion enhancement reported by others is actually a misinterpretation of a complex fluorescence quenching artefact. The FCS experiments reported within this thesis find no evidence for active enzyme diffusion enhancement.

To completely rule out the possibility of fluorescence artefacts, another diffusion measurement technique, which does not require labeling, was adapted for enzymes: Diffusion nuclear magnetic resonance (NMR) spectroscopy. Hence, the work presented in this thesis reports the first diffusion NMR experiment of an active enzyme. Earlier reports by others claim that enzymes, which catalyze endothermic reactions, self-propel and cause enhanced diffusion of molecules in their surroundings. The diffusion NMR studies in this

thesis reveal that neither the enzymes themselves nor tracer molecules in the solution show enhanced diffusion.

These findings of enzyme diffusion NMR measurements and the unraveling of artefacts in FCS enzyme measurements seriously question the hypothesis that enzymes are active matter and experience enhanced diffusion. The publication of these results was the first to experimentally question this hypothesis. Since then, several reports by others have appeared that support the predictions and experimental observations presented herein.

In addition to the academic interest, there is also a practical interest in enzymes due to their fascinating properties as catalysts. Biocatalysis, which uses enzymes or whole organisms for organic synthesis, has several advantages over conventional catalysis. Enzymes are, for instance, highly selective and efficient, can be operated in mild conditions and are safe to dispose of. The natural enantioselectivity of enzymes in some reactions is of special interest for the synthesis of pharmaceuticals, which are often homochiral. However, reuse of enzymes by recovery from reaction mixtures is problematic due to their small size and fragility. Immobilization of enzymes onto microparticles simplifies the recovery step, but often lowers the enzymes' catalytic activity. In this thesis, a novel nanoconstruct is presented, which allows easy recovery of enzymes with a magnet, but still ensures high enzymatic activity. In this construct, filamentous viruses are utilized as intermediate immobilization templates between the microparticle and the enzyme. This novel nanoconstruct has been termed enzyme-phage-colloid (E-P-C). Within this thesis two applications for E-P-Cs are presented. The first application is the repeated use of E-P-Cs as biocatalysts with easy magnetic recovery between each reaction cycle. Activity assays showed that the enzyme had even higher catalytic turnover, when it was immobilized on the E-P-C. In the second application, E-P-Cs were used to construct an enzymatic micropump, which is a microdevice that creates convective flows due to density differences with a locally catalyzed reaction. E-P-Cs can conveniently be immobilized with a magnet onto the wall of a microcontainer to form an enzymatic micropump. These E-P-C micropumps are shown to generate the fastest flow speeds of an enzymatic micropump to date. Additionally, it is shown that urease E-P-Cs can pump blood at physiological urea concentrations, which might enable medical lab-on-a-chip applications.

The last part of this thesis considers the diffusion of active molecular catalysts. These synthetic catalysts have recently been reported to show enhanced diffusion in diffusion NMR experiments similar to enzymes. This is particularly surprising as the molecular catalysts exhibit catalytic turnover rates that are orders of magnitude lower than the ones of enzymes. In addition to the proposed self-propulsion, it was claimed that the molecular catalysts transfer kinetic energy to the surrounding solvent and substrate molecules, thereby causing their diffusion enhancement. However, re-examination of these diffusion NMR studies showed that there is no diffusion enhancement of molecular catalysts and the misinterpretation of the diffusion NMR experiments is caused by a complex artefact due to intensity changes over the course of the diffusion experiment, which are caused by relaxation phenomena. These results have recently been submitted for publication.

Kurzzusammenfassung

Enzyme sind faszinierende Nanomaschinen und katalysieren alle Reaktionen, die essentiell für das Leben sind. Die Forschung an Enzymen ist daher notwendig für die Biologie und Medizin, aber die katalytischen Fähigkeiten von Enzymen finden auch Anwendung in der organischen Synthese. Diese Arbeit befasst sich mit der Frage, ob die enzymatische Katalyse bei Enzymen selbst erhöhte Diffusion hervorrufen kann. Außerdem untersucht sie, ob es möglich ist für Enzyme kollektiv Strömungen in Flüssigkeiten hervorzurufen, wenn sie in funktionale Biohybrid-Nanostrukturen eingebunden sind.

Das Diffusionsverhalten von Enzymen ist wichtig für ihre Verteilung in Zellen und beeinflusst die Fähigkeit von Enzymen Substrate zu erreichen. Mehrere Berichte innerhalb des letzten Jahrzehnts behaupten, dass Enzyme ihre Diffusion um bis zu 80 % erhöhen, wenn sie katalytisch aktiv sind. Dies hätte große Auswirkungen auf unser Verständnis der biologischen Funktion von Enzymen. Die erwähnten Berichte basieren auf Messungen der Fluoreszenzkorrelationsspektroskopie (FCS), bei der die Fluoreszenzfluktuationen einzelner Fluorophore (hier, fluoreszenzmarkierte Enzyme) gemessen werden, während diese sich durch ein kleines fokales Volumen bewegen. FCS ist eine wertvolle Methode für Diffusionsmessungen, wird aber von foto- oder biophysikalischen Prozessen beeinflusst. Diese Arbeit beinhaltet theoretische Voraussagen darüber wie diese Prozesse zu Fehlinterpretationen speziell im Fall von FCS Messungen aktiver Enzyme führen können. Zusätzlich wurden diese Simulationen von Multidetektor-FCS Messungen bestätigt, was zu der Erkenntnis führte, dass die berichteten 80 % Diffusionserhöhung tatsächlich auf einer Fehlinterpretation eines komplexen Fluoreszenzlöschungsartefakts basieren. Die FCS Messungen in dieser Arbeit zeigen daher keine Diffusionserhöhung aktiver Enzyme.

Um Fluoreszenzartefakte komplett ausschließen zu können, wurde eine weitere Methode zur Diffusionsmessung, welche keine Probenmarkierung benötigt, auf Enzymmessungen erweitert. Hierbei handelt es sich um die Methode der Diffusionskernspinresonanz-spektroskopie (Diffusions-NMR-Spektroskopie). Diese Arbeit beinhaltet daher die ersten Diffusions-NMR-Messungen aktiver Enzyme. Frühere Arbeiten anderer Forschungsgruppen

berichten von erhöhter Diffusion von Enzymen, die endotherme Reaktionen katalysieren, was außerdem angeblich zu erhöhter Diffusion von Molekülen in der Umgebung des Enzyms führen soll. Diffusions-NMR-Messungen in dieser Arbeit zeigen jedoch weder für Enzyme selbst noch für Moleküle in der Reaktionslösung Diffusionserhöhung.

Diese NMR-Ergebnisse zur Enzymdiffusion und die Entdeckung von Artefakten in FCS-Messungen von Enzymen widersprechen der Hypothese, dass Enzyme aktive Materie sind und aktiv ihre Diffusion erhöhen. Die Veröffentlichungen dieser Ergebnisse stellen als erste experimentelle Studien diese Hypothese in Frage. Weitere Publikationen anderer Forschungsgruppen untermauern die hier präsentierten Ergebnisse.

Zusätzlich zu dem akademischen Interesse gibt es auch ein wirtschaftliches Interesse an den beeindruckenden katalytischen Fähigkeiten von Enzymen. Die Biokatalyse, welche Enzyme oder vollständige Organismen zur organischen Synthese verwendet, hat mehrere Vorteile gegenüber der konventionellen Katalyse. Enzyme sind zum Beispiel hoch selektiv, effizient, können unter milden Bedingungen arbeiten und sind sicher in der Entsorgung. Die natürliche Entioselektivität von Enzymen in einigen Reaktionen ist von besonderem Interesse für die Synthese pharmazeutischer Wirkstoffe, da diese oft homochiral sind. Jedoch ist die Wiedergewinnung von Enzymen aus Reaktionsmischungen problematisch aufgrund ihrer kleinen Größe und Sensibilität. Immobilisierung von Enzymen hingegen kann die Wiedergewinnung stark vereinfachen, erniedrigt aber oft die Enzymaktivität. Diese Arbeit präsentiert ein neuartiges Nanokonstrukt, welches die einfache Wiedergewinnung von Enzymen mit einem Magneten erlaubt und dabei die katalytische Aktivität des Enzymes erhält. Das Konstrukt besteht aus filamentösen Viren, welche als intermediäre Immobilisierungstemplate Mikropartikel und Enzym verbinden. Dieses neuartige Nanokonstrukt wird als Enzym-Phagen-Kolloid (E-P-C) bezeichnet. Innerhalb dieser Arbeit werden zwei Anwendungen von E-P-Cs demonstriert. Die erste Anwendung ist der wiederholte Einsatz von E-P-Cs als Biokatalysatoren mittels magnetischer Rückgewinnung zwischen den Reaktionszyklen. Aktivitätsmessungen zeigten, dass die katalytische Aktivität der Enzyme sogar erhöht ist, wenn sie auf dem Nanokonstrukt immobilisiert sind. Die zweite Anwendung beinhaltete die Verwendung von E-P-Cs in enzymatischen Mikropumpen, welche

Konvektionsströme durch Dichtegradienten mittels lokaler Katalyse erzeugen. Um eine enzymatische Mikropumpe herzustellen, können E-P-Cs mit einem Magneten einfach an der Wand eines Mikrocontainers lokalisiert werden. Die enzymatischen Mikropumpen, welche auf diese Art hergestellt wurden, erzeugen die schnellsten Flussgeschwindigkeiten, die bis heute in enzymatischen Mikropumpen gemessen wurden. Zusätzlich wurde gezeigt, dass E-P-Cs aus Urease Blut mittels seines natürlichen Harnstoffgehaltes durch enzymatische Mikropumpen bewegen können. Dies könnte in Form von mikrofluidischen Lab-on-a-Chip Systemen Anwendung in der Medizin finden.

Der letzte Teil dieser Arbeit befasst sich mit der Diffusion von aktiven molekularen Katalysatoren. Vor kurzem veröffentlichte Studien basierend auf Diffusions-NMR-Messungen berichten von erhöhter Diffusion dieser synthetischen Katalysatoren in einer ähnlichen Weise wie es für Enzyme vermutet wird. Diese Hypothese ist besonders verwunderlich, da molekulare Katalysatoren um Größenordnungen niedrigere Aktivitäten aufweisen als Enzyme. Zusätzlich zu der vermeintlichen Eigendiffusionserhöhung wird auch von einem Übertrag der kinetischen Energie der molekularen Katalysatoren auf die sie umgebenden Substrat- und Lösungsmoleküle berichtet, was wiederum zu deren Diffusionserhöhung führen soll. Der Teil dieser Arbeit, welcher sich diesem Thema widmet, wurde vor Kurzem zur Veröffentlichung eingereicht. Es war möglich zu zeigen, dass weder die molekularen Katalysatoren noch die Moleküle in ihrer Umgebung Diffusionserhöhung erfahren und dass die Fehlinterpretationen der Diffusions-NMR-Messungen auf komplexen Artefakten basiert, welche durch Signalintensitätsänderungen basierend auf Relaxationsphänomenen während des Diffusionsexperiments hervorgerufen werden.

1 Introduction

Enzymes are nature's smallest machines and essential for life itself. From the production of ATP by ATP synthase in mitochondria to the motion of muscles by myosin all life is controlled by enzymes. These examples are motor proteins, which convert chemical energy into rotational or linear motion during their catalytic cycle. Motor proteins are embedded in membranes or moving along protein fibers to transfer their motion, but the majority of enzymes is freely diffusing in cells or other biological fluids. Several reports within the last decade have claimed that these freely diffusing enzymes may also be able to propel themselves, i.e., swim, when they are catalytically active.[7–10] If this claim would be true, it would change our fundamental understanding of the function and motion of enzymes and therefore would have far-reaching implications for the biology of the cell, pharmaceuticals and medicine. One part of this thesis is therefore dedicated to testing the active enzyme diffusion hypothesis with advanced diffusion measurement techniques.

The initial reports in support of this hypothesis were based on measurements of fluorescence correlation spectroscopy (FCS). This technique, which is discussed in detail in Chapter 4.1, measures the diffusion of fluorophores (here, fluorescently labeled proteins) through the confocal volume in a microscope. The time needed to pass through this volume is therefore a measure of the samples diffusion coefficient. Artefacts, which change the photophysical behavior of the fluorophore or the diffusing sample, can, however, lead to misinterpretations of the otherwise very precise FCS method, as is shown in Chapter 7 (Ref. 1). Therefore, multi-detector FCS measurements together with careful enzyme purification studies were performed within this thesis to thoroughly test the active enzyme diffusion hypothesis. Initially, two enzymes (alkaline phosphatase and F_1-ATPase) were tested and the results are presented in Chapter 7 (Ref. 1).

To avoid any possible photophysical artefacts, a label-free technique was also chosen to test the active enzyme diffusion hypothesis. Therefore, diffusion nuclear magnetic resonance (NMR) measurements were introduced within this thesis as an alternative to FCS

measurements to carefully measure the diffusion of enzymes and solvent molecules. This is demonstrated for the enzyme aldolase (see Chapter 8, Ref. 2), which is of particular interest, since it has been reported[9] to show enhanced diffusion even though no heat is released during its catalyzed reaction. All experiments presented in Chapter 7 and 8 (Ref. 1 and 2) found no evidence of enhanced enzyme diffusion. Rather, it was possible to identify measurement errors in earlier publications by other groups,[11,8,9,12] which had reported enhanced enzyme diffusion. The implications of the results presented in Chapter 7 and 8 (Ref. 1 and 2) for the field of enhanced enzyme diffusion are summarized in Chapter 11.

In addition to the academic interest in enzymes for a deeper understanding of biochemical processes, there is also interest in enzymes as biocatalysts for applications in synthesis. This is due to several advantages that enzymes have over conventional chemical catalysts (see Chapter 6.1). Enzymes are highly selective and efficient catalysts, which can be operated in mild conditions and are safe to dispose of, just to name a few advantages. The natural enantioselectivity of enzymes in some reactions is of special interest for pharmaceuticals which are often homochiral. In recent decades, biocatalysis has reached increasing importance due to novel efforts in directed enzyme evolution, which increases the spectrum of reactions enzymes can catalyze. However, separation of enzymes from the reaction mixture is problematic due to their small size and fragility, but their recovery is of economic interest. Immobilization of enzymes onto microparticles simplifies their recovery, but often lowers their catalytic activity, since the enzyme can be locked in certain conformational positions due to tight binding to a solid template or the supply of substrate his hindered, if the enzyme's active site is facing the surface of the template. In this thesis, a novel nanoconstruct is presented, which allows easy recovery of the enzyme with a magnet, but still ensures high enzyme activity. In this construct, filamentous viruses, which are harmless to humans, have been utilized as templates to immobilize enzymes. The viruses were then anchored onto a magnetic carrier particle. There is no reduction of the enzyme's activity, since virus and enzyme both consist of proteins and since the virus is a much more flexible template than a solid surface and allows sufficient substrate diffusion to the enzyme due to

its filamentous structure. This novel nanoconstruct has been termed enzyme-phage-colloid (E-P-C) and is introduced together with two of its applications in Chapter 9 (Ref. 3).

Recently, similar claims to the active enzyme diffusion hypothesis have been made about (synthetic) molecular catalysts.[13] It has been claimed that the transfer of energy from catalysts to substrate and solvent molecules in their surrounding is a common phenomenon in chemical reactions. This would have substantial impact on our understanding of the chemical reaction. So far it was believed that energy released by chemical reactions (including catalyzed reactions) is dissipated on small length scales and fast timescales via vibrational energy relaxation processes. The authors of Ref. 13 suggest that a substantial amount of energy from the reaction is transferred into enhanced diffusion without significant temperature change of the solution, which seems unphysical since the molecules move within the highly overdamped low Reynolds number regime (Chapter 2.1). Additionally, the claims in Ref. 13 are especially extraordinary, since molecular catalysts have orders of magnitude lower catalytic turnover rates than enzymes, which leads to a much lower power output of the catalyst. Since the enhanced diffusion of enzymes has been deemed thermodynamically impossible by some,[14] this would make enhanced diffusion for molecular catalysts even more unlikely. Nevertheless, it is necessary to find an explanation for the observations in Ref. 13. Therefore, the experiments in Ref. 13 have been reproduced as part of this thesis, but with thorough controls, which could reveal an entirely different explanation, which is in fact not at all connected to active diffusion, but to intensity changes due to relaxation phenomena during the NMR experiment (Chapter 10, Ref. 4).

This thesis is structured as follows. First, the fundamental aspects of motion at the nanoscale are described in Chapter 2. This includes the requirements for swimming at low Reynolds numbers and a description of Brownian motion. Active motion at the nanoscale is then discussed in Chapter 3, where self-phoresis is introduced. Additionally, Chapter 3 includes an overview of the experimental and theoretical reports on the active enzyme diffusion hypothesis and active molecular catalyst diffusion. The advanced diffusion measurement techniques, which are used in this thesis, are presented in detail in Chapter 4, which mainly includes FCS and diffusion NMR. Detailed knowledge of enzyme properties is needed for

enzyme diffusion measurements and to utilize enzymes in biocatalysis. Therefore, the properties of the enzymes studied in this thesis are presented in Chapter 5. How to utilize and immobilize enzymes is discussed in Chapter 6. These introductory chapters are followed by the published experimental results (Refs. 1–4) which are briefly summarized and reprinted in Chapters 7-10.