Developing Immobilized Biocatalysts with Advanced Interface for Applications in Organic Synthesis

vorgelegt von

M.Sc. Chemie

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Von der Fakultät II -Mathematik und Naturwissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften
-Dr. rer.nat.-

genehmigte Dissertation

Promotionsausschuss:

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Berichter: Professor Dr. Udo Kragl

Tag der wissenschaftlichen Aussprache: 27.09.2011

Berlin 2011

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谨以此文献给我的父母，
感谢他们的养育之恩！

Acknowledgement

To mentor:

- Professor Dr. **Marion B. Ansorge-Schumacher**: Thank you so much to give me the chance to join your group at TU Berlin, and to finance and support me to study such interesting topics. I am deeply impressed by your keen insights and great passions to science, and your endless patience and trust to me are much appreciated. I will not forget that every time after discussion I left your office with being motivated and excited to my future research. Anyway, thank you to inspire me to be my best!

To dissertation defense committee

- Professor Dr. **Udo Kragl**: I am really grateful that you could agree to come to Berlin for my PhD defense, and appreciate your time to correct my PhD dissertation.
- Professor Dr. **Michael Gradzielski**: Thank you to aggree to be the chairman in my PhD defense. Also I am grateful that you allowed me to make several experiments in your lab in the past years.

To organizations

- **UniCat**: Many thanks to UniCat, the Cluster of Excellence, to finance me for three years to complete my PhD research. My special appreciation to those people who organized hundreds of lectures and activities in the past for "Unifying Concepts in Catalysis".
- **BIG-NSE**: I am truly grateful to BIG-NSE, the outstanding "Berlin International Graduate School of Natural Science and Engineering", to accept me as a fellow at the beginning of my PhD. Within BIG-NSE, I am lucky to know many bright PhD students, especially these from batch 2008: **Subhamoy Bhattacharya**, **Sara Bruun**, **Manuel Harth**, **Kirstin Hobiger**, **Stanislav Jaso**, **Sardor Mavlyankariev**, **Sylvia Reiche**, **Carlos Carrero**, and **Lars Lauterbach**. I thank Professor Dr. **Reinhard Schomäcker** (TU Berlin) and Dr. **Jean-Philippe Lonjaret** (TU Berlin) for organization.

To collaborators

- Professor Dr. **Helmuth Möhwald** (MPI Potsdam): Thank you to support your student and group leader to collaborate with us for two projects.
- Professor Dr. **Regine von Klitzing** (TU Berlin): Many thanks to you for valuable discussion, and appreciate your permission for me to use several instruments in your group.
- Professor Dr. **Dayang Wang** (University of South Australia): Thanks for your fruitful discussion, and your great supports to our collaborations.
- Dr. **Shuo Bai (**MPI Potsdam**)**: It is my pleasure to cooperate with you to complete two projects. Especially thank you to implement some characterization of our samples.
- **Kornelia Gawlitza** (TU Berlin): Thanks for your contributions to our cooperation. It is my happy experience to complete the experiments, posters, presentations, and papers together with you**.**

To team players

- **Dirk Schlienz** (FU Berlin): Thanks for your hard and creative diploma work to make one challenging project successful.
- **Jonas Hanske** and **Erik Walinda** (FU Berlin): Thanks for your choice to come to the group of Professor Ansorge-Schumacher, and believe me to guide your short but successful internship.

To colleagues

- I thank all our former and current colleagues in the group of Professor Ansorge-Schumacher (TU Berlin) for your helps and discussion. Your helps made me easy to adapt and live in Berlin.
- Especially I am grateful to the kind friendship offered from you: Dr. **Lars Wiemann**, Dr. **Nora Bieler**, Dr. **Andy Maraite**, Dr. **Valantina Yazbik, Juliane Ratzka**, **René Nieguth**, **Alexander Scholz**, **Shenay Sali**, and **Christoph Loderer**. Particularly, I thank Juliane Ratzka and Christoph Loderer for German translation in this dissertation.

To buddies:

- Dr. **Tanguy Chau** (MIT Boston): Thank you, Tanguy! Thanks for your countless suggestions and discussion to help me make several important decisions of my family.
- **Jian Li**, **Mengjun Xue**, **Zhengfang Zhang**, Dr. **Jingyu Chen**, Dr. **Susan Kelleher** (TU Berlin): Nice to meet all of you in Berlin. I enjoyed every moment together with you.

To sister and brother:

- Thank you my elder sister (**Mengzhi Wu**) and my younger brother (**Changbao Wu**). Your love and supports to me will never be forgotten.

To family:

- **Wife:** Thank you so much to come to Berlin with me. Your love and patience mean a lot to me!
- **Son:** You are the biggest result and the best present of Dad's PhD period. I love you forever!

To parents:

- **Dad:** Thank you Dad (**Xiangui Wu**) for your patience, understanding, and hard working to raise and support me all the time!
- **Mom:** Thank you Mom (**Xiuhua Deng**) for your unconditional and everlasting love. Your perseverance taught me to never give up anything important to us; your dedication inspired me to sacrifice for something we love. Thanks for all you gave to me!

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Abstract

Biocatalysis by immobilized enzymes in organic solvent media has achieved tremendous importance for chemical and pharmaceutical synthesis within the last number of decades. However, in practical applications, their success is often compromised by serious mass transfer limitations during reactions. Limitations vary with different carriers, but a common feature is the formation of a poor interface between carriers, enzyme phase or reaction media, which greatly reduces the catalytic efficiency. Thus, improving the interface is crucial to enhance the biocatalytic performance of immobilized enzymes in organic solvents. In this thesis, three promising carriers (hydrophilic agar gels, hydrophobic silicone beads, and Pickering emulsions) were selected as representative study objects. Different strategies were employed to improve the poor interface of these carriers.

Typically, hydrophilic agar gels were obtained by dropping liquid agar solutions into hexane. Hydrophobic silicone beads were produced through suspension polymerization, i.e. by immersing hydrophobic silicone precursors in polyvinyl alcohol (PVA) solutions with constant stirring. Pickering emulsions were stabilized by silica nanoparticles. These carriers were used to immobilize different enzymes including lipase A and B from *Candida antarctica* and benzaldehyde lyase (BAL) from *Pseudomonas fluorescens*.

Esterification of octanol and octanoic acid was performed to assess lipase activity. Benzoin condensation was employed for BAL catalyzed reaction. Optimal reaction conditions were set up for each reaction as literature. Gas chromatography (GC) was used to determine substrates and products for calculation of enzyme activity and reaction conversion.

For hydrophilic agar gels, a solvent exchange process was used to improve the solubility of agar gels loaded with lipase B from *Candida antarctica* (CalB) in organic phase. By this approach, CalB labeled with fluorescence dye was transfered into cores of agar gels. This indicates that this method is able to wet the surface of hydrophilic gels and to concentrate enzymes into the gel cores to avoid solvent inactivation. It was also found that the stability and reusability of immobilized CalB were improved after the solvent exchange, compared to native enzymes. These results illustrate that the solvent exchange process is a good method to improve the solubility of hydrophilic carriers in organic solvents, thus improving their interface for catalysis.

For hydrophobic carriers, lipase A from *Candida antarctica* (CalA) was entrapped in biocatalytic active silicone beads (BASE), in which the aqueous enzyme solution was emulsified as numerous micro-pools. By calculation, these micro-pools have a large surface area, more than 300 times larger than they form as one sphere of liquid. Encouraged by this calculation, optimization of these silicone beads for catalysis was executed to further increase aqueous enzyme phase in silicone. Interestingly, a larger volume of aqueous phase in silicone beads resulted in a greater number of smaller micro-pools, which contributed to an even higher apparent activity of silicone beads. An optimal composition of the silicone beads consists of 8.8 g silicone and 5 mL enzyme solutions. Afterwards the water content in the defined ratio of silicone (g) to aqueous phase (mL) was further optimized. In addition, mass transfer limitation was assessed by penetration progression of dyed heptane in silicone beads. Thus through these studies, the interface of the hydrophobic silicone carriers was improved by optimizing them in terms of composition and water content for biocatalysis.

For Pickering emulsions, silica nanoparticles were used to encapsulate CalB and BAL, respectively. Surprisingly, assessment of the two enzyme activity revealed that CalB activity had increased more than 300 times, and BAL activity had enhanced 8 times. These results demonstrate that Pickering emulsions are a good system for both stable enzymes like CalB and susceptible ones like BAL. The enhanced activity of enzymes in Pickering emulsions is due to the large interface of these emulsions in organic phase.

During research, agar gels, silicone beads, and Pickering emulsions are selected as targeted carriers to immobilize enzymes for biocatalysis in organic media. Different strategies were used to improve the solubility of agar gels in solvents, and to extend the interfacial area of aqueous enzyme phase within the silicone beads, and to produce large surface area of Pickering emulsions in organic solvents. With these findings in three carriers, the contributions to improve the interface of diverse immobilization matrices for biocatalysis in organic media were demonstrated.

Zusammenfassung

Die Biokatalyse in organischen Lösunsgmitteln unter Verwendung immobilisierter Enzyme hat in den letzten Jahrzehnten eine enorme Bedeutung für die chemische und pharmazeutische Synthese erlangt. In technischen Anwendungen ist ihr Erfolg jedoch oft durch Stofftransferlimitierungen während der Reaktionen beeinträchtigt. Diese Einschränkungen variieren mit unterschiedlichen Trägern, ein gemeinsames Merkmal ist jedoch die Ausbildung einer geringen Grenzfläche zwischen dem Träger, der Enzymphase oder dem Reaktionsmedium, was die katalytische Effizienz stark reduziert. Aus diesem Grund ist die Verbesserung der Grenzfläche entscheidend, um die biokatalytische Leistung von immobilisierten Enzymen in organischen Lösungsmitteln zu erhöhen. In dieser Arbeit wurden drei vielversprechende Träger (hydrophile Agargele, hydrophobe Siliconperlen und Pickering-Emulsionen) als Gegenstand einer repräsentativen Studie ausgewählt. Verschiedene Strategien wurden dabei zur Verbesserung der geringen Grenzflächen dieser Träger verwendet.

Üblicherweise wurden hydrophile Agargele durch das Eintropfen einer flüssigen Agarlösung in Hexan erhalten. Hydrophobe Siliconperlen wurden durch Suspensionspolymerisation hergestellt, das heißt, durch Eintauchen hydrophober Siliconvorstufen in Polyvinylalkohol (PVA)-Lösungen unter ständigem Rühren. Pickering-Emulsionen wurden durch Silicat-Nanopartikel stabilisiert. Diese Träger wurden dazu verwendet, verschiedene Enzyme, einschließlich der Lipase A und B aus *Candida antarctica* (CalA und CalB) und der Benzaldehydlyase aus *Pseudomonas fluorescens* (BAL) zu immobilisieren.

Die Veresterung von Octanol und Octansäure diente der Bestimmung der Lipase-Aktivität. Die Benzoinkondensation wurde als BAL-katalysierte Reaktion eingesetzt. Für jede Reaktion wurden entsprechend der Literaturangaben optimale Reaktionsbedingungen eingesetzt. Die gaschromatographische Analyse diente der Bestimmung der Substrate und Produkte zur Berechnung der Enzymaktivität und des Reaktionsumsatzes.

Für hydrophile Agargele wurde ein Lösungsmittelaustauschverfahren verwendet, um die Löslichkeit von Agargelen, die mit CAlB beladen waren, in der organischen Phase zu verbessern. Mit diesem Ansatz wurde die CalB mit Fluoreszenz-Farbstoff markiert und in die Kerne von Agargelen übertragen. Dies deutete darauf hin, dass anhand dieser Methode, die Oberfläche des hydrophilen Gels benetzt werden kann und Enzyme in den Gelkernen konzentriert werden können, um eine Lösunsgmittelinaktivierung zu vermeiden. Zudem

konnte die Stabilität und Wiederverwendbarkeit der immobilisierten CalB nach Austausch des Lösungsmittels im Vergleich zum nativen Enzym verbessert werden. Diese Ergebnisse verdeutlichen, dass das Lösungsmittelaustauschverfahren eine gute Methode darstellt, um die Löslichkeit von hydrophilen Trägern in organischen Lösungsmitteln und somit deren Oberfläche für die Katalyse zu verbessern.

Für hydrophobe Träger wurde die CalA in biokatalytisch aktiven Siliconperlen (BASE) eingeschlossen, in denen die wässrige Enzymlösung in Form zahlreicher Mikroeinsätze emulgiert wurde. Anhand von Berechnungen besitzen diese Mikroeinsätze eine große Fläche, die mehr das als 300-fache einer Sphäre in flüssiger Form beträgt. Basierend auf diesen Berechnungen wurde eine Optimierung dieser Siliconperlen für die Katalyse durchgeführt, um die wässrige Enzymphase in Silicon weiter zu vergrößern. Interessanterweise führte ein größeres Volumen der wässrigen Phase der Siliconperlen zu einer Vergrößerung der Anzahl und Verringerung der Größe der Mikroporen. Dies hatte eine Erhöhung der enzymatischen Aktivität zur Folge. Die optimale Zusammensetzung der Siliconperlen betrug 8,8 g Silicon und 5 mL Enzymlösung. Anschließend wurde eine weitere Optimierung des Wassergehaltes bei dem definierten Verhältnis von Silicon (g) und der wässrigen Phase (mL) durchgeführt. Darüber hinaus wurde die Stofftransferlimitierung über den Verlauf der Penetration von eingefärbtem Heptan in die Siliconperlen beurteilt. Durch diese Studien konnte die Schnittstelle des hydrophoben Silicon-Trägers durch Optimierung hinsichtlich der Zusammensetzung und des Wassergehaltes für die Biokatalyse verbessert werden.

Für Pickering-Emulsionen wurden Silicat-Nanopartikel verwendet, um die CalB bzw. die BAL zu verkapseln. Überraschenderweise zeigte die Bewertung der beiden Enzymaktivitäten eine 300-fach erhöhte Aktivität für die CalB und eine 8-fach erhöhte Aktivität für die BAL. Dieses Ergebnis zeigt, dass Pickering-Emulsionen ein gutes System für stabile Enzyme wie die CalB und anfällige wie die BAL darstellen. Die erhöhte Aktivität von Enzymen in Pickering-Emulsionen lässt sich auf die große Oberfläche dieser Emulsionen in der organischen Phase zurückführen.

Im Rahmen Untersuchungen wurden Agargele, Siliconperlen und Pickering-Emulsionen gezielt als Träger ausgewählt, um Enzyme für die Biokatalyse in organischen Medien zu immobilisieren. Es wurden verschiedene Strategien angewendet, um die Löslichkeit von Agargelen in Lösungsmitteln zu verbessern und die Grenzfläche der wässrigen Enzymphase

innerhalb der Siliconperlen zu vergrößern sowie eine große Oberfläche von Pickering-Emulsionen in organischen Lösungsmitteln zu erzielen. Mit diesen Ergebnissen für die drei Träger wurde ein Beitrag zur Verbesserung der Grenzfläche verschiedener Immobilisierungsmatrizen für die Biokatalyse in organischen Medien geleistet.

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Symbols and Abbreviations

ΔE	Interfacial energy
°C	Celsius
AA	Acrylic acid
AARs	Amino acid residues
$A_{\text{one spherical droplet}}$	Total surface area of one spherical liquid
$A_{\text{total micro-pools}}$	Total surface area of micro-pools
a_w	Water activity
BAL	Benzaldehyde lyase
Bar	Unit of pressure
BASE	Biocatalytic active static emulsions
BSA	Bovine serum albumin
CalA	Lipase A from *Candida antarctica*
CalB	Lipase B from *Candida antarctica*
CD	Circular dichroism
CLE	Cross-linking enzymes
CLEAs	Cross-linked enzyme aggregates
CLECs	Cross-linked enzyme crystals
CLSM	Confocal laser scanning microscopy
cm	Centimeter
C_o	Concentration of original QDs
CPG	Controlled-pore glass
d_i	Diameter of each micro-pool
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
E_{CalA}	CalA loading efficiency
FID	Flame ionisation detector
FITC	Fluorescein isothiocyanate
g	Gram
GC	Gas chromatography
h	Hour
I	Intensities of the maximal absorbance bands of the CdTe QDs
IPA	Isopropanol
kDa	Kilodalton (10^3 Dalton)
KPS	Potassium peroxodisulfate
L	Liter
M	Molar concentration ($mol \cdot L^{-1}$)
MBA	N,N'-methylenebisacrylamide

mg	Milligram
MIBK	Methyl isobutyl ketone
mL	Milliliter
mM	Millimolar concentration ($mmol \bullet L^{-1}$)
MPI Potsdam	Max-Planck-Institut für Kolloid- und Grenzflächenforschung, Potsdam
MPs	Microparticles
MTBE	Methyl tert-butyl ether
N_A	Avogadro constant
nm	Nanometer
N_{mp}	Numbers of CdTe QDs loaded in PNIPAM MPs
NPs	Nanoparticles
O/W	Oil-in-water emulsion
PBS	Phosphate buffered saline
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PMMA	Macroporous poly(methyl methacrylate)
PNIPAM	Poly(N-isopropylacrylamide)
PVA	Polyvinyl alcohol
QDs	Quantum dots
r	Particle radius
rcf	Relative centrifugal force
rpm	Revolutions per minute
SEM	Electron microscope
SPLS	Single particle light scattering
TCD	Thermal conductivity
TEOS	Tetraethyl orthosilicate
THF	Tetrahydrofuran
TMODS	Trimethoxy (octadecyl) silane
TU Berlin	Technische Universität Berlin
U	One unit of activity
UV-Vis	Ultraviolet–visible spectroscopy
v/v	Volume percent (volume per volume)
$V_{aqueous\ phase}$	Volume of aqueous solutions
V_{CdTe}	Volume of the original QD solution
W/O	Water-in-oil emulsion
w/w	Weight percent (weight per weight)
W_e	Water content of the enzyme
W_s	Water content
wt/v	Weight in volume
ZELMI	Zentraleinrichtung Elektronenmikroskopie (TU Berlin)

γ_{OW}	Interfacial energy from interface of oil/water
γ_{PO}	Interfacial energy from interface of particle/oil
γ_{PW}	Interfacial energy from interface of particle/water
γ_w	Water activity coefficient
$\delta_{solubility}$	Solubility parameters ($cal^{1/2} \bullet cm^{-3/2}$)
µL	Microliter
µm	Micrometer
χ_w	Mole fraction of water

1. Introduction

1.1. Enzyme Immobilization

1.1.1. History of Enzyme Immobilization

Enzymes are widely considered to be remarkable catalysts with excellent properties for many applications: high capability to accept a wide range of complex molecules as substrates, exquisite selectivity to catalyze reactions with unparalleled chiral (enantio-) and positional (regio-) selectivities, mild reaction conditions (e.g. physiological pH and temperature), etc.[1, 2] These unique properties offer extensive interest for enzymes applied in such industrial fields as pharmaceutics, chemicals, food, organic synthesis, agrochemistry, and detergents. However, the intrinsic drawbacks of enzyme instability often prevent them from large-scale applications in industrial conditions (e.g. extreme pH, high temperature, solvent toxicity, and high shear force in reactors). Fortunately, immobilizing enzymes can improve their practicability for applications, obviously enhancing their stability and reusability, increasing their activity and specificity, and facilitating the separation process. Enzyme immobilization can also distinctly improve enzyme performance under the optimal process reaction conditions (e.g. acidity, alkalinity, organic solvents, and elevated temperatures), which frequently prevent enzymes from industrial applications.[3] Immobilized enzymes were firstly defined in 1971 as “enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously” in the first Enzyme Engineering Conference.[4] Figure 1.1 calculates the number of the published papers or issued patents relating to enzyme immobilization since 1900. It illustrates the rapid rise in activities on enzyme immobilization since 1970. Especially in the last 20 years, there is an almost linear increase in the number of literature, which reflects the fact that more scientists and engineers are now recognizing the real value of enzyme immobilization to the catalysis industry.

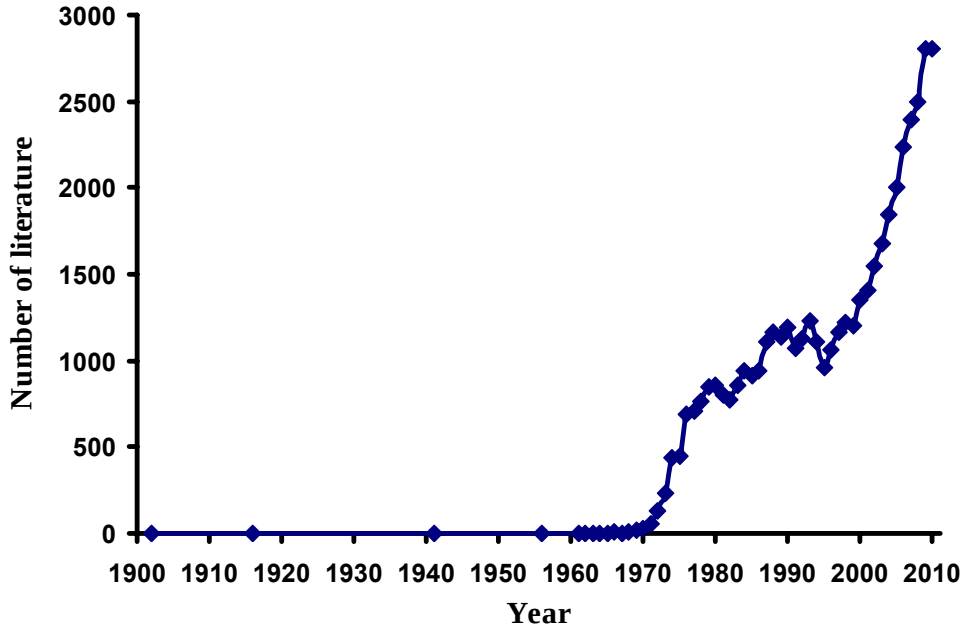


Figure 1.1. Number of literature from 1990 to 2010 (Searching via "SciFinder Scholar" with the key word "enzyme immobilization").

Reviewing the development history of enzyme immobilization, six historic phases are particularly defined according to recent Cao's book:[5] "the early phase (1916-1940s), the underdeveloped phase (1950s), the developing phase (1960s), the developed phase (1970s), the post-developed phase (1980s), and rational design of immobilized enzymes (1990s-present)".

In the time of 1916-1940s, Nelson and Griffin are widely accepted to set the earliest record in enzyme immobilization history. They found the catalytic activity of invertase adsorbed in $Al(OH)_3$ and charcoal.[6] Unfortunately, at that time, the value of this method was not really recognized so that in the subsequent 40 years it was rarely reported on catalysis using these water insoluble forms of enzymes.[7] The bio-immobilization technologies were then only employed for protein isolation via adsorption of inorganic carriers.[5]

In the 1950s, immobilization techniques were most limited to physical methods (e.g. from non-specific physical adsorption to specific ionic adsorption). A progressive example of adsorption was from the work of Grubhofer and Schleith, who first intended to improve enzymatic performance through immobilization in 1953.[7, 8] Covalent binding was later established as an alternative immobilization method, for example, lipases and other proteins

were bound to polyaminostyrene. In addition, sol-gel techniques were initially introduced in 1955 to entrap myoadenylate deaminase in silicic-acid derived glass with catalytic functions.[9] But the importance of sol-gel entrapment was not immediately recognized at that time, and its further development was delayed about 40 years. Meanwhile there were more carriers available, such as nature polymer derivatives (e.g. CM-cellulose and DEAE-cellulose), inorganic materials (e.g. carbon, glass, kaolinite, and clays), few synthetic polymers (e.g. aminopolystyrene and polyisocyanate), and synthetic ionic adsorbents (e.g. Amberlite XE-97, Dowex-2, and Dowex 50), etc.[5]

In the 1960s, immobilization techniques were further advanced. The focus of the time was to develop various covalent-binding strategies to immobilize different enzymes. The traditional techniques of adsorption and entrapment were gradually advanced with more options as well. Besides, in 1964 Chang first established the encapsulation method to immobilize erythrocyte hemolysate and carbonic anhydrase into semipermeable microcapsules (nylon membranes).[10] The technique of cross-linking enzymes (CLE) also emerged at that time. With these techniques available, enzymes were not only immobilized into/onto carriers, but also formed as carrier-free immobilisates by cross-linking of crystalline enzymes or dissolved enzymes. In the developing phase of 1960s, an increasing number of enzymes were immobilized with high potential for applications in pharmaceutical and chemical industry. Meanwhile, much attention was paid to carrier selection and optimization, e.g. catalytic performance of enzymes was studied in carriers with different physical or chemical properties, which were specifically explored in terms of microenvironment, hydrophobicity, surface charge, and the binding chemistry, and the influence these properties had on enzyme activity and stability.[5, 11, 12] Furthermore, more carriers were developed to favour specific enzymes for immobilization. In particular, hydrophilic insoluble carriers with defined geometry (e.g. agarose, and cellulose beads) were broadly applied. In the end of 1960s, the first immobilized enzyme (ionically bound l-amino acid acylase) was successfully applied in industry by a Japanese company.

In the 1970s, an important event of enzyme immobilization was “the first Enzyme Engineering Conference” held in 1971 at New Hampshire (USA) where immobilized enzymes were the key theme and therein were officially defined. Since then, the developed phase of the time was witnessed by rapidly increasing number of publications and patents (Figure 1.1). One of significant advancements at that time was the continuous efforts to immobilize

enzymes to enhance enzyme practicability. For examples,[5] enzyme leaching was obviously reduced by entrapping them in gel-matrix following with a cross-linking treatment; chemically modified enzymes with enhanced stability or activity were achieved through being adsorbed on cationic exchanger or entrapped in a polymeric matrix. Moreover, immobilized enzymes were gradually applied into different solvent media and not solely limited to aqueous media. In the beginning, low concentrations of water soluble solvents (e.g. acetone) were employed for hydrolysis of *p*-nitrophenyl phosphate by wheat germ acid phosphatase.[13] Later, higher concentrated solvents were optimized under reaction conditions for hydrolysis of lysyl dipeptides by trypsin bound to porous glass.[14] The subsequent pioneering review and research by Klibanov emphasized the importance of immobilized enzymes in organic solvents, such as, the synthesis of N-acetyl-L-tryptophan ethyl ester by adsorbing chymotrypsin on porous glass.[15] In the end of the 1970s, more than hundred publications appeared with different immobilization methods and enzymes used for applications in non-aqueous media. A more detailed introduction of immobilized enzymes applied in organic solvents is addressed later in this thesis. Other advancements of immobilization techniques in the 1970s reflect on more sophisticated techniques to prepare tailor-made carriers with diverse properties. These carriers either possess different physical and morphological properties (e.g. hydrophobicity and particle size) or chemical functional groups for binding specific enzymes. Besides, much attention has been given to study the enzymatic performance by effects of the microenvironment of carriers,[16-18] the spacer or arm, different modes of binding (chemistry, position and number), enzyme loading, diffusion constraints, etc.[5] Significantly, two commercial applications appeared at that time:[19] Immobilized penicillin acylase was used in England to catalyze penicillin G or V in the production of 6-amino penicillanic acid; fructose was converted from glucose by immobilized glucose isomerase in the USA.

In the 1980s, the interest of enzyme immobilization research intensively gained on application potentials.[2, 5] Immobilized enzymes with high selectivity and specificity were explored as excellent candidates to produce enantiomerically pure compounds in pharmaceuticals and agrochemicals. Under the drive towards green, sustainable methodologies for chemical manufacture, immobilizing biocatalysts was studied as alternative solutions for biocatalytic processes under mild reaction conditions and environmentally acceptable solvents (e.g. water). Furthermore, the immobilization of enzymes in/on carriers was extensively applied in organic solvent media to increase enzyme stability and to catalyze large quantities of chemical

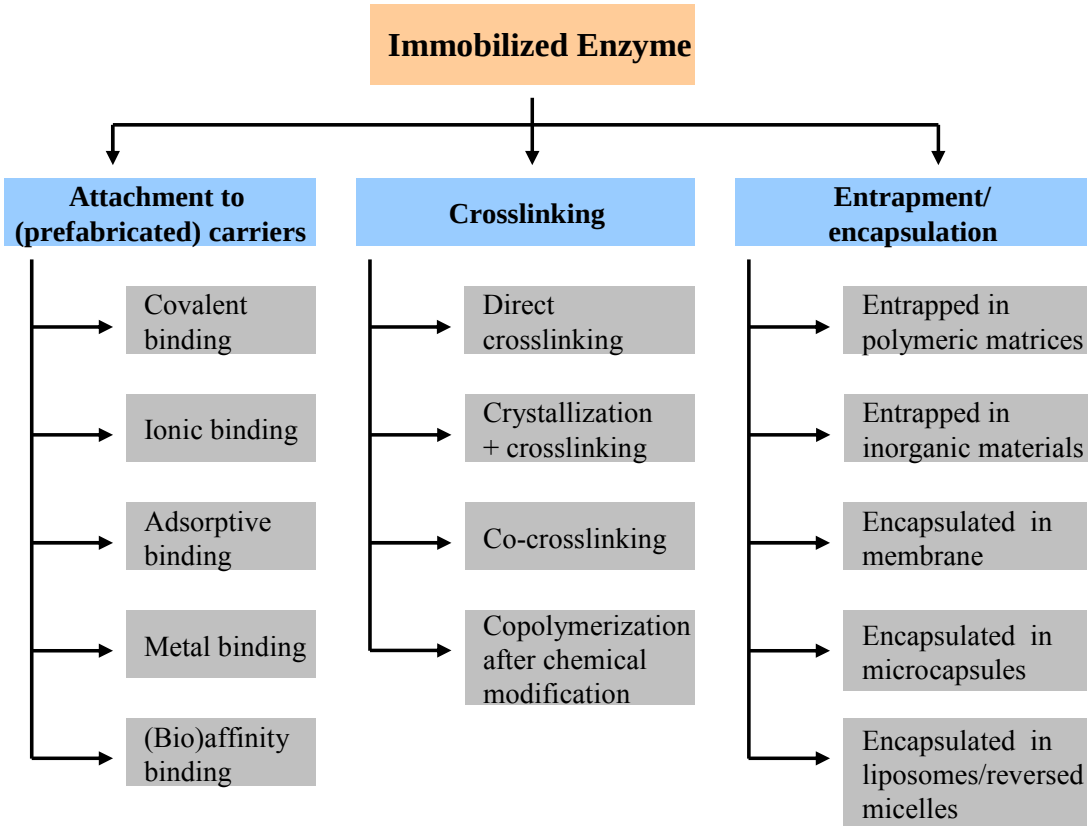
reactions that are not feasible in aqueous media.[20] In organic media, the immobilized enzymes were studied for topics on water activity[21, 22], mass transfer[23, 24], and interfacial activation[25]. Meanwhile, dual immobilized enzyme systems were established for electrode analysis[26] and production of maltose[27], even for more complex cofactor regeneration[28].

Since the 1990s, enzyme immobilization has been much more rationally designed for specific application demands, enzyme properties, and their catalytic performance (e.g. activity, stability, and selectivity). It is impossible here to present all achievements regarding the thousands of literature since 1990, but it is worth noting several technical milestones in the past 20 years: 1) Inspired by cross-linked enzyme crystals (CLECs) in 1960s, enzymes were precipitated as aggregates and cross-linked to form so called "cross-linked enzyme aggregates (CLEAs)". This new method was used to avoid laborious enzyme crystallization, and proved more stable against denaturation during heating and in organic solvents than free enzymes, and later, it was successfully commercialized.[2, 5] 2) Although enzyme entrapment in sol-gel was invented in 1955, the great interest and attention was only raised since 1995 when Reetz *et al.* increased lipase activity up to 800 % for esterification in organic solvents by using hydrophobic silica gels.[29-31] 3) A new concept of solidifying enzyme solution for biocatalysis in organic solvents was recently introduced by the group of Ansorge-Schumacher.[32] For example, polyvinyl alcohol (PVA), a hydrophilic hydrogel, was employed to entrap aqueous solution of alcohol dehydrogenase or benzaldehyde lyase for enantioselective synthesis.[2, 32] Later, the same group invented biocatalytic active static emulsions (BASE) to emulsify enzyme solution with large catalytic interfacial area within silicone spheres, and this method has been proved particularly effective for lipase esterification in organic media.[20, 33, 34] 4) Besides these, there were many other techniques that have been developed for improving biocatalyst performance, such as, the use of dendrimeric (or tentacle) carriers, post-immobilization methods, molecular imprinting techniques, and solvent exchange approach for loading enzymes.[5, 35] In general, the tremendous efforts in the past 20 years have been devoted to make any available enzyme capable of being immobilized, and has been dedicated to bringing these immobilized biocatalysts with excellent catalytic performance to industry.

1.1.2. Methods of Enzyme Immobilization

In past three decades, immobilization methods have been reviewed and classified by many reviews and books.[36] In 1983, Klibanov summarized these methods available as five types:

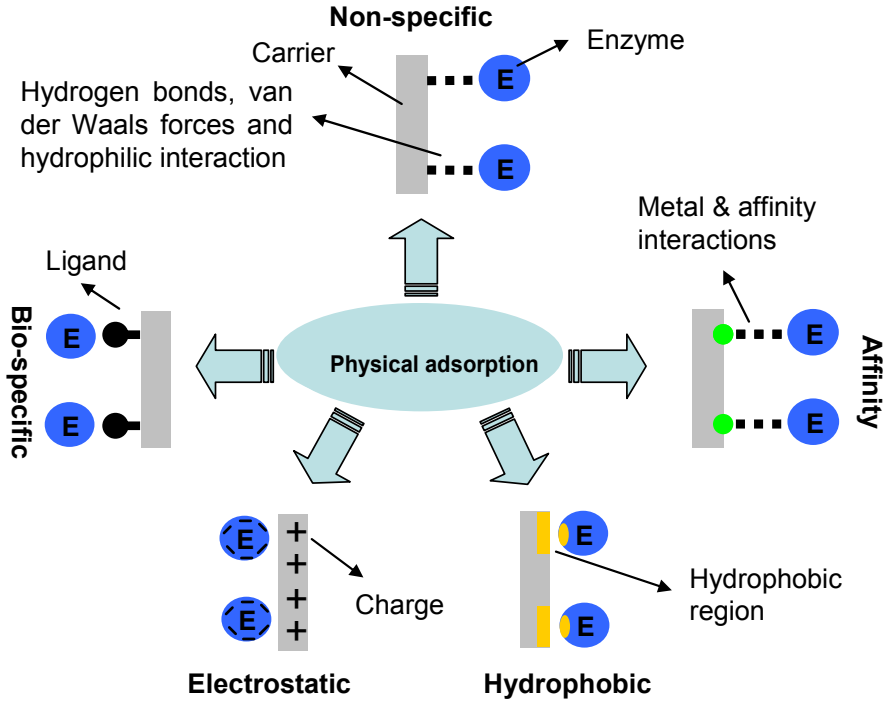
adsorption, covalent attachment, entrapment, cross-linking, and encapsulation.[37] On the other hand, immobilization methods could be sorted in different ways according to different point of views such as: 1) chemical binding and physical retention,[38] 2) support binding, entrapment, and cross-linking,[2] or 3) attachment and entrapment.[36] Because of emerging new techniques introduced to date, a detailed classification is demonstrated in Scheme 1.1, which is summarized and modified according to the recent reviews by Tischer and Wedekind in 1999 and Ansorge-Schumacher in 2008.[36, 39] However the difference in immobilization methods is defined, the conventional five methods (adsorption, covalent attachment, entrapment, encapsulation, and cross-linking) are still considered as the most important techniques to immobilize enzymes for the time being.[5, 40]



Scheme 1.1. Classification of immobilization methods, modified according to literature.[36, 39]

Adsorption of biocatalysts in/on solid materials is a relatively simple and inexpensive method with wide applicability. It was the first method used in history through physically adsorbing invertase onto charcoal with retention of biological activity,[6] and also the first one applied industrially via immobilizing amino acid acylase onto DEAE-cellulose.[5] Adsorption during

enzyme immobilization results from various interactions such as hydrophobic, polar, electrostatic, and chelating interactions.[3, 36] Cao classified these interactions into five categories (Scheme 1.2):[5] 1) non-specific adsorption including van der Waals forces, hydrogen bonds, and hydrophilic interactions; 2) bio-specific adsorption through use of immobilized ligands to adsorb enzymes; 3) affinity adsorption, realized by embedding specific metals or dyes into carriers to anchor corresponding enzymes (e.g. attachment of His-tagged proteins to Ni^{2+} in agarose)[36, 41, 42]; 4) electrostatic interaction, taking effects when there are different charges between carriers and proteins; 5) hydrophobic interactions, existing in the hydrophobic area of carriers and enzymes. Obviously these interactions are intrinsically different from the covalent binding because all of them are physical interactions and do not permanently fix enzymes in/on carriers. The properties of non-covalent adsorption give this method four unique advantages: 1) simplicity and cost-effective, which can be achieved by simply mixing enzymes and suitable absorbents under appropriate conditions (e.g. pH or ionic strength); 2) reversibility, which is realized through repeatedly loading or desorbing enzymes onto/out of carriers, and enables the reuse of enzymes and carriers; 3) high loading efficiency (about one gram protein per gram of matrix); 4) higher retention activity, which is compared to covalent binding method. These advantages determine the adsorption method that is most widely used in reality.

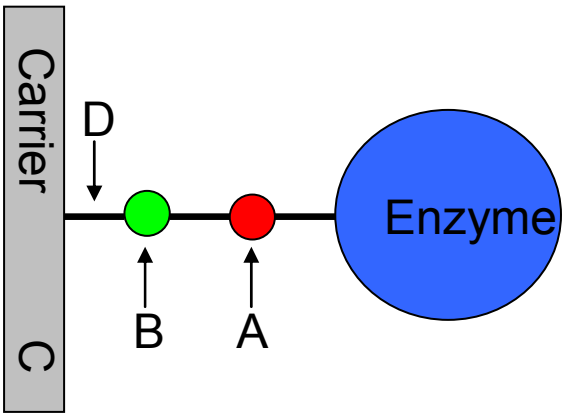


Scheme 1.2. Interactions of physical adsorption of enzyme on carriers.

Due to these advantages, various types of materials, ranging from polymers (both natural and synthetic) to inorganic carriers (e.g. silicate, zeolite, and clay), have been used to adsorb biocatalysts.[36] Under particularly applicable circumstances, these materials are further modified to improve applicable performance (such as mechanic strength and resistance against shear force in reactor, high temperature and extreme pH). A good example is the well-known carriers of "Sephadex" and "Sepharose" (Pharmacia, Uppsala, Sweden), which are obtained through cross-linking epichlorhydrin and hydrophilic polymer chains in organic polymer dextran and agarose.[36] Other widely used carriers include macroporous acrylic polymer resins (e.g. Amberlite XAD-7 and VPOC 1600 (Bayer)) and silica-based materials (e.g. modified aerogels and Celite). In the market, lipases are favorably adsorbed onto/into different carriers, such as in commercial products of Novozym 435 (Novozymes) and Chirazyme (Roche Molecular Biochemicals).[3] The drawback of adsorption technique is the enzyme leaching during applications because of the weak interaction between carriers and enzymes. But enzyme leaching can be reduced by combination of adsorption with other methods, e.g. adsorption–cross-linking, modification–adsorption, selective adsorption–covalent attachment, and adsorption–coating. In the past, some theories were also developed to understand catalytic performance of adsorbed biocatalysts. One example is the monolayer loading theory: It is believed that a minimal monolayer loading of enzyme (approximately 2 – 3 mg protein per square metre) is required on the carrier surface because less coverage led to more confirmation change of the enzymes. This principle is especially subjected to "soft" enzymes (unstable enzymes), and for "hard" enzymes (e.g. CalB) where it is not easy to observe the effects of monolayer loading.[5] Other theories are also considered, such as the effect of microenvironment and geometry constraints to immobilized enzymes. For instance, most lipases require interfacial activation,[36, 43, 44] and modification of surface hydrophobicity (e.g. functionalization of silica with octyltriethoxysilane[45]) can dramatically increase their activity.

Covalently binding enzymes onto carriers has been extensively studied since the 1950s and it is believed to provide the strongest linkage between enzymes and carriers, resulting in super stable matrix without leaching of biocatalysts.[5, 37] In contrast to non-covalent adsorption, however, only a small amount of enzymes can be immobilized into carriers (approx. 0.02 g

protein per gram of matrix). Although the low loading efficiency, the appeal for less enzyme leaching has driven scientists to develop various strategies to bind enzymes in this manner. Covalent binding generally occurs between amino acid residues on the enzyme surface (a) and active groups on the carrier surface (b) (shown in Scheme 1.3).[5]



Scheme 1.3. Covalently binding enzyme on the carrier: (a) active amino acid residue, (b) binding functionality of the carrier, (c) carrier, and (d) spacer. Modified according to literature.[5]

Concerning enzyme part, the use of specific amino acid residues (AARs) for coupling is dependent on availability, reactivity, exposure of enzyme surface, and stability of coupling bonds. According to Table 1.1, exposure of AARs and their reactivity during reaction are the crucial factors to determine their usefulness, and for this, lysine with $–NH_2$ group is often taeken as the best candidate. There are a variety of other AARs with special functional groups used for enzyme grafting. The reactivity of nucleophiles for these AARs generally follows the sequence:[46] $–S^-$ > –SH > $–O^-$ > $–NH_2$ > $–COO^-$ > –OH >> $–NH^{3+}$. Therefore, before any coupling, it is highly recommended to check the availability and reaction possibility of the specific AARs using for the individual covalent reaction.

In the case of carrier part, the physical and chemical properties should be carefully considered to improve catalysis. Carriers with different physical properties, e.g. surface area, shape, size, internal structure, porosity and pore size distribution, can couple with enzymes leading to different performance.[5] For example, higher activity presents for glucose oxidase covalently immobilized in silica with large pore size (CPC-silica carrier, 37.5 nm) than small pore size (Silica gel with 0.4, 4 and 10 nm).[47] Likewise, chemical properties of carriers can also

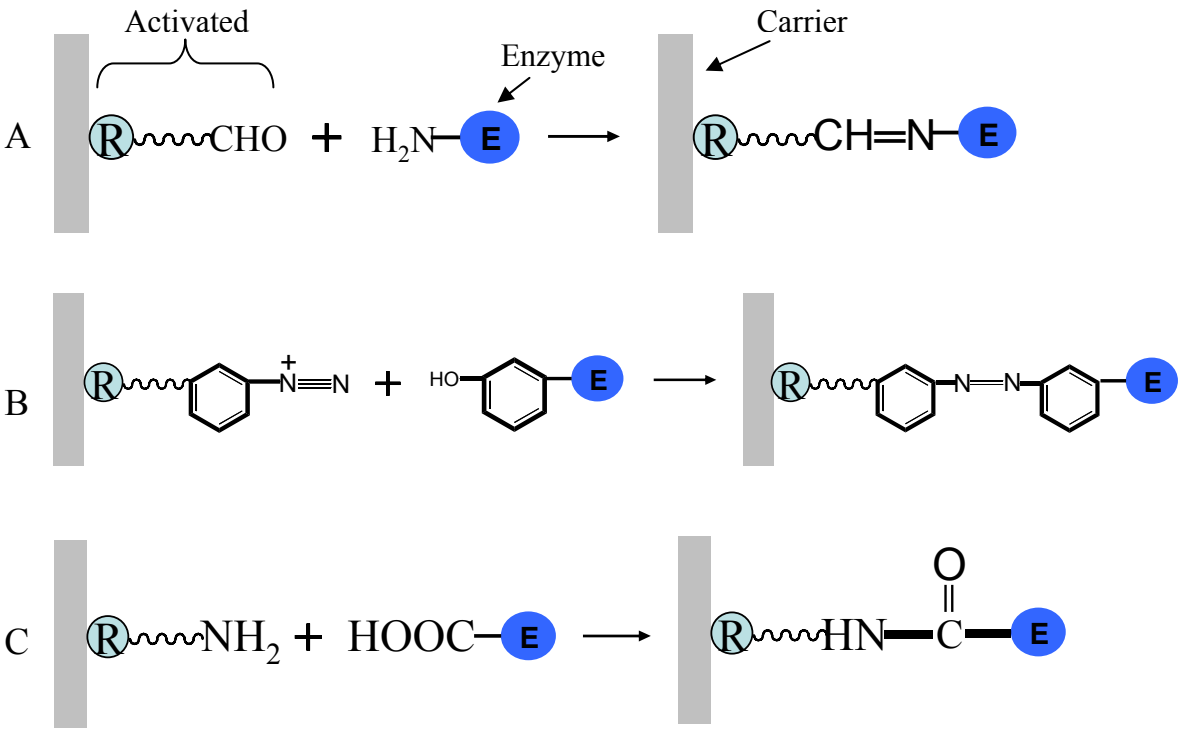
influence activity and stability of covalently immobilized enzymes. The influence may result from the difference of surface chemistry (e.g. hydrophilicity and hydrophobicity), microenvironment, chemical nature of active groups and spacers, etc. Thus, selecting carriers for binding enzymes is a complex process which takes into account both physical and chemical properties. Sometimes, the "ready to use" carriers are often not the ones best suited for coupling enzymes with good catalytic performance.[5]

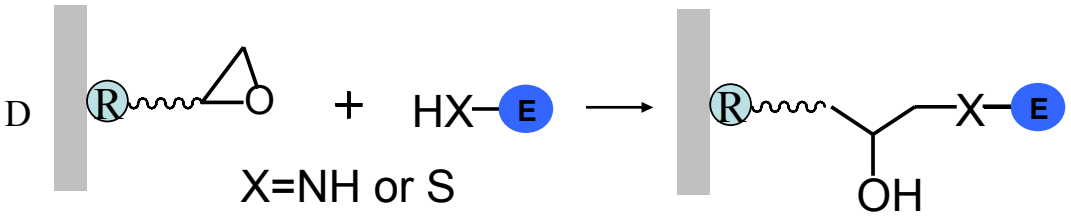
Table 1.1. Relative usefulness of amino acid residues for covalent coupling: - very poor, - ~ + poor, - ~ ++ not good, + good, ++ very good, NA not available. Modified according to literature.[46]

Residue	Content	Exposure	Reactivity	Stability of couple	Use	Rank
Lysine	++	++	++	++	++	1
N terminus	-	++	++	++	+	2
Glutamate	+	++	+	+	+	3
Aspartate	+	++	+	+	+	4
C terminus	-	++	+	+	+	5
Histidine	NA	++	+	+	+	6
Tyrosine	+	-	+	- ~ +	+	7
Arginine	+	++	-	NA	-	Poor
Carbohydrate	- ~ ++	++	+	+	NA	Poor
Serine	++	+	NA	+	NA	Poor
Threonine	++	NA	NA	+	NA	Poor
Cysteine	-	NA	++	-	-	Poor
Cystine	+	-	NA	NA	-	Poor
Methionine	-	-	NA	-	-	Poor
Tryptophan	-	-	-	NA	-	Poor

Until now, there have been dozens of synthetic strategies available to bind enzymes to carriers.[5, 48] The most used reactions are five types:[5, 37, 48, 49] namely Schiff base formation (Scheme 1.4a), azo coupling (Scheme 1.4b), carbodiimide coupling (Scheme 1.4c), ring opening reaction of epoxide (Scheme 1.4d), and thioether formation (Scheme 1.4d). Schiff base formation involves two steps,[37] namely activation of carriers and covalent binding enzymes onto functionalized carriers. This strategy especially suits silica-based carriers. For

example, glass and silica nanoparticles can be first functionalized with 3-aminopropyltriethoxysilane ($(C_2H_5O)_3Si(CH_2)_3–NH_2$) to provide carriers with free $–NH_2$ group; subsequently glutaraldehyde ($OCH–(CH_2)_3–CHO$) is added to react with $–NH_2$ but remains some active –CHO unreacted; after the activation, $–NH_2$ on enzymes can easily react with –CHO on carriers — Schiff base formation.[37, 50] Azo coupling strategy is especially suitable for enzymes with available tyrosine residues. For instance, an arylamine support can be reduced into diazonium form which then easily reacts with tyrosine by azo coupling. Carbodiimide coupling is another widely used method and generally includes two steps to couple enzymes: hydroxylic matrix (agarose or cellulose) can be first modified with $–NH_2$ using cyanogen bromide (CNBr) or chloro-sym-triazinyl groups; and carbodiimides are subsequently formed between carriers ($–NH_2$) and enzymes (–COOH).[49] The ring opening reaction has been extensively applied for enzyme immobilization on epoxide polymers which can readily react with $–NH_2$ or –SH groups on the surface of enzymes. A good example of epoxide polymer for covalent immobilization is the commercial support Eupergit® and Sepabeads which are activated with epoxide. Reviewing the all above methods, the choice of a certain synthetic strategy for enzyme binding depends on many factors, especially the properties of carriers and selected enzymes.





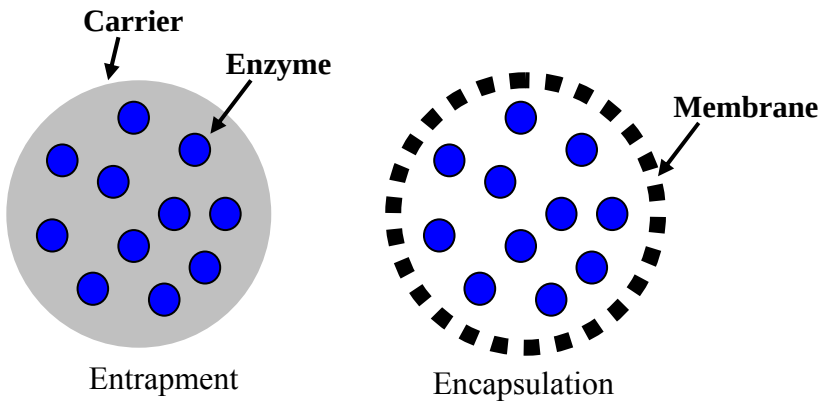
Scheme 1.4. Most commonly used strategies to covalently bind enzymes in/on carriers: a) Schiff base formation, b) azo coupling, c) carbodiimide coupling, and d) ring open reaction and thioether formation.

Enzyme entrapment is typically referred to as free enzymes entrapped within interstitial spaces of a cross-linked, water-insoluble polymeric matrix.[51] The techniques used for entrapment include thermal gelation, ionotropic gelation, photo-induced cross-linking, precipitation, polymerization, and polycondensation.[36] The thermal gelation is a very simple and widely used method where, through heating and subsequent cooling of dissolved polymers, enzymes are entrapped within a matrix, mainly made from natural (e.g. agar, agarose, κ-carrageenan, and gelatin) or synthetic polymers (e.g. poly(vinyl alcohol)). The problems of thermal gelation method include the low melting point and poor mechanic resistance of the matrix, and deactivation of enzymes during heating polymers for dissolving enzymes. Ionotropic gelation obviously avoids heating enzymes and frequently occurs between some natural polymers (e.g. alginate) with cross-linking ion (e.g. Ca^{2+}). But the resulting gels sometimes become unstable in media with other chelating compounds (e.g. phosphate, citrate, EDTA, and lactate) or anti-gelling cations such as Na^{+} or Mg^{2+}.[36] Furthermore, gels formed by ionotropic gelation always have poor mechanic stability under reaction conditions. Mechanic stability is greatly improved in synthetic polymers by employing polymerization process during enzyme entrapment. Polyacrylamide is the first synthetic polymer to entrap various enzymes through different polymerization strategies, such as initiation of potassium persulfate, X- or γ- radiation, and ultraviolet radiation.[51] Many other used synthetic polymers include acrylic acid with glycidylacrylate, 2-hydroxyethylmethylacrylate, N-vinylpyrrolidone, 2-hydroxylpropylamide, 2-hydroxypropylacrylate, poly(ethylene glycol) methylacrylate, butanediolacrylate, and ethyleneglycoldiacrylate.[5] The drawback of using polymerization processes for enzyme entrapment is the retention of toxic monomers. Silicone polycondensation, however, is a non-toxic process, and has been broadly applied since 1955. This approach has drawn much

attention since Reetz *et al.* demonstrated a 80 folder activity increase of lipases that were entrapped in sol-gel.[36] Overall, the advantages of enzyme entrapment are the simple immobilization process, the low cost of materials, and the less deactivation of enzymes during the process. Additionally, entrapment technique can provide enzymes with custom-tailored microenvironment in a matrix (e.g. hydrophobicity) for biocatalysis. However, a major disadvantage of the method is the mass transfer limitation for substrates diffusing through gel matrices.

Enzyme encapsulation is similar to the entrapment method because in both cases biocatalysts are entrapped in a defined region. But the difference (Scheme 1.5) is that encapsulation method is used to encapsulate enzymes within semi-permeable membranes rather than carrier matrix. Encapsulation technique was pioneered by Chang in 1964 through encapsulating enzymes within a nylon membrane.[10] Later on, a number of approaches were created to encapsulate enzymes into various membranes such as polymers, liposomes, and colloids. But three major principles are most commonly employed to prepare enzyme encapsulation: phase inversion, interfacial process, and mini/micro-emulsions.[5, 36] Membranes created by phase inversion typically involve two steps: 1) mixing enzyme solutions and water immiscible organic solvents dissolved with polymer ingredients, and 2) introducing another water immiscible solvent to induce phase separation and finally rendering polymers (e.g. cellulose nitrate, ethyl cellulose, and nitrocellulose) to fusion around aqueous enzyme droplets as semi-permeable membrane. Interfacial process can be realized by both physical and chemical methods in order to form a membrane at the interface between the enzyme aqueous sphere and the water immiscible solvent: Physical approaches often use physical gelation; chemical approaches usually need to introduce hydrophilic monomers into aqueous phase and hydrophobic ones into organic phase, and then polymerization starts at interface. Aqueous enzyme droplets can also be encapsulated by small molecules of surfactants which have amphiphilic groups to assemble as molecule membrane at interface of two phases.[52] They can even be encapsulated by nanoparticles (e.g. silica nanoparticles) which have amphiphilic property to stabilize aqueous droplets in organic phase.[53, 54] Up to now, various membranes (e.g. polysulfones, cellulose acetate, and acrylic copolymers) have been applied for encapsulation and most popular formations are hollow fibers and ultrafiltration.[36] A commercial example is that Snamprogetti uses hollow fibers to encapsulate penicillin acylase, lactase, and aminoacylase.[37] Compared to the entrapment method, enzyme encapsulation can

easily retain a vast aqueous phase, providing the enzyme with sufficient water activity, and the semi-permeable membrane has substantive pores which facilitate mass transfer of substances. On the other hand, this method suffers from two main drawbacks of mechanical weakness and enzyme leaching. To improve them, sometimes other immobilization strategies are required, for example, cross-linked or entrapped enzymes that are encapsulated in membranes.

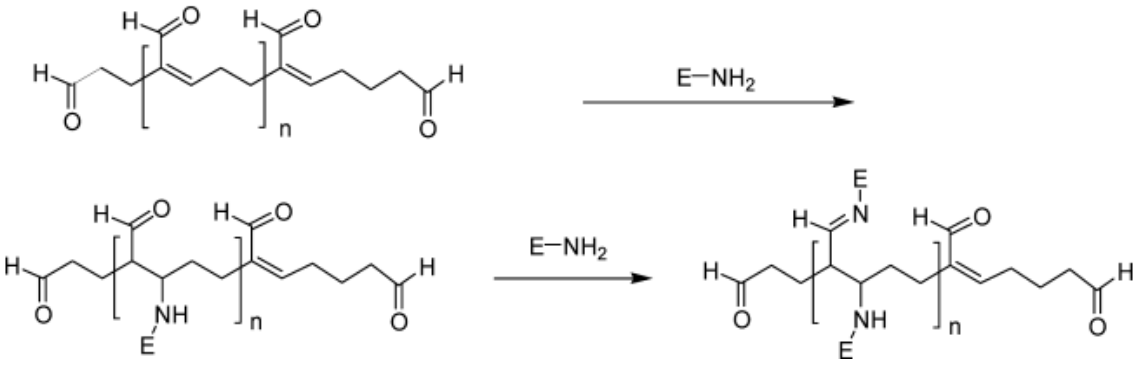


Scheme 1.5. Entrapment and encapsulation.

Cross-linking method is defined as enzymes are cross-linked as aggregates or crystals by bifunctional reagents to form carrier-free macroparticles.[2] The technique was introduced in the 1960s for study of solid phase protein with bifunctional cross-linker, typically through mixing enzyme solutions and glutaraldehyde.[55] But its further applications were seriously delayed due to the poor performance of activity retention, reproducibility, and mechanical stability from its gelatinous nature.[56] In the 1990s Altus Biologics created a new trademark CLEC® by cross-linking crystal enzymes to form as solid microporous structure.[36] This new technique can be applied to most enzymes with such advantages as good operation stability, ease of recycling, and high productivity. Its main problem is the laborious crystallization of enzymes, resulting in increased cost and required instrument facilities. Since 2000, Sheldon's group has gradually established a new concept of cross-linked enzyme aggregates (CLEAs). CLEAs are created through physically aggregating enzymes by addition of salts or organic solvents or nonionic polymers, and subsequently cross-linking by bifunctional reagents.[56, 57] This new idea simplifies the cross-linking process for applications, compared with CLEC.[58] But the weakness of this technique is the poor mechanic strength, which was later improved by combining with other methods like copolymerization to form a silica-CLEA composite. The employment of cross-linking method for immobilizing enzymes can increase enzyme

performance (e.g. space-time yield and productivity) and simplify the immobilization process as carrier-free form. But its disadvantage is the mechanic weakness which often needs to be reinforced by other carries.[2, 36]

Cross-linking technique relies on use of crosslinkers to form network among enzymes. These crosslinkers include dialdehydes (e.g. glutaraldehyde and dextrandialdehyde) for combining exposed $-NH_2$ group on enzyme surface, bis-maleimides (e.g. N,N′-methylenebismaleimide) for targeting group of –SH, diisocyanates (e.g. hexamethyldiisocyanate), and diisothiocyanates (e.g. p-phenylene-diisothiocyanate).[36] Among these, glutaraldehyde is most frequently used because it is inexpensive and readily available, as well as having a high efficiency to react with lysine residues on enzyme surface. However, its detailed mechanism of cross-linking enzymes is not fully understood yet. Two main mechanisms are hypothesized: Schiff's base formation between glutaraldehyde and $-NH_2$ group, and a Michael-type 1,4 addition to α, β-unsaturated aldehyde moieties by aldol condensations of glutaraldehyde (Scheme 1.6).[56] In addition to selection of crosslinkers, a good protocol of cross-linking technique also needs the optimization of other preparation parameters including temperature, pH, concentration of enzymes, ionic strength of solutions, and time of reactions.[36, 56]



Scheme 1.6. Possible reactions for enzyme cross-linking by glutaraldehyde.[56] Reprinted with permission from Org. Process Res. Dev.[56] Copyright © 2010 American Chemical Society.

Overall, it is not realistic to simply compare the above five immobilization methods to reach a universal conclusion. Each method has its own advantages and drawbacks. The enzyme performance is determined not only by the immobilization strategies, but also by many other parameters such as microenvironment of different carriers, enzyme structure-activity relations,

and particular reaction conditions. However, from a technical viewpoint, a series of comparison was simply listed in Table 1.2. It should be clarified here, that the research status of enzyme immobilization today is still more empirical and in laborious screening procedure.[36] The emerging concept of rational design has tried to reasonably find one or several immobilization methods in integrated and sophisticated ways to enhance biocatalyst performance. But the complex of immobilization process not only depends on the perspectives of material science through physically or chemically combing enzymes into carriers, but also relies on biological properties of enzymes. This combined complex makes it extremely difficult to develop general theories to guide the whole field of enzyme immobilization. Nevertheless, the current achievements of available methods have provided scientists with more options to immobilize enzymes to work as stable, easy-to-use, and high active forms.

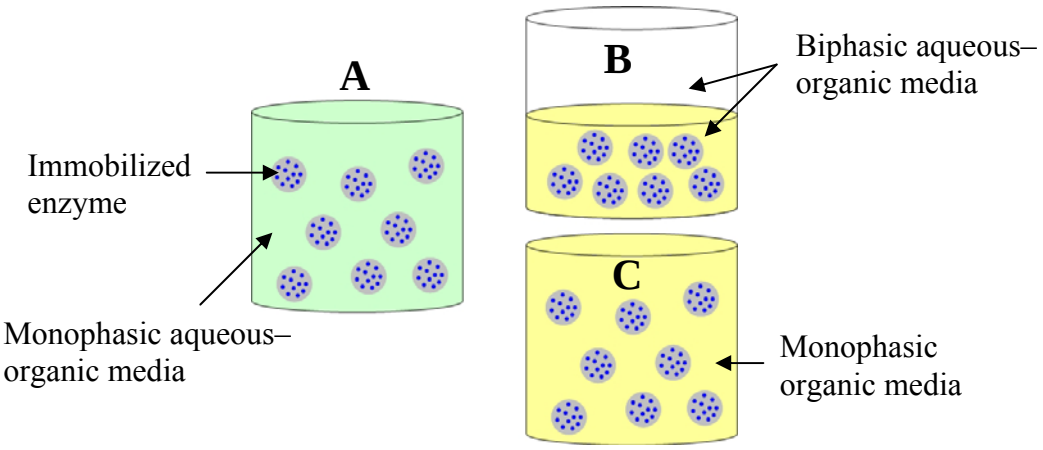
Table 1.2. The technical comparison for different immobilization methods. Modified according to literature.[46]

Characteristics	Adsorption	Covalent binding	Entrapment	Encapsulation	Cross linking
Preparation	Simple	Difficult	Moderate	Difficult	Difficult
Cost	Low	High	Moderate	High	High
Binding force	Variable	Strong	Weak	Strong	Moderate
Enzyme leakage	Yes	No	Yes	No	Yes
Applicability	Wide	Selective	Wide	Wide	Selective
Running problem	High	Low	High	High	High
Matrix effect	Yes	Yes	Yes	No	No
Mass transfer	No	No	Yes	Yes	No
Microbial protection	No	No	Yes	Yes	No

1.2. Immobilized Biocatalysts for Organic Synthesis in Non-aqueous Media

1.2.1. General Considerations

The importance of organic synthesis by enzymes has been widely recognized for the production of various valuable compounds in industries of pharmaceutics, agrochemicals, and foods. The biocatalytic synthesis often takes places in non-aqueous media to improve solubility of reactants and products. However, the employment of non-aqueous media readily denatures enzymes, thus enzymes prefer to be immobilized before entering into the media. In the past decades, three principle non-aqueous media were developed for biocatalytic synthesis of immobilized enzymes (Scheme 1.7): 1) monophasic aqueous–organic media (water–water miscible solvents), 2) biphasic aqueous–organic media (water–water immiscible solvents), and 3) monophasic organic media (immobilization matrix in only organic solvents).



Scheme 1.7. Immobilized enzymes in non-aqueous media.

Monophasic aqueous–organic media (Scheme 1.7a) are formed by mixing water-miscible solvents, water, and immobilized biocatalysts. The monophasic systems particularly fit to organic synthesis with water miscible reactants whose ratio to water can range from 0 % (v/v) to nearly 100 % (v/v). Other advantages of the systems are the cheap cost of water as a solvent, and provision of water activity to enzymes. The problem in this case is the poor material compatibility. Usually, some hydrophobic carriers (e.g. polydimethylsiloxane) are mechanically stronger against solvents than hydrophilic ones (e.g. hydrogels), but they are restricted for use due to their poor solubility in hydrophilic phase. Hydrophilic polymers like hydrogels are considered as good candidates for these systems, but in many cases, some of them are not stable and can be decomposed by the applied hydrophilic solvents. For example, agarose gels are readily decomposed in tetrahydrofuran (THF). Another problem is that these media can denature enzymes comparably fast due to their easy penetration into hydrophilic

carriers and facile access to enzymes afterwards. Also, considerable enzyme leaching is inevitable because of good solubility of enzymes in hydrophilic phase.

Biphasic aqueous–organic systems (Scheme 1.7b) are mainly designed for organic reactions between aqueous and water immiscible phase. The advantage of these systems is that reactions with substrates and products with different hydrophobicity can be suitably carried out through partitioning them in separate phases. For example, hydrolysis of esters can be catalyzed by immobilized lipases in organic phase where substrates and immobilized matrix are dissolved, and the products of smaller molecules are continuously diffused into water phase, and vice versa for esterification reaction. But a major disadvantage of these systems is the mass transfer limitation, which is due to either the diffusion limitation of substrates into carriers or that of products into different phases. Basically, it is not recommended to perform a reaction where reactants and immobilized biocatalysts are not in the same phase, otherwise a serious mass transfer limitation is present.

Monophasic organic media (Scheme 1.7c) only comprise of organic solvents as reaction media for synthesis catalyzed by immobilized enzymes. In fact, these media can be further subdivided as: 1) Monophasic hydrophobic media, which are used especially for the reaction of both hydrophobic substrates and products during reactions; 2) monophasic hydrophilic media, which are designed for biocatalysis with hydrophilic substrates and products; 3) monophasic solvent media with the mixture of hydrophilic and hydrophobic solvents, which is created to improve solubility of products and substrates with different hydrophobicity. In addition, carriers before or after enzyme immobilization, can be selected or modified to adapt to the particular solvent phase in order to reduce mass transfer limitation. So far, most organic synthesis by immobilized enzymes is performed in monophasic organic media because the majority of organic reactions take place in either hydrophobic or hydrophilic solvents or the mixture of both. Compared to the other two non-aqueous media, the monophasic organic phase, however, can not provide large amount of water phase for immobilisates in the system.

With the availability of these non-aqueous media, biocatalysts can be designed to catalyze the organic synthesis with a diverse range of substrates and products. The carriers used for immobilization in these systems can distinctly enhance stability and reusability of biocatalysts, and simplify separation process. Therefore, the combination of immobilization

techniques and non-aqueous media reinforces the practicability of enzymes for organic synthesis.

1.2.2. Key Parameters of Immobilized Biocatalysts for Organic Synthesis

Similar to free enzymes applied in non-aqueous media, the performance of immobilized enzymes in non-aqueous media can be mainly influenced by such parameters as water activity, mass transfer, enzyme stability, mechanic strength of carriers, and enzyme leaching.[59]

Water activity

The traditional notion believed enzymes only function in aqueous medium. But since the 1980s, emerging applications have illustrated the possibility and promising future of biocatalysis in non-aqueous media, and even in anhydrous solvents.[60, 61] Unfortunately, subsequent studies confirmed that enzymes lost tremendous activity in organic media compared to in water, e.g. in anhydrous octane compared to in water there was $10^4 – 10^5$ times lower activity for the proteases α-chymotrypsin and subtilisin.[62] The decrease in activity was explained by lack of water which acts as lubricant or plasticizer to offer enzymes the sufficient conformational flexibility needed for catalysis.[62] To exploit water influence, several famous investigations were conducted by Zaks and Klibanov in the 1980s and they reached the following three conclusions:[21, 59] 1) Higher water content leads to higher activity on unrelated enzymes, 2) less water is required to reach the maximal activity in hydrophobic solvents than in hydrophilic ones (Figure 1.2a), and 3) A monolayer of "essential" water bound on enzymes is responsible for necessary flexibility of enzymes for catalysis (Figure 1.2b). Later on, Halling *et al.* introduced the concept of water activity (a_w), expressed as $a_w = \gamma_w \bullet \chi_w$ (γ_w is water activity coefficient, and χ_w is mole fraction of water), to thermodynamically predict biocatalysis in non-aqueous media.[63, 64]

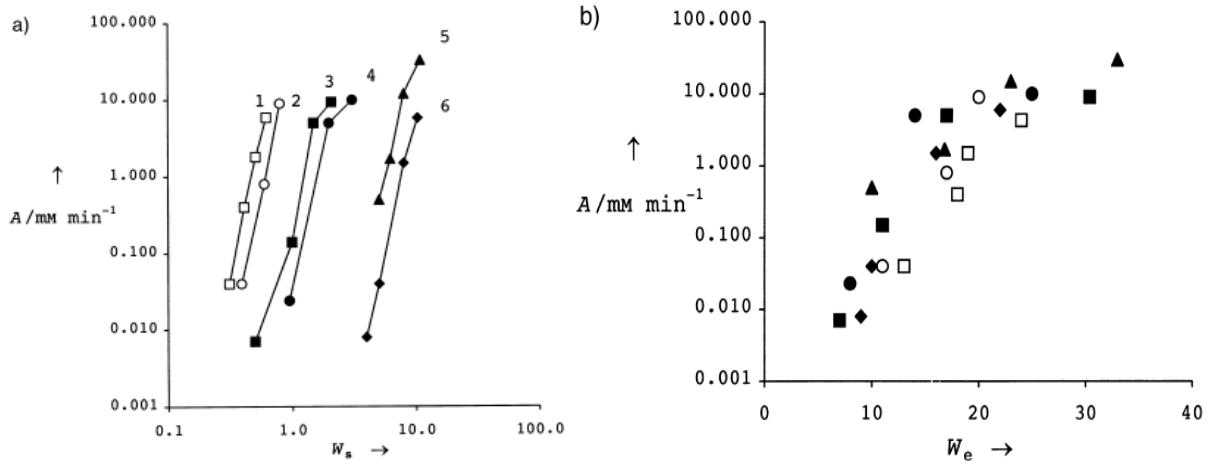


Figure 1.2. The activity (A) of yeast alcohol oxidase activity: a) in organic solvent with different water content (W_s in % (v/v)), b) on the water content of the enzyme (W_e in % (w/w)). In the figure: 1 = Diethylether (□), 2 = butyl acetate (○), 3 = ethyl acetate (■), 4 = n-octanol (●), 5 = tert-amyl alcohol (▲), 6 = 2-butanol (◆).[59] Reprinted with permission from Angew. Chem., Int. Ed.[59] Copyright © 1999–2011 John Wiley & Sons, Inc.

The effect that water has on immobilized enzymes is more complicated than free enzymes in non-aqueous media because carriers act as intermediate between solvents and enzymes. So the influence that water has on enzymes is highly dependent on many other parameters, especially carrier hydrophobicity, mass transfer, and enzyme loading efficiency. For example, Reslow *et al.* found that α-chymotrypsin adsorbed on hydrophobic materials of hexyl-CPG is easier to be activated by lower water content than that on less hydrophobic glucose-CPG (Figure 1.3a).[65] This was explained by the hydrophobic carriers being less competitive to strip water from solvents than the less hydrophobic counterparts. In contrast to water content, however, if applying water activity (a_w) to six supports with different hydrophobicity, there is no obviously different relative activity versus optimal water level for *Rh. miehei* lipase adsorbed on them (Figure 1.3b).[66] This finding on the other hand, illustrates that water activity (a_w) is a thermodynamic parameter independent to individual adsorption isotherms during enzymatic catalysis. Interestingly, it was observed that high enzyme loading on/in supports could decrease sensitivity of immobilized enzymes to water activity, as it was believed that excessive enzymes themselves could influence each other and facilitate the conformation mobility for catalysis.[66] In addition, carrier porosity and geometry influence the adsorption and distribution of water in a matrix, and thus affect the water contribution for catalytic performance of immobilized enzymes.

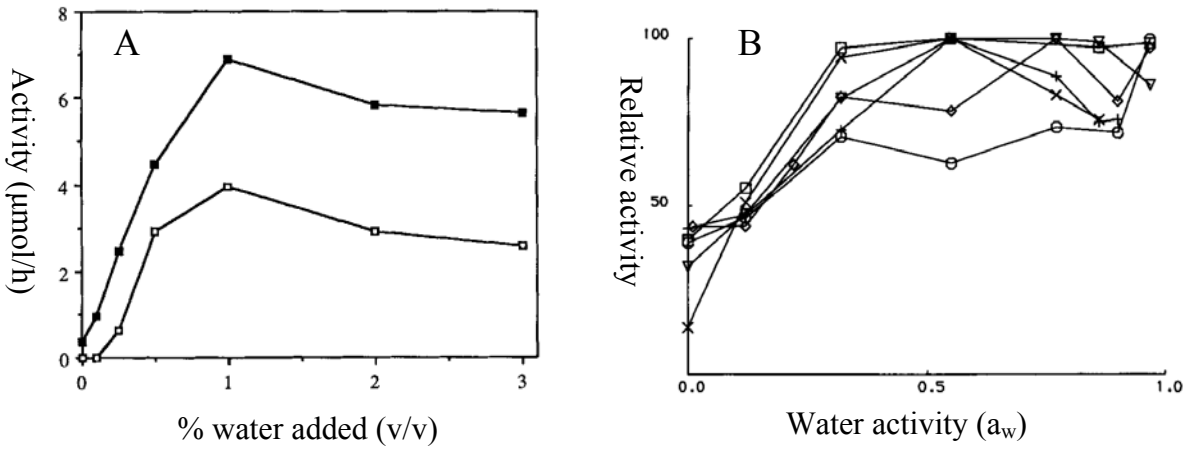


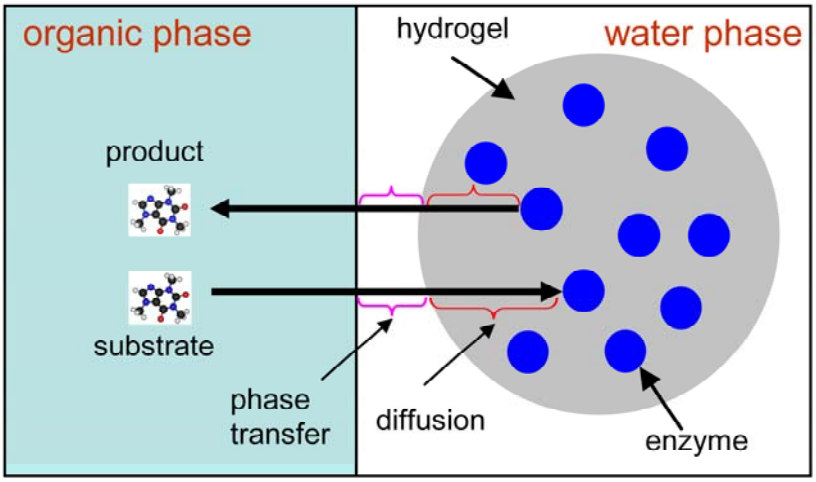
Figure 1.3. a) Esterification activity of chymotrypsin immobilized on glucose-CPG (□) and hexyl-CPG (■) under the similar immobilization conditions. b) Activity/profiles for *Rh. miehei* lipase adsorbed on different support materials. The support materials were polypropylene (▽), anion-exchange resin (+), hydrophobic porous glass (◊), polyamide (○), celite (□) and anion-exchange silica (×).[65, 66] Reprinted with permission from Eur. J. Biochem.[65, 66] Copyright © 1999–2011 John Wiley & Sons, Inc.

Mass transfer

An efficient application of immobilized biocatalysts in non-aqueous media requires substrates and products to be quickly transported through different phases, i.e. organic and aqueous phases, and liquid and solid phases (non-aqueous media and carriers). A slow diffusion results in many serious problems, such as decreasing reaction rate, precipitating products, blocking carrier pores, and inducing undesirable pH gradients.[67] Mass transfer behaves quite differently in different systems, depending on types of non-aqueous media, properties of carriers and enzymes, solubility of substrates and products, etc.

For hydrophilic carriers, reactions preferably take place in monophasic aqueous–organic or monophasic hydrophilic organic media, with polar substrates and products used. In this case, mass transfer resistance is minimized because of the solubility compatibility of substrates and products among different phases. But in many other cases, non-polar substrates or products are widely applied and require a biphasic aqueous–organic or monophasic hydrophobic organic medium to be employed for better solubility. For example, a model reaction of stereoselective carboligation of two 3,5-dimethoxy-benzaldehyde catalyzed by benzaldehyde lyase (BAL) immobilized in κ-carrageenan was recently reported in a biphasic medium.[68, 69]

The mass transfer limitation in that case mainly attributes to two facts: 1) transfer limitation of substrates and products between organic and water phase, 2) transfer resistance of them between aqueous phase and solid phase (Scheme 1.8). Though mass transfer limitation doesn't change the reaction equilibrium, it can greatly slow down the reaction rate.[70] Modified kinetic models are often produced according to individual case.[69, 70]



Scheme 1.8. Mass transfer limitation in the biocatalytic reaction of hydrogel with immobilized enzymes.

For hydrophobic carriers, monophasic hydrophobic organic media are often used to reduce the diffusion resistance between liquid and solid phases. But if hydrophilic substrates or products are present, co-solvents or two phase system can be introduced to improve solubility of hydrophilic substances and to facilitate mass transfer.[71] Therefore, the main strategy to minimize mass transfer limitation for biocatalytic synthesis by immobilisates is to optimize the comparability and solubility among carriers, solvents, substrates and products.

Stability

A driving force in the area of immobilizing enzymes is to improve enzyme stability against solvents, which leads to their numerous applications in non-aqueous media. Klibanov attributed the improved enzyme stability by immobilization to two aspects: 1) elimination of aggregation effects, 2) avoiding some conformation change.[72] As the natural process of enzyme aggregation denatures enzymes when they are in presence of organic solvents, immobilizing them can avoid the aggregation and result in better stability. The conformational change is accepted as the main reason responsible for enzyme inactivation in organic solvents.

Immobilizing enzymes in/on carriers increases their rigidity which reduces the possibility of unfolding from organic solvents. In addition, the carrier surface can partially exclude organic solvents from enzymes, which leads to a lower concentration of solvent around enzymes and thus improves the stability.

While much attention has been devoted to studying free enzyme stability in various non-aqueous media,[73, 74] little is yet reported to explore the stability of immobilized enzymes in different non-aqueous media. This could be due to the complex system of immobilisates in non-aqueous media. For example, water content and solvent hydrophobicity in non-aqueous media are the two key conditions to inactivate free enzymes.[75, 76] However these two conditions are not the only facts to influence stability of immobilized enzymes, which can be greatly affected by many other crucial conditions, e.g. carrier difference and mass transfer efficiency. Thus the diversity of immobilization strategies and carriers causes the widely different stability and activity of enzymes even in a same non-aqueous medium.

Mechanical stability & enzyme leaching

Mechanical stability is one of determinable parameters for immobilisates successfully applied in bioreactor conditions with long-term operation. The mechanical stability of immobilisates often relates to types of reactors, carriers, and solvent media. For examples, Stantos and Vogelsang *et al.* studied the abrasion stability of hydrogels in glass bubble column, and found that the abrasion was related to fatigue of the gel material, but not to their stiffness or fracture stress.[77, 78] In addition, organic solvents can swell and dissolve immobilisates to a different extent, depending on their solubility parameters ($\delta_{solubility}$, $cal^{1/2} \bullet cm^{-3/2}$).[79] If $\delta_{solubility}$ of both solvents and carriers is similar, solvents readily transfer and swell through carriers to facilitate mass transfer of reactants, but it increases the risk to decompose the carriers. In conclusion, the mechanical stability of immobilisates in non-aqueous media is determined by specific application conditions (e.g. reactor type, medium type, and properties of carriers and solvents).

An efficient immobilisate for biocatalysis in non-aqueous media requires minimizing enzyme leaching during applications and recycling. Enzyme leaching depends on immobilization strategy, enzyme properties, medium type, and carriers. For example, enzymes covalently bound on carriers are hard to leach out due to the strong bonds between carriers and enzymes.

But enzymes bound by physical adsorption are prone to leach differently in different non-aqueous media. In polar solvent media, most of adsorbed enzymes are easily leached due to good soluble enzymes in these media, but in non-polar solvent media, enzyme leaching is not easily observed, except with lipases which have strong hydrophobic regions.[44] Enzyme leaching is also highly dependent on carrier porosity.[80] Carriers with high porosity and large pore size are desirable for diffusion of substances, but increase the possibility of enzyme leaching. Coating immobilisates can substantially reduce the leaching.[80] However, it increases mass transfer limitation. Thus considering economic aspects, one has to find an optimal balance between reducing enzyme leaching and mass transfer limitation for immobilisates.

1.2.3. Lipase Immobilization for Esterification in Organic Solvents

Basic of lipases

Lipases (triacylglycerol acylhydrolase, E.C.3.1.1.3) are versatile biocatalysts that have been widely applied in food, dairy, detergent, chemical and pharmaceutical industries.[81, 82] Up to now there have been more than 50 lipases identified, purified, and characterized from various resources, e.g. plants, animals, and (native or genetically engineered) microorganisms.[83] About 5 % of the global enzyme market is shared by lipases due to their industrial versatility and unique catalytic performance, and an even increasing market occupation is expected.[84] Lipases belong to the sub-class of hydrolases, and hydrolyze long-chain triacylglycerols, such as natural substrates, into fatty acids and glycerol at lipid-water interface. Under non-aqueous conditions, lipases enable reverse reactions of hydrolysis for synthesis.[81] The capacity of lipases for both hydrolysis and synthesis renders them as biocatalysts with high chemo-, regio- and enantioselectivity for diverse substrates. The major reactions by lipases are summed up as follows:[82]

- Hydrolysis:

$R_1COOR_2 + H_2O \longleftrightarrow R_1COOH + R_2OH$

- Synthesis (including esterification and transesterification)

a) Esterification:

$R_1COOH + R_2OH \longleftrightarrow R_1COOR_2 + H_2O$

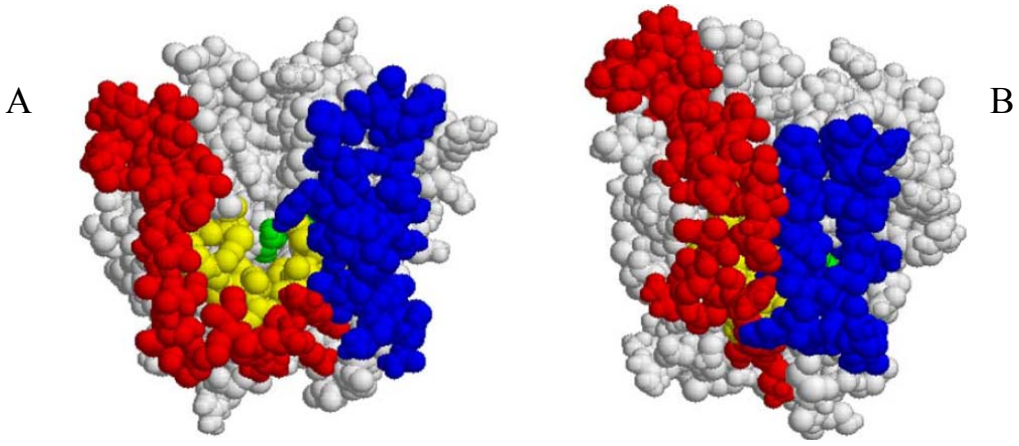
b) Transesterification:

$R_1COOR_2 + R_3COOR_4 \longleftrightarrow R_1COOR_4 + R_3COOR_2$ (Interesterification)

$R_1COOR_2 + R_3OH \longleftrightarrow R_1COOR_3 + R_2OH$ (Alcoholysis)

$R_1COOR_2 + R_3COOH \longleftrightarrow R_1COOR_3 + R_2COOH$ (Acidolysis)

A principal feature of lipases is their interfacial activation at oil/water interface, a characteristic distinguishing them from esterases.[85] A Ser-His-Asp catalytic triad comprises the active site of lipases and is covered by one or more relative hydrophobic polypeptide lids. A hydrophobic side of the lid faces the catalytic site, and the hydrophilic side is opposite to it. When a hydrophobic surface or substance approaches the vicinity of active site, a lid movement is triggered to expose the active site, resulting in interfacial activation. But if lipases are placed in aqueous media, the active site is buried by lids to protect it from environment. A simulation model of lid movement in *P. aeruginosa* lipase is demonstrated in Scheme 1.9.[86] The interfacial activation takes effects in most of lipases, for examples, in *R. miehei* lipase, thermoalkalophilic lipase,[87] and *P. glumae* lipase.[88] However an exception is lipase B from *Candida antarctica* (CalB), which possesses only a small lid with high mobility and shows no interfacial activation. Distinct from CalB, lipase A from *Candida antarctica* (CalA) displays the obvious effect of interfacial activation.



Scheme 1.9. A qualitative representation of lid movement in *P. aeruginosa* lipase: a) the open form of lipase, and b) the closed form of lipase. Active site is colored as yellow, and two lids are red and blue, respectively.[86] Free reprint permission of PLoS Comput. Biol. under the terms of the Creative Commons Attribution License.[86]

Lipase immobilization techniques

In general, lipases have been used to date as the most successful enzymes for immobilizations, and engage all available immobilization techniques (e.g. adsorption, covalent binding, entrapment, encapsulation, and cross-linking). Among these techniques, adsorption and entrapment are widespread employed for lipase immobilization. Adsorption is exceptionally widely used for different lipases, of which Novozym 435 is a prominent example of adsorbing CalB onto macroporous poly(methyl methacrylate) (PMMA) with extensive applications for synthesis and polymerizations.[36, 80] A great number of examples show that lipases can be adsorbed onto hydrophilic carriers (e.g. silica, hydrogels, glass, celite, sepharose, and sephadex) owing to their broad availability and simplicity of preparation. However, Fernandez-Lafuente *et al.* systematically reviewed that lipase adsorption on hydrophobic supports works more efficient because matrix hydrophobicity resembles their nature products, inducing the active site open.[89] Additionally, the interactions between lipases and hydrophobic carriers are found to be much stronger than that between lipases and hydrophilic carriers. In addition to hydrophobicity, the final performance of lipase adsorption on carriers relies on many other parameters, e.g. pH, ionic strength, isoelectric point of the lipases, surface and protein properties, and the dependence of lipase-adsorption kinetics.[90]

Entrapment is another technique broadly used for lipase immobilization. There are many non-porous natural and synthetic polymers used to entrap lipases. Significant progress on lipase entrapment was made by Reetz's group through the entrapment of different lipases onto various porous hydrophobic silica sol-gels to obtain up to 80 times higher relative activity.[30, 36] They found that the gel matrix from more hydrophobic monomers of the type $RSi(OCH_3)_3$, where R is alkyl groups, resulted in obviously increasing activity of lipases (Figure 1.4).[30] This finding proves that the hydrophobic matrix favors lipase entrapment, which indicates to optimize sol-gels of lipase entrapment in terms of hydrophobicity of carriers. Interestingly, the same group later studied the influence of added additives in matrix to lipase catalysis, and realized that addition of synthetic polymer (e.g. polyethylene glycol (PEG) and polyvinyl alcohol (PVA)) and other proteins (e.g. albumin and gelatin) could enhance lipase activity. The resultant phenomena were speculated as these additives avoid inactivation of lipases and change the microenvironment in the defined silica gels.[30] Recently, "biocatalytic active static emulsions (BASE)" were invented as a novel method of lipase entrapment, by which lipases are emulsified as micro-water droplets inside polydimethylsiloxane (PDMS).[20, 33] The great enhancement of lipase activity compared with sol-gel system was found in BASE, which

mainly attributes to the numerous micro aqueous pools in BASE to enhance interfacial area. Another advantage of BASE is the strong mechanic strength of PDMS matrix used. All these advantages should drive BASE to a bright future for broad applications.

Indeed, there is also plenty of literature reported on lipase immobilization by covalent binding,[91] encapsulation,[92] and cross-linking.[93] Due to space limitation of this thesis, immobilization of lipases by other methods is not introduced here because of their smaller importance for lipases than the methods of adsorption and entrapment.

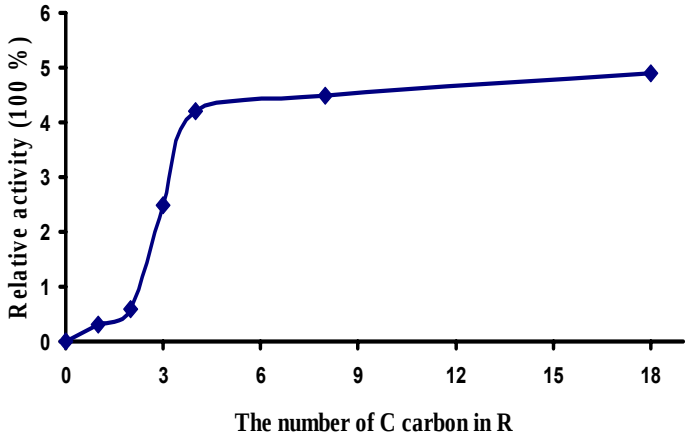
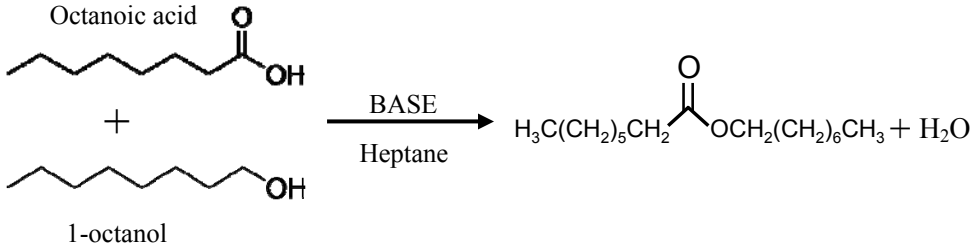


Figure 1.4. Influence of the chain length of R on the activity of immobilized *Ps. cepuciu* lipase in gels of the type TMOS/RSi(OMe)$_3$ (1:l).[30] Reprinted with permission from Adv. Mater.[30] Copyright © 1999–2011 John Wiley & Sons, Inc.

Esterification by immobilized lipases in non-aqueous media

Immobilized lipases are extensively applied in non-aqueous media for organic synthesis with the main purpose to enhance enzyme stability. Among different syntheses, esterification has been accepted as a particularly important reaction for many applications (e.g. food industry) and thus established as one of standard reactions for catalytic assay of immobilized lipases.[20, 30, 31, 33, 34] For example, Scheme 1.10 illustrates an esterification of 1-octanol and octanoic acid catalyzed by BASE for production of octyl octanoate.[20, 33, 34] Esterification applications can also be found by other immobilized lipases (e.g. Novozym 435) in solvents or even solvent free media.[80, 94] Generally, water activity, solvent selection, and carrier hydrophobicity are considered as three crucial parameters to any successful esterification catalyzed by immobilized lipases.



Scheme 1.10. Esterification of 1-octanol and octanoic acid by BASE in heptane.

1.3. Pickering Emulsions and Janus Catalysts

1.3.1. Basic of Pickering Emulsions

Pickering emulsions are referred to as emulsions stabilized by solid particles adsorbing at interface between immiscible liquids, with a typical feature in Scheme 1.11a.[95] A century ago, Pickering and Ramsden pioneered this concept, and originally observed paraffin-water emulsions stabilized by various solid particles (e.g. basic sulphides of iron, copper and nickel).[96] These emulsions stabilized by particles possess excellent properties, e.g. irreversible adsorption of particles, low toxicity, and low cost. As a consequence of this, much attention has been paid for their use in applications ranging from synthesizing materials (e.g. preparation of porous materials, composite microspheres, and hollow microspheres) to oil separation, cosmetic preparation and waste water treatment. In 1980, Pieranski theoretically concluded that the decrease of interfacial energy (ΔE) by localizing particles at interface is the intrinsic reason of forming Pickering emulsions (equation 1.1 and Scheme 1.11b):[97]

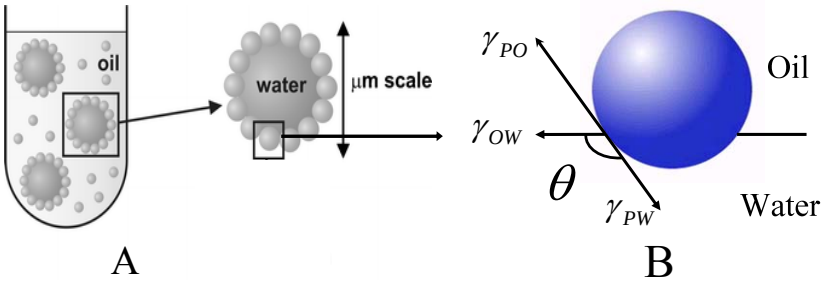
$$\Delta E = -\frac{\pi r^2}{\gamma_{OW}}\left[\gamma_{OW} - (\gamma_{PW} - \gamma_{PO})\right]^2 \qquad (1.1)$$

Where γ_{OW}, γ_{PW} and γ_{PO} represent interfacial energy from interface of oil/water, particle/water, and particle/oil, respectively, and r is particle radius. From equation 1.1, the stabilization capacity of particles at interface is highly size-dependent. Presuming particle size is smaller than few microns in diameter, the effect of gravity of particles is negligible. Thus, particle stability in a given emulsion system (i.e. with fixed γ_{OW}, γ_{PW} and γ_{PO}) is proportional to r^2, and bigger particles result in more decrease of total free energy (ΔE) than smaller ones. By this, microscopic particles can irreversibly localize at interface and produce super stable Pickering emulsions, while nanoscopic particles (< 20 nm) are unstable at interface and

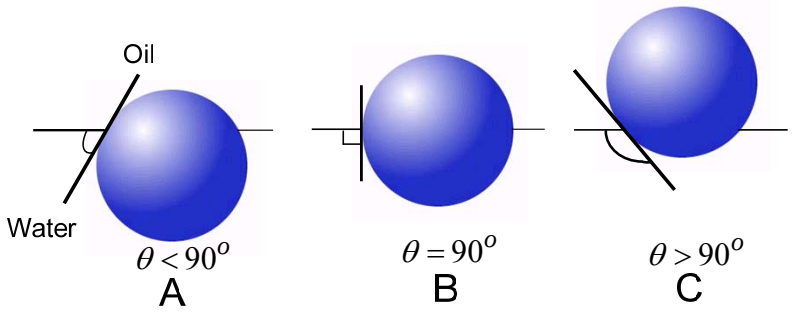
equilibrate between liquids and interface.[98] In addition, the stability of Pickering emulsions also highly relates to particle wettability, which is quantitatively reflected by contact angel (θ). According to Young equation, the contact angel (θ) of particles at interface is subjected to equation 1.2 (Scheme 1.12). Equation 1 can thus be expressed with contact angel as equation 1.3.[99] When θ = 90° (Scheme 1.12b), there is the lowest total energy of the system, which results in the thermodynamically stable Pickering emulsions. If θ < 90° (Scheme 1.12a), an oil-in-water (O/W) emulsion is stable; if θ > 90° (Scheme 1.12c), a water-in-oil (W/O) emulsion is favored. However, the emulsion system is unstable if θ locates between 0 and 20° or 160° and 180°, which indicates particles that are too hydrophilic or hydrophobic and therrfore unsuitable for stabilizing immiscible liquids.

$$\cos\theta = \frac{\gamma_{PW} - \gamma_{PO}}{\gamma_{OW}} \qquad (1.2)$$

$$\Delta E = -\pi r^2 \gamma_{OW} (1 - |\cos\theta|)^2 \qquad (1.3)$$



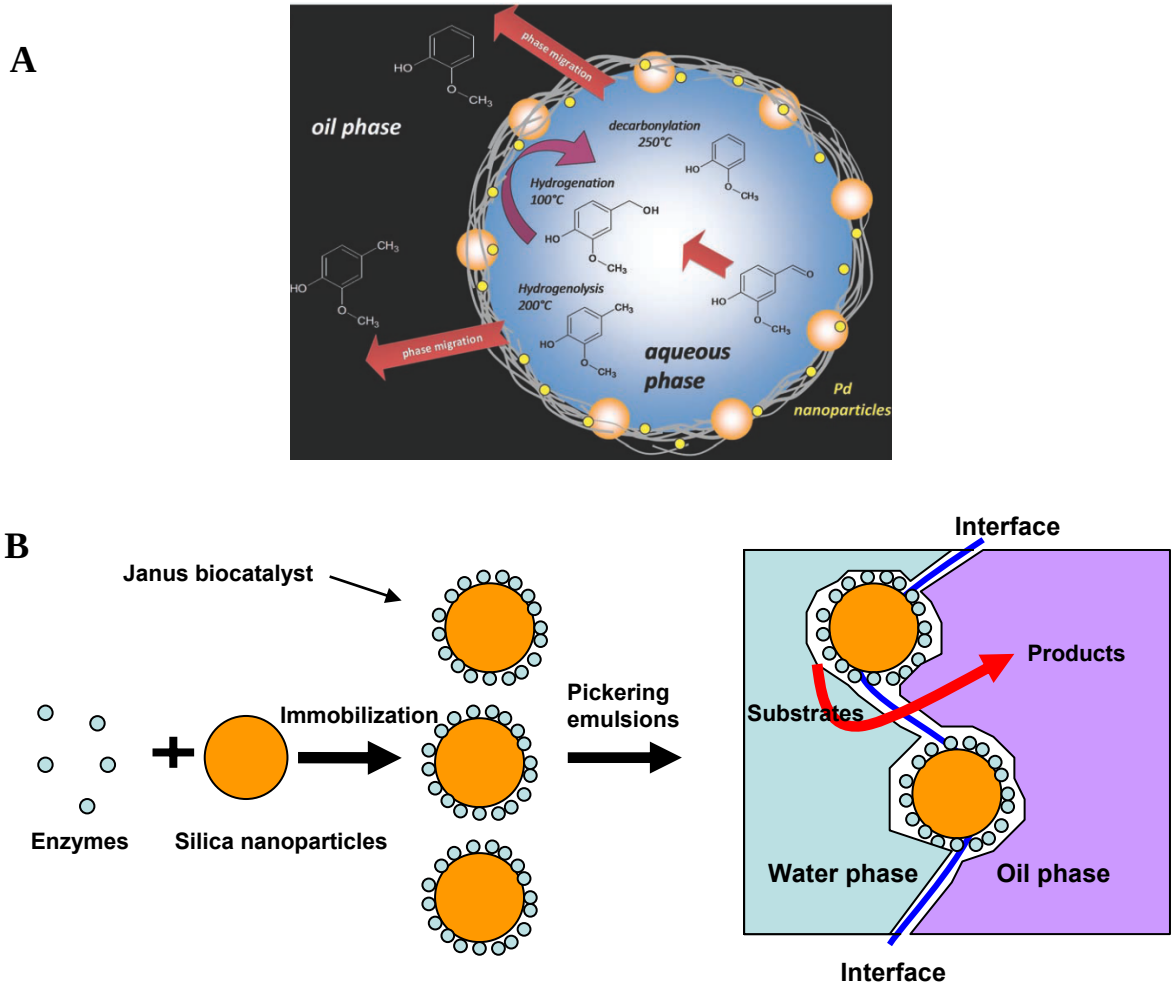
Scheme 1.11. a) A typical feature of water-in-oil (O/W) emulsions stabilized by solid particles.[95] Reproduced by permission of The Royal Society of Chemistry.[95] b) A particle adsorbed at interface involving various interfacial energy (γ_{OW}, γ_{PW}, and γ_{PO}) and contact angel (θ).[100] Modified accordint to literature.[100]



Scheme 1.12. In immiscible phases of oil and water, particle behavior at interface with different contact angles (wettability): a) $\theta < 90^o$, b) $\theta = 90^o$, and c) $\theta > 90^o$.

1.3.2. Janus Catalysts and Janus Biocatalyts

Janus catalysts were termed by Cole-Hamilton in 2010 as reactions catalyzed by Janus particles which, like the Roman god Janus, present two faces with one of the hemispheres hydrophilic and the other hydrophobic.[101] In these Janus catalysts, catalytically active metals are embedded into one region of particles for the function of catalysis, while amphiphilic properties of particles lead them localized at interface of immiscible liquids.[102] Applications of Janus catalysts can obtain several advantages:[101, 103] 1) large interfacial area of aqueous phase, 2) facilitated mass transfer of molecules between biphasic phases, and 3) simplified reaction/separation process with recoverable solid catalysts used instead of surfactants. The Resasco group is pioneering at the research of Janus catalysis. In early 2010, they successfully deposited palladium (Pd) onto Janus catalysts consisted by hydrophobic carbon nanotube and hydrophilic oxide nanoparticles (SiO_2 and MgO) to catalyze biomass refining reactions (e.g. hdyrodeoxygenation) in a water-decailin immiscible system (Scheme 1.13a).[101, 104] Alternatively, the catalytically active Pd could be selectively deposited into either hydrophobic or hydrophilic face of the Janus hybrid, which could be chosen for conversion of the desired reaction with different solubility of reactants and products in the immiscible system. Later on, the same group employed Janus silica nanoparticles loaded with Pd on either hydrophobic or hydrophilic face for hydrogenation in biphasic systems.[103] A "phase selectivity" phenomenon was observed by controlling the desired reactions on one side where catalytic active Pd was loaded. Due to the elucidated excellence of Janus catalysts, the arisen interest and applications of them are being expected.



Scheme 1.13. a) A representation of Janus catalyst with two faces for catalysis. Reprinted with permission from Science.[104] Copyright © 2011 American Association for the Advancement of Science. b) Janus biocatalyst for biocatalysis

Conventional inorganic catalysts are classified as either homogeneous or heterogeneous catalysts, depending on whether catalysts and substrates are in the same phase. Biocatalysts (enzymes) are often considered as a separate group. But strictly speaking, the soluble enzymes in aqueous media belong to homogeneous catalysts, while immobilized enzymes are categorized into heterogeneous catalysts. As introduced before, heterogeneous biocatalysts (i.e. immobilized enzymes) are often applied in organic media for the sake of improved solubility of substrates and products. The problems in these media are the mass transfer limitation from immiscible phases, and sometimes lack of water phase to enzymes.[20] Emulsified biphasic system can provide sufficient water content to enzymes and reduces diffusion resistance of substances. But most investigated emulsions are microemulsions or

reverse micelles, stabilized by amphiphilic surfactants which not only denature enzymes but cause them close restricted in water pools and inaccessible to reactants. The recent concept of Janus catalysts is inspiring us to envision the next generation of biocatalysts — Janus biocatalysts (Scheme 1.13b). Theoretically, a hydrophobic particle (< 1 μm) is capable to adsorb/bind any enzyme to form two faces as Janus particles, which is similar to the reported Janus catalysts with two faces. A few advantages should be expected in Janus biocatalysts: high interface area, simple separation of biocatalysts, and almost all accessible catalytic sites. Unfortunately, none of the research on Janus biocatalysts has been reported yet, which may be due to the just freshly emerging idea of Janus catalysts, let alone Janus biocatalysts. However due to benefits catalyzing reactions at interface, Janus biocatalysts should be considered as approaches to eliminate diffusion resistance and offer enough water activity for enzymes, bridging the gap between homogeneous and heterogeneous biocatalysts.

1.4. Research Motivation

The study of this thesis aims to improve the interface of immobilized biocatalysts in organic solvent media. As it is well known, organic solvent media are beneficial to the organic synthesis catalyzed by immobilized enzymes.[62] However, their practical applications often encounter serious mass transfer limitation, which renders their catalytic performance remarkably decreased and thus restricts their practicability. All mass transfer limitation essentially originates from a same fact – the formation of a poor interface among carriers, enzyme phase and solvent medium. To solve these problems, different strategies should be developed to improve the interface formed by different immobilisates, typical which are enzymes in hydrophilic, or hydrophobic carriers, or emulsions. In each case, it is necessary to find the reason for the formation of the poor interface and to deliver a corresponding solution in order to improve the interface for better catalytic performance.

Hydrophilic hydrogels are widespread employed for enzyme immobilization due to their easy availability and good biocompatibility. However, if they are presented in organic solvents, two main problems occur: 1) the hydrophilic carriers are insoluble in most hydrophobic solvent media, and 2) hydrophobic substrates and products are difficult to diffuse into/out of these hydrophilic carriers. These two solubility problems are due to the poor interface existing between hydrophilic carriers and hydrophobic solvents. According to the previous finding, some water-miscible solvents enable to wet hydrogel surface and subsequently to adapt

hydrogels in hydrophobic organic solvent media, which is called solvent exchange process.[105] Encouraged by its advantages, the solvent exchange method is applied here to improve the interface of hydrophilic immobilisates in organic solvent media. To demonstrate generality, this method is designed to improve the interface of two different hydrogels (agarose and PNIPAM gels) immobilized with three different enzymes in heptane. The reusability and stability of immobilisates is studied to check catalytic performance after solvent exchange. Success of this method will provide a general idea for improving the interface of hydrophilic immobilisates for better catalytic performance in organic solvents.

For hydrophobic carriers, there is no solubility incompatibility between carriers and hydrophobic substances. But in industrial applications, facilitated separation process generally requires large particle size (> 100 μm) to be used, which leads to small interface area of these immobilisates and thus decreases their catalytic efficiency.[5] Within the size limit, one of the solutions for better catalytic performance is to extend internal surface area of these immobilization matrices. Interestingly, an emulsified enzyme solution in hydrophobic carrier was recently reported and named as BASE.[20, 33] With this technique, two main advantages are achieved, namely, increased internal surface area and additional provision of water activity to enzymes. This system has proved exceptionally good for esterification catalyzed by lipase immobilisate in hexane.[33] However, the resultant system is still in a preliminary stage and not optimized yet, with their structure and morphology undisclosed as well. It is important to take BASE as a representative example for further optimization, demonstrating the contribution to improve the interfacial area of aqueous enzyme phase for biocatalysis in these hydrophobic carriers.

Emulsifying aqueous enzyme solutions in organic solvents is believed as a very effective method to obtain large contact area of aqueous enzyme phase to reactants. For example, microemulsions stabilized by surfactants are reported to form enzyme water droplets at diameter 1 – 20 nm in organic solvents, resulting in high enzyme activity. But applications of these microemulsions are often compromised in practice owing to their separation difficulty and surfactant inactivation to enzymes. Different from microemulsions, Pickering emulsions are the emulsions stabilized by particles at interface to form micron-sized water droplets in solvents. Not only do they possess high interface area of aqueous phase, but particles can be easily separated from system with simple methods such as filtration and centrifugation. Thus

encapsulating enzymes for catalysis in Pickering emulsions will benefit advantages of both separation and large interfacial area. Unfortunately, this kind of enzyme encapsulation has not been reported to date. Their advantages, however, exactly satisfy the interest to pursue an emulsion system with the advanced interface of immobilizing enzymes for catalysis.

To summarize, improving the interface of immobilisates in organic media for biocatalysis is the research objective of this thesis. For this purpose, three classic immobilisates (i.e. hydrophilic and hydrophobic matrices, and emulsions) were selected to analyze the reason for their intrinsically poor interface that they form in organic solvents, and to provide corresponding methods for improvement. These methods can be: general, like solvent exchange process for hydrophilic immobilisates; specific, like BASE system; and novel, like enzyme encapsulation in Pickering emulsions. This thesis has studied targets to present methods and ideas for various immobilisates with improved interface for biocatalysis in organic solvent media.

2. Experimental

2.1. Materials

2.1.1 Chemical Materials

Chemical	Source	Chemicals	Source
Acrylic acid (AA)	Sigma-Aldrich (Munich, Germany)	Calcium chloride	Sigma-Aldrich (Munich, Germany)
Agarose (Type XI, gelling temperature ≤ 17 °C)		3-mercaptopropionic acid (MPA) (99%)	
Cadmium chloride hemi (pentahydrate) ($CdCl_2$, 99 %)		Tetraethyl orthosilicate (TEOS) (≥ 99 %)	
N-isopropylacrylamide (≥ 97%) (NIPAM)		Trimethoxy (octadecyl) silane (TMODS) (90 %)	
Sodium carbonate		Methyl tert-butyl ether (MTBE) (≥ 99.5 %)	Carl Roth (Karlsruhe, Germany)
Octanoic acid (≥ 99%)		Sodium chloride	
1-octanol (≥ 99%)		Hexane	
Oleylamine (technical grade, ≥ 70%)		Heptane	
Tellurium powder (-200 mesh, 99.8 %)		Methyl isobutyl ketone (MIBK)	
Polyvinyl alcohol (PVA, Mowiol 10-98, Mw ~ 61,000)		Cyclohexane	
Fluorol yellow 088		Toluene	
Sudan red B		Chloroform	
Octyl octanoate (≥ 98 %)		Tetrahydrofuran (THF)	
Benzaldehyde (≥ 99 %)		Sulfoxide (DMSO)	
Benzoin (≥ 99 %)		Sylgard 184 (silicone elastomer kit including Syl-Off® 4000)	Dow Corning (Wiesbaden, Germany)
Thiamine diphosphate		N,N'-methylenebisacrylamide (MBA) (≥ 99,5%)	Fluka (Munich, Germany)
Acetonitrile		Potassium peroxodisulfate (KPS) (≥ 99%)	

Isopropanol (IPA)		The fluorescein isothiocyanate (FITC) labeling kit	EMD Biosciences (Nottingham, UK)

2.1.2 Biological Materials

Enzymes and biocatalysts	**Source**
Lipase A from *Candida antarctica* (CalA, Novocor ADL, protein content 1.28 % (w/w))	Novozymes A/S (Bagsvaerd, Denmark)
Lipase B from *Candida Antarctica* (CalB, Lipozyme CALB L, protein content 0.24 % (w/w))	
Novozym 435	
Bovine serum albumin (BSA, 98 %, lyophilized powder, ~ 66 kDa)	Sigma-Aldrich (Munich, Germany)
Benzaldehyde lyase (BAL)	Purification according to literature[106]

2.2. Immobilization of Enzymes in Diverse Carriers

2.2.1. In Hydrophilic Hydrogels

Agarose microparticles (MPs) with sizes in the range of 5 – 50 μm were obtained according to a modified protocol reported by Mao et al.[107] Typically, 15 mg agarose were dissolved in 1 mL water at 80°C; the agarose concentration was 1.5 % (w/w). The resulting hot aqueous solution was dropped into 20 mL hexane in the presence of oleylamine (5% wt/v) as stabilizer under sonication at ambient condition. Oleylamine was removed by washing with tetrahydrofuran (THF) for at least 3 times. The agarose MPs were consecutively washed with isopropanol (IPA) for 3 times and with water for 3 times and finally stored in water.

PNIPAM MPs with diameters of about 600 nm were synthesized via surfactant free emulsion polymerization at 70 ºC. PNIPAM MPs with diameters of about 1.5 μm were synthesized via surfactant free emulsion polymerization under deliberate temperature control.[108] Typically, 1.8 g NIPAM and 0.06 g MBA were dissolved in 125 mL water. After deaeration with N_2 for 1 h, the aqueous mixture was slowly heated to 45 °C, followed by addition of 5 mL KPS aqueous solution (0.078 M) under gentle stirring. Subsequently, the reaction medium was heated to 65 °C at a rate of 0.5 °C/min and maintained at 65 °C overnight under stirring to

guarantee completion of the polymerization. After removal of aggregates via filtration through glass wool, the resulting PNIPAM MPs were purified by 4 times repetition of 1 h centrifugation at 8965 g, decanting the supernatants, and redispersion in water. They were collected via lyophilization at - 85 °C under 1 x 10^{-3} bar for 48 h.

2.2.2. In Hydrophobic Silicone BASE

Entrapment of enzymes in BASE employed with two different mixing systems. The initial mixing system consisted of a 400 mL beaker-glass with a 4 cm magnetic stir bar. This was placed on a magnetic stirrer. The advanced mixing system consisted of a 600 mL beaker-glass equipped with four baffles and a motor-controlled impeller with four blades (each 1 cm in length). This set-up was placed on a heating plate (Figures 2.1 and 2.2).

For preparation of standard BASE 2 mL CalA dissolved in water were added to a mixture of 8 g Sylgard® compound A (α, ω-divinyl terminated polydimethylsiloxan) and 0.8 g Sylgard® compound B (copolymer of methylhydrosiloxane and dimethylsiloxane). 4 mL Syl-Off® 4000 in chloroform (1.46 % (w/w)) was introduced to initiate polymerization. After 25 min gentle shaking on an over-head shaker, the solution was poured into 300 mL aqueous solution of PVA (4 % (w/w)) and stirred at 1000 rpm and 45 °C for 2 h. The solidified beads were separated by filtration. Protein content in the filtrate was determined via Bradford assay.[109] BASE particles were transferred to hexane and stored at room temperature until use. For optimization water content and enzyme concentration were varied as described in the chapter 4.

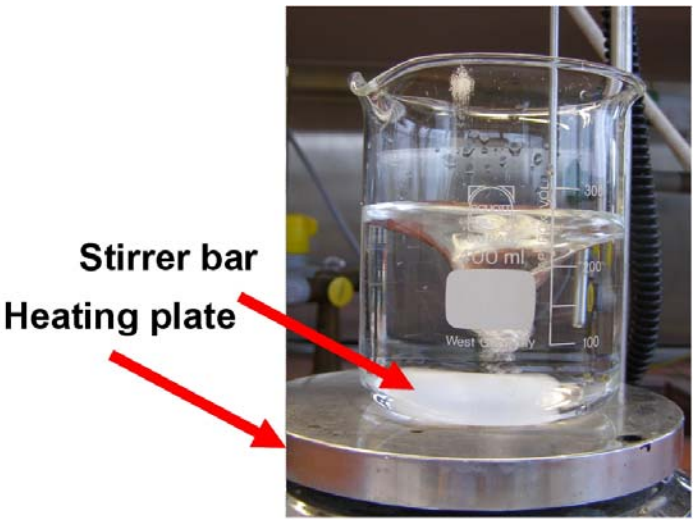


Figure 2.1. Photo of plain beaker glass using a magnetic stir bar for preparation of prototype BASE.

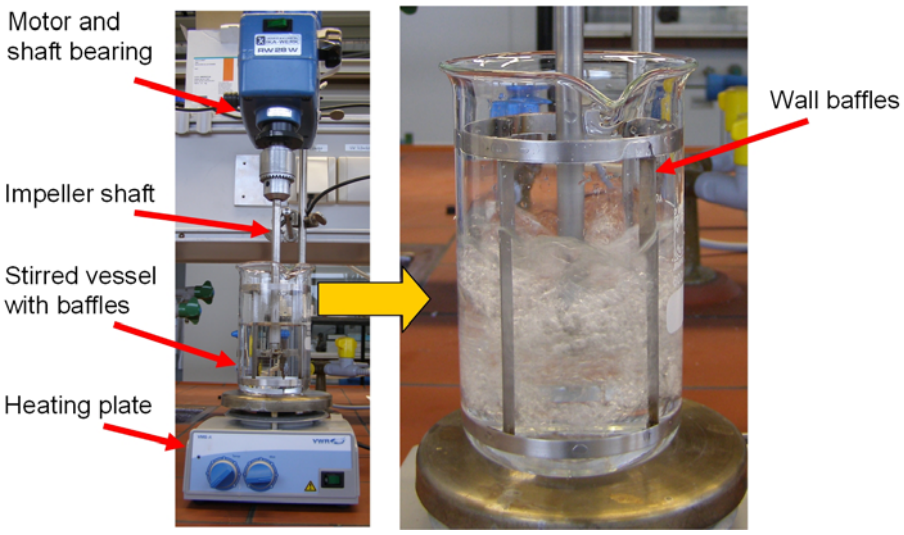


Figure 2.2. Photos of an advanced mixing system of stirred vessel with baffles: the left photo is the whole advanced mixing system equipped by motor, impeller, heating plate, and stirred beaker glass with baffle; the right photo is an amplified photo of stirred glass with baffle.

Dried BASE were obtained by two-month drying prototype BASE in air. Supplying low-water system to the dried BASE was controlled by incubating them in closed desiccators, where saturated salt solutions or distilled water was placed in to provide different water activity (a_w). Table 2.1 lists a_w in different saturated salts and distilled water.

Table 2.1. Saturated salt solutions used for different water activity.

Saturated salt solutions	$CaCl_2$	NaCl	Na_2CO_3	Distilled water
Equilibrium relative humidity (ERH) (%)	32	76	92	100
Water activity (a_w)	0.32	0.76	0.92	1.00

2.2.3. In Pickering Emulsions

To obtain Pickering emulsions, modifying silica nanoparticles with TMODS (SiO_2@TMODS) is required. Monodisperse SiO_2 NPs with diameters of 140 nm were synthesized from TEOS in ethanol according to the Stöber method.[110] The resulting SiO_2 NPs were collected by centrifugation at 9000 rcf for 5 min. After the supernatants were decanted, the SiO_2 NPs were dispersed in ethanol. The cycle of

centrifugation/decanting/redispersion was repeated for three times to remove the excess TEOS and ammonia. For hydrophobization of nanoparticles, 40 μL TMODS was added into 2 mL SiO_2 NP dispersion in ethanol (3 % (w/w)), followed by heating at 60 °C overnight. After washing with ethanol via three times repetition of centrifugation/decanting/redispersion, the resulting SiO_2@TMODS NPs were redispersed into heptane or MTBE.

In order to immobilize enzymes in Pickering emulsions, 100 μL aqueous stock solution of native CalB or BAL (its volumetric activity of benzoin condensation is 500 U/mL) were added into 2 mL dispersions of SiO_2@TMODS NPs in heptane or MTBE at 0 °C, followed by homogenization. To immobilize enzymes into jellified Pickering emulsions, 15 mg agarose with a gelling temperature of ≤ 17 °C were dissolved in 1 mL buffer solutions at 50 °C. After cooling down to 25 °C, the resulting agarose solutions were mixed with 1 mL aqueous stock solution of native CalB or BAL (its volumetric activity is 500 U/mL). 100 μL enzyme/agarose mixture solutions were homogenized in 2 mL dispersion of SiO_2@TMODS NPs in heptane or MTBE at 0 °C, leading to immobilization of the enzymes in the jellified Pickering emulsion droplets.

2.3. Characterization of Diverse Immobilization Systems

2.3.1. Characterization of NPs and Enzymes in Hydrophilic Hydrogels

Phase transfer and encapsulation of hydrophilic CdTe QDs into hydrogel MPs

3-mercaptopropionic acid-stabilized CdTe quantum dots (QDs) with different sizes were synthesized in water according to the method reported by Zhang et al.[111] They were used to characterize the loading of nanoparticles in hydrogels. Typically, freshly prepared aqueous NaHTe solutions were injected into 20 mmol•L^{-1} N_2-saturated $CdCl_2$ solutions in the presence of various thio-ligands as stabilizers to form the QD precursors at pH 11. The molar ratio of Cd^{2+} : thiol : HTe^- was set as 1 : 2.4 : 0.5. The resultant precursor solutions were refluxed at 100 °C to prepare CdTe QDs. The QD sizes were controlled to be 2.8, 3.3, and 4.0 nm by increase of the reflux time, and the sizes were estimated from the first electronic transition in the absorption spectra of the QDs.

Hydrogel MPs were incubated in aqueous dispersions of CdTe QDs for 2 h to ensure homogeneous and extensive diffusion of the QDs into the hydrogel MPs. After decanting the aqueous QD dispersion, the MPs were collected by centrifugation and incubated in

isopropanol for 1 min. After centrifugation, the MPs were readily dispersed into various water-immiscible organic solvents such as chloroform, toluene, and hexane, and thus the QDs were transferred into the organic solvents and trapped within agarose or PNIPAM MPs soaked by the organic solvents. Large agarose MPs were easily collected by simple sedimentation or by slight centrifugation at 2000 g for 5 min, and small PNIPAM MPs were collected via centrifugation at 18 000 g for 12 min. In order to improve the dispersibility of QD-loaded hydrogel MPs in water-immiscible organic solvents, they were incubated in THF for 1 min after IPA incubation, which was usually a need in the case of PNIPAM MPs.

Phase transfer and encapsulation of enzymes into hydrogel MPs

For visualization of the enzyme loading with fluorescence microscopy and spectroscopy, the enzymes were labeled with FITC according to the instructions in the FITC labeling kit. All chemicals for labeling, including FITC vials, the solvent reagents to dissolve FITC, and carbonate and PBS buffers, were provided by EMD Biosciences (Nottingham, UK). Typically, 10 mL aqueous solution of proteins was dialyzed overnight in carbonate buffer. The purified protein solutions were concentrated into 2 mg/mL. 850 μL solvent reagents were added into one FITC vial, which was thoroughly mixed to obtain 1 mg/mL FITC solutions. 500 μL resulting FITC solution was mixed with 1.25 mL protein solutions (2 mg/mL protein) on an overhead-shaker for 2 h at ambient condition. Note that in the entire labeling process, the mixture solutions were protected by aluminum foil. The FITC labeled proteins were dialyzed twice with PBS buffer. The purified FITC labeled proteins were kept at - 20 °C.

For phase transfer of BSA, CalB, and BAL, hydrogel MPs were incubated overnight in the buffered enzyme solutions (0.1 M potassium phosphate buffer, pH 7) at 4 °C. In order to minimize the adverse effect of water-miscible organic solvents on the enzymes, large agarose MPs, which could be rapidly separated from the organic solvents, were used as carriers and host for phase transfer and isopropanol (IPA) was used for BSA and CalB loading and dimethyl sulfoxide (DMSO) for BAL loading.

Characterization of transfer process and MP and NP properties

The sizes of CdTe QDs were determined by UV-vis absorption spectroscopy (Cary 50 UV-vis spectrophotometer, Varian, Inc.). Hydrogel MPs in water were characterized by dynamic light scattering and confocal microscopy (Leica DM IRBE confocal laser scanning microscope (CLSM) with a 30 W UV lamp (λ = 350 nm) as the light source). Transfer and encapsulation

of hydrophilic NPs into hydrogel MPs via solvent exchange were analyzed by CLSM. The photoluminescence variation of CdTe QDs during transfer and encapsulation of hydrogel MPs via solvent exchange was analyzed by using a Fluoromax-4 spectrophotometer. The secondary structures of enzymes were analyzed by circular dichroism (CD) spectroscopy (JAS.C.O J-715 spectropolarimeter). The concentration of CdTe QDs loaded in hydrogel MPs was estimated with the help of UV-vis absorption spectroscopy. The number of hydrogel MPs was determined by using single particle light scattering (SPLS).[112] The concentration of CalB immobilized in agarose MPs was quantified via Bradford assay.[109]

2.3.2. Characterization of Hydrophobic Silicone BASE

Determination of BASE size distribution

Particle sizes were determined by sorting the beads with sieves of different mesh sizes.[80] Mesh sizes were 100, 150, 300, 400, 500, 600, 800, 1000, and 1400 µm (ISO 3310-1, Retsch, Haan, Germany).

Immobilization yield

The amount of immobilized CalA was estimated by subtracting the protein amount determined in the filtrate after BASE preparation from initial amount of protein introduced into the system.

Microscopic studies

Scanning electron microscopy (SEM) was performed on a Hitachi S-2700 instrument (Hitachi, Nissei Sangyo GmbH, Ratingen, Germany) at an acceleration voltage of 20 kV. Cross sections of BASE were obtained by crashing beads after treatment in liquid nitrogen for 2 min. Confocal microscopy was performed on a Leica TCS SP5II confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 512 ~ 582 nm. Emission was detected by a photomultiplier. Endoscopic images were taken by a probe equipped with a 7 mm thick endoscope in front of a CCD camera as microscope lens. 5 min after introduction of the emulsion of enzyme solution in siloxane precursors into the stirred PVA solution, the endoscope probe was carefully inserted into the solution and semi-automatically captured 100 photos per minute. Fluctuations due to the influence of stirring were minimized by use of a quick flash light. Detailed set-up and theory of this measuring device can be found in literature.[113, 114]

Mechanic strength and protein leaching between BASE and Novozym 435

500 mg BASE and 500 mg Novozym 435 were stirred at 500 rpm and 40 °C in 5 mL heptane for one day. The optical photos were taken before and after stirring to compare their mechanic strength. After stirring, heptane in both BASE and Novozym 435 were completely evaporated in fume hood for one week. Then they were re-dispersed in 5 mL distilled water for 2 min, and the particles were finally filtrated. The protein leaching in 5 mL filtrate was analyzed according to Bradford assay.

2.3.3. Characterization of Enzyme Encapsulation by Pickering Emulsions

CalB and BAL were labeled with FITC, respectively. The size of silica NPs were determined by dynamic light scattering. Aqueous or jellified enzyme droplets stabilized by NPs in organic solvents were characterized by CLSM (Leica DM IRBE confocal laser scanning microscope with a 30 W UV lamp (λ=350 nm) as the light source) and environmental scanning electron microscopy (ESEM). The secondary structures of enzymes were analyzed by circular dichroism (CD) spectroscopy (JAS.C.O J-715 spectropolarimeter). The concentration of enzymes immobilized in agarose MPs was quantified via Bradford assay.

2.4. Catalytic Assay of Immobilized and Free Enzymes

2.4.1. Activity Assay of Immobilized CalB in Hydrogels or as Free Enzymes

The catalytic performance of CalB was determined via the esterification of 1-octanol and octanoic acid in heptane (as shown in Scheme 1.11). Typically, 50 μL droplets of the aqueous solution of native CalB (0.12 mg of protein) or 500 mg of agarose MPs loaded with CalB (0.42 mg protein) were thrown in 5 mL of substrate solution in heptane containing 100 mmol•L^{-1} 1-octanol and 100 Mm octanoic acid. These dispersions were stirred at 400 rpm for 50 min at 40 °C. During the reaction, 50 μL aliquots of the reaction solutions were taken every 10 min and subjected to gas chromatography (GC) (Table 2.2). The concentration of the product octyl octanoate was calculated at a typical retention time of 10.01 min. All reactions were performed in triplicate. One unit per mg (U/mg) of specific activity of free or immobilized CalB was defined as 1 μmol of product produced per min per mg of free or immobilized CalB.

For determination of enzyme reusability, agarose MPs loaded with CalB were separated from old substrate media, washed three times with 10 mL of heptane, and redispersed in fresh

substrate media for the next experimental run. Enzyme stability was measured by analyzing the residual catalytic activity after storage of agarose MPs loaded with CalB in hexane at 30 °C for 10 days under gentle shaking. All experiments were performed in triplicate.

2.4.2. Determination of BASE Activity and Conversion

100 mg BASE were introduced to a solution of 5 mL substrate solution (100 mmol•L^{-1} 1-octanol, 100 mmol•L^{-1} octanoic acid) in heptane. The mixture was stirred for 50 min at 400 rpm and 40 ºC. Every 10 min 50 µL of solution were withdrawn and analyzed for ester concentration via gas chromatography (Table 2.2).[35] The concentration of the product octyl octanoate was calculated from the peak area at a typical retention time of 10.01 min. All reactions were performed in triplicate. One unit of catalytic activity corresponds to the production of 1 µmol octyl octanoate per minute. Apparent activity was expressed in units per gram of bead ($U•g^{-1}_{BASE}$). Specific activity was expressed as unit per milligram of protein ($U•mg^{-1}_{protein}$).

BASE conversion experiments were performed in triplicate under the same conditions as activity assay. Conversion was determined by calculating the amount of substrates (1-octanol, 3.25 min retention time at GC; octanoic acid, 4.11 min retention time at GC, table 2.2) converted after 17 hour reactions. The analysis conditions by GC were the same as activity assay.

2.4.3. Catalytic Performance of Encapsulated Enzymes in Pickering Emulsions

Assessment of the catalytic performance of free and immobilized CalB

The catalytic performance of CalB was determined via the esterification of 1-octanol and octanoic acid in heptane. Typically, 1 mL substrate solution, containing 400 mmol•L^{-1} 1-octanol and octanoic acid in heptane, was added into 1 mL Pickering emulsion containing CalB. The esterification reactions were carried out on a rotating shaker with 80 rpm at 30 °C. After 10 min, the Pickering emulsion droplets were removed from the organic reaction medium via centrifugation at 14100 rcf and the product concentration in the organic phase was analyzed by gas chromatography (GC) (Table 2.2). One unit of activity (U) was defined as 1 µmol product produced within one min. The specific activity was related to the amount of CalB (U/mg). The activity of native and immobilized CalB in agarose particles was evaluated under the same conditions. All reactions were repeated twice.

Assessment of the catalytic performance of free and immobilized BAL

BAL activity was determined via benzoin condensation. Reactions in emulsion systems were started by adding 1 mL substrate solution (200 mmol•L^{-1} benzaldehyde in MTBE) to 1 mL Pickering emulsion containing BAL and carried out on a rotating shaker at 80 rpm and 30 °C. After 30 min, the organic phase was separated from the Pickering emulsion by centrifugation and analyzed for product formation via GC. Reactions with native BAL were performed in a biphasic system containing 1 mL aqueous solution of dissolved BAL and 1 mL solution of benzaldehyde in MTBE (200 mmol•L^{-1}). The system was set on a platform shaker (Vibramax 110) at 2500 rpm and the reaction was run for 30 min. The formation of benzoin in MTBE was detected via GC. All experiments were performed in triplicate, and BAL stock solutions were cooled with ice prior to use. The GC conditions were the same as for the determination of CalB activity except for the temperature program (Table 2.2). The typical retention time of benzoin was 9.56 min. The activity was defined as the production of benzoin (μmol) within one hour. Distinct from CalB, active BAL is usually not evenly distributed in dried enzyme powders. The volumetric activity (activity related to 1 mL of enzyme stock solution) was therefore used to quantify the enzyme amount prior to immobilization, and the absolute activity rather than the specific activity was used to describe the catalytic performance.

Prior to BAL immobilization, the added BAL was quantified with the same amount of volumetric activity. For this, Stock BAL solutions were diluted to the BAL volumetric activity of 500 U/ml (μmol•h^{-1}•mL^{-1}) before using for Pickering emulsion process. The reactions were performed in biphasic system (1 mL BAL solution with 1mL substrate solution (200 mmol•L^{-1} benzaldehyde in MTBE)) with 2500 rpm shaking on platform shaker (Vibramax 110) for 30 min. The formations of benzoin in MTBE were detected under the same GC conditions as described above for benzoin analysis. All experiments were performed in triplicate, and BAL stock solution was cooled with ice before use. One U/mL of volumetric activity was referred as 1 μmol benzoin produced per hour per ml of stock BAL solutions.

Table 2.2. GC conditions for analysis of compounds.

Reaction	Analysis of	Retention time (min)	Injector/ detector (°C)	Temperature program	GC/ Column type
Esterification	Octyl octanoate	10.01	275/300	• Start: 80.0°C, hold for 0.5 min • Ramp #1: 20 °C/min from 80 to 170 °C • Ramp #2: 5 °C/min rise from 170 to end temperature 200 °C	Shimadzu 2010/ BPX5 column from SGE
	Octanoic acid	4.11			
	1-octanol	3.25			
Benzoin condensation	Benzoin	9.56	275/300	• Start: 60.0°C, hold for 0.5 min • Ramp #1: 20 °C/min from 60 to 170 °C • Ramp #2: 5 °C/min rise from 170 °C to end temperature 260 °C	

3. Using Hydrogel Microparticles to Transfer Hydrophilic Enzymes to Organic Media via Stepwise Solvent Exchange

3.1. Introduction

Colloidal nanoparticles (NPs) ubiquitously exist in a dazzling diversity of chemical nature and morphology, such as slurries, inks, dusts, fogs, micelles, latex paints, enzymes, bacteria, and erythrocytes. They have been important research objects of both fundamental science and industrial applications as in ceramics, information storage, catalysis, pigments, medicine and many others in the past decades. According to the dispersion media, colloidal NPs can be simply classified into two categories: hydrophilic NPs dispersed in aqueous media and hydrophobic NPs in organic media. Owing to easy and environmentally benign synthesis protocols and easily accessible precursors, synthesis of inorganic and organic NPs in aqueous media has been a scientific fascination for more than a century.[115-119] Nevertheless, hydrophilic NPs usually are polycrystalline and polydisperse in size and shape. Their surfaces are usually not coated by additional stabilizing ligands, thus leading to a poor passivation. Hence there are surface defects that deteriorate the intrinsic properties of hydrophilic NPs, which becomes particularly obvious when quantum confinement such as photoluminescence is involved. Therefore, hydrophilic NPs are less promising in applications such as nanoelectronics in which excellent quantum confinement properties are crucial.[120-122] On the other hand, the bare surfaces of hydrophilic NPs being rich in surface defects can benefit a number of applications in which high surface activity is the key issue, such as sensing and catalysis.[123] As a matter of fact, the use of hydrophilic NPs of noble metals, transition metals and metal oxides for catalysis of organic reactions has already been extensively studied before the advent of nanoscience.

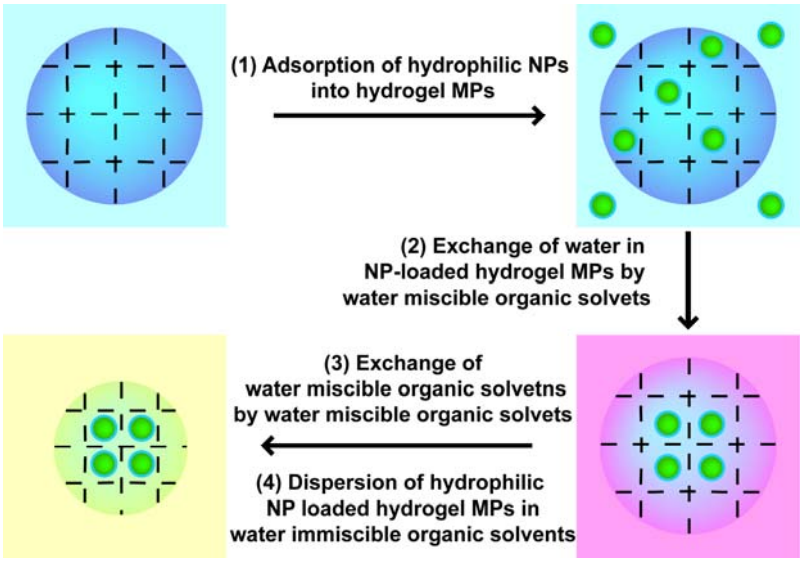
In order to adapt to different technical applications, NPs, both hydrophilic and hydrophobic, must be able to be easily transferred to a variety of environments without agglomeration and deterioration of intrinsic and surface properties. Up to date, numerous methods have been successfully developed for phase transfer of hydrophobic NPs to aqueous media in order to exploit their biomedical applications.[124-127] In contrast, coating with amphiphilic molecules remains the only way to transfer hydrophilic NPs from aqueous to organic media with the intent of better surface passivation for the enhancement of the intrinsic properties of the

NPs.[128, 129] As results of surface passivation, the access of substrates to the surfaces of NPs is blocked, which is unsatisfactory in applications such as catalysis. Up to date, to my best knowledge, there is no methodology allowing transfer of hydrophilic NPs to organic media without surface modification. Crooks et al. have recently synthesized hydrophilic metallic NPs inside dendrimers and created dendrimer-encapsulated metallic NPs.[130] They have demonstrated that the dendrimers can efficiently stabilize the metallic NPs. At the same time, their nanoporous structures enable the nanoparticle surfaces to interact with the surrounding substrates and thus catalyze organic reactions in both aqueous (original dispersion media) and organic media (after phase transfer by modification of dendrimers with alkane carboxylic acid).[130] However, this dendrimer encapsulation protocol should be materials-specific and its applicability is limited especially by the deliberate design of dendrimers. Furthermore, the surface modification of nanoparticle guests during modification of the dendrimer hosts is inevitable. Herein hydrogels are employed as generic carriers and hosts for hydrophilic NPs for phase transfer without modification of the NPs or hydrogels.

A hydrogel is a three-dimensional, physically and/or chemically cross-linked, network of hydrophilic natural or synthetic polymers. As the name suggests, it usually is in a form of water-swollen jelly-like solid. Hydrogels have been extensively exploited for various technical uses, as superabsorbents, sensors, contact lenses, medical electrodes, scaffolds for tissue engineering, dressings for wound healing, implants, just to name a few.[131, 132] The highly porous and biomimetic aqueous interior environment and the stimuli-responsive swelling and shrinking behavior make hydrogels ideal candidates for encapsulation and release of hydrophilic drug NPs and proteins in a controlled manner.[133] This encapsulation strategy has recently been extended to various hydrophilic inorganic NPs, such as CdTe quantum dots (QDs) and gold NPs, and opened up new pathways to produce multifunctional nanocomposites.[134-136] In order to guarantee encapsulation of hydrophilic NPs within hydrogels, attractive interaction and size matching between the particle guests and the pores of the hydrogel hosts are needed.[133-136]

It can be easily understood that hydrophilic polymers can be soluble in a wide range of organic solvents of different polarity by taking into account the fact that the molar fractions of their hydrophobic segments are at least comparable to those of the hydrophilic segments. Möhwald and co-workers have recently demonstrated that the dual solubility of the hydrophilic polymer coating can impart NPs with dispersibility in both water and most

organic solvents and even the capability of reversible transfer between aqueous and organic media across the interfaces.[137, 138] This concept has also been extended to transfer hydrogel microparticles (MPs), loaded without and with hydrophilic inorganic NPs, from aqueous to organic media via stepwise exchange of the water initially swollen in the hydrogels with water-miscible organic solvents such as ethanol and tetrahydrofuran (THF) and eventually with water-immiscible organic solvents such as toluene and hexane.[105, 134] Inspired by these successes, here a new approach of using hydrogel MPs to directly encapsulate and transfer hydrophilic NPs into organic media via stepwise solvent exchange (Scheme 3.1) is presented. Different from the previous studies,[133-136, 139] hydrophilic NPs are embedded within hydrogel MPs in the course of solvent exchange and phase transfer. They are confined within the hydrogel hosts soaked with organic solvents after solvent exchange by the incompatibility of the NP guests with the organic surrounding media. As results, chemical modification of the surfaces of hydrophilic NPs and/or the hydrogel networks is avoided. Furthermore, no size matching or chemical affinity of the hydrophilic NPs to the hydrogel networks is required. Thus, the present approach is independent of the chemical nature of hydrophilic NPs and hydrogels. This study has successfully employed both agarose and poly(N-isopropylacrylamide) (PNIPAM) hydrogel MPs with different sizes as carriers and hosts to encapsulate hydrophilic NPs with very different properties such as CdTe QDs and lipase B from *Candida antarctica* (CalB), and transfer them to organic media with little change of their intrinsic behavior, such as photoluminescence and catalytic activity.



Scheme 3.1. Schematic illustration of using hydrogel MPs as carriers for phase transfer of hydrophilic NPs from water to water-immiscible organic solvents and thus encapsulation via solvent exchange.

3.2. Results and Discussion

3.2.1. Using Hydrogel MPs for Phase Transfer and Encapsulation of CdTe QDs

Fluorescent CdTe QDs were used as models to demonstrate the present methodology because they allowed both fluorescence and absorption based detection of the QD loading and transfer, and their fluorescence behaviour is very sensitive to changes in surface chemistry. Because of the high water content of hydrogel MPs (98-99 % (w/w)) in the current work), CdTe QDs can easily enter hydrogel MPs. This was demonstrated by the fact that after 2 h incubation followed by fast separation from the aqueous dispersions of CdTe QDs, agarose MPs became fluorescent and the fluorescence was homogeneously distributed over the MPs (Figure 3.1a). This easy and fast diffusion of CdTe QDs through agarose MPs suggests that the pores of the agarose hydrogel network (agarose concentration of 1.5 % (w/w)) are much larger than the QDs size (in the range of 2.8 nm – 4.0 nm). Since the stabilizing ligands of the QDs, MPA, are completely deprotonated under the applied conditions (pH 11), which renders the QD surfaces negatively charged, the hydrogen bonding between the QDs and the network of the agarose hydrogel network is expected too weak to confine the QDs within the hydrogel MPs. As results, the redispersion of CdTe QD-loaded agarose MPs back into water caused a fast release of the QDs out of the agarose MPs via diffusion; no QDs were left after washing the agarose MPs with water (Figure 3.2).

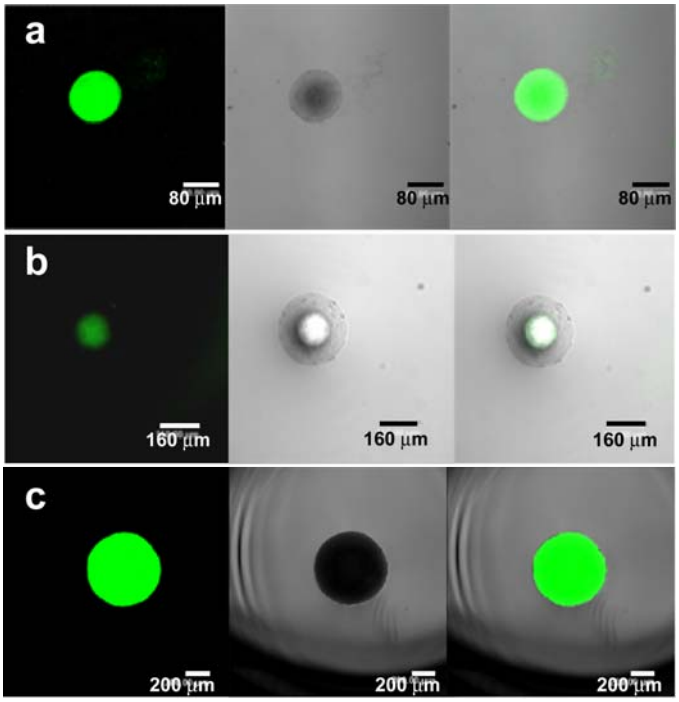


Figure 3.1. CLSM images of agarose MPs loaded with hydrophilic CdTe QDs dispersed in water (a), isopropanol (b), and hexane (c), in the course of solvent exchange. The fluorescence, transmission, and their overlay images are shown in the left, middle, and right panel, respectively. Part a was shot immediately after separation of the gel MPs from the aqueous dispersion of the QD.

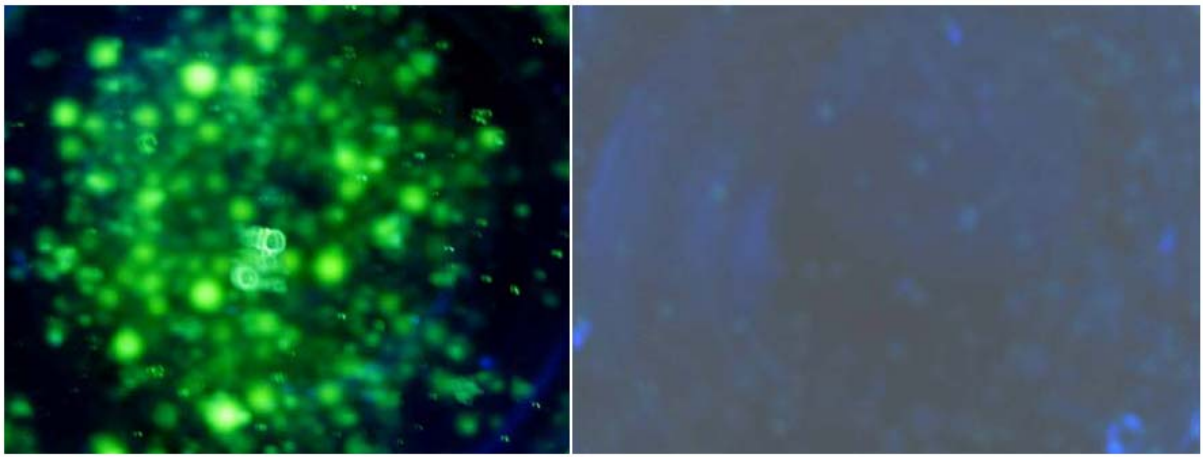


Figure 3.2. Left panel: Fluorescence photograph of hydrophilic CdTe QDs-loaded agarose MPs redispersed in water after separation from aqueous dispersions of CdTe QDs. The image was shot immediately after redispersion in water. Right panel: Fluorescence photograph of CdTe QDs-loaded agarose MPs obtained after washing with water.

In order to confine CdTe QDs in the hydrogel network, after CdTe QD-loaded agarose MPs were separated from the aqueous dispersion of the CdTe QDs, they were immediately dispersed in polar water-miscible organic solvents, such as ethanol, IPA, acetone, tetrahydrofuran (THF), dimethylformamide (DMF) and DMSO. The incompatibility of the negatively charged surfaces of the CdTe QDs with these solvents forced the QDs to remain within the agarose MPs, as shown in the left panel of Figure 3.1b.

The fluorescence and transmission overlay imaging reveals a non-fluorescent periphery of all CdTe QDs-loaded agarose MPs, dispersed in for instance IPA, suggesting that the organic polar solvents dispel the QDs to the center of the agarose MPs (the right panel of Figure 3.1b). In the current work, agarose hydrogel blocks were used to assess the effect of organic polar solvents on the migration and intrinsic fluorescence behavior of CdTe QDs loaded in the hydrogels. Noteworthy, polar water-miscible solvents such as IPA are usually used to precipitate CdTe QDs from their aqueous dispersions accompanied with the fluorescence quenching of the QDs. However, Figure 3.3 shows that after IPA is brought on the top of a CdTe QDs loaded hydrogel block, the CdTe QDs did not precipitate but moved towards the bottom of the hydrogel block. The hydrogel block was clearly divided into two layers, the colorless and non-fluorescent upper layer and the yellow and fluorescent lower layer. The strong fluorescence of the lower layers suggests that they remain rich in water rather than organic polar solvents; otherwise decomposition and fluorescence quenching of CdTe would be observed. The colorlessness of the upper layers suggests no presence and decomposition of the CdTe QDs inside these upper layers; otherwise they would be either yellow due to the QD presence or dark blue due to the QD decomposition or shape transformation.[140] Intriguingly, the thickness of the newly formed colorless upper layer increases with the dielectric constant of the organic polar solvent (Figure 3.4). The migration of CdTe QDs in the presence of organic polar solvents is reminiscent to the process of thin layer chromatography.[141]

Figure 3.3. Optical and fluorescent photographs of an agarose hydrogel block loaded with hydrophilic CdTe QDs after a defined amount of isopropanol was added atop for 3 h.

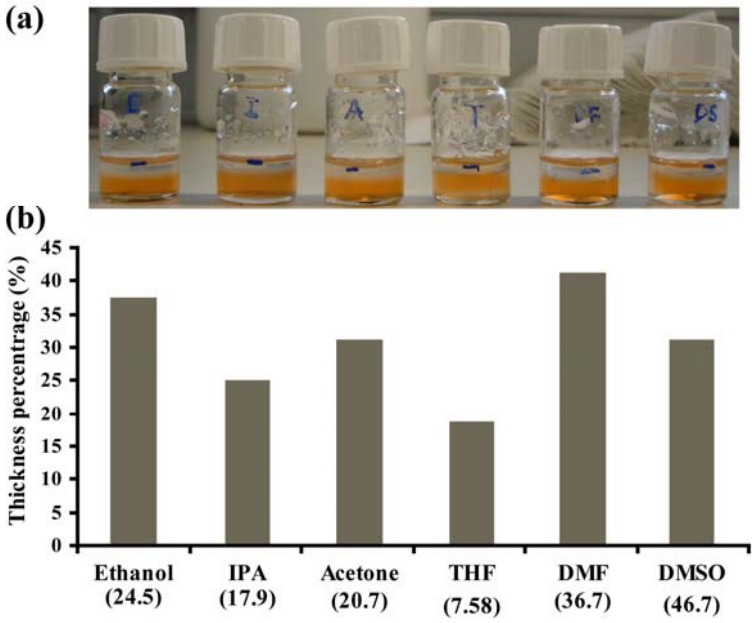


Figure 3.4. (a) Optical photographs of glass vials in which different water-miscible polar organic solvents are added atop agarose hydrogel blocks loaded with hydrophilic CdTe QDs. From left to right, the organic solvents used are ethanol, IPA, acetone, THF, DMF, and DMSO. The photographs were shot after 3 h incubation. (b) Chart of the thickness percentages of the colorless upper layers in the hydrogel blocks versus different water miscible organic solvents.

After dispersion of CdTe QD-loaded agarose MPs in polar water miscible organic solvents such as IPA, their peripheral shells are expected to be rich in the organic solvents and thus

more compatible with water immiscible organic polar and even apolar solvents. Therefore, after incubation in IPA, CdTe QDs-loaded agarose MPs were able to readily disperse in various water immiscible organic solvents, e.g. chloroform, toluene, hexane and many others. Figure 3.1c shows that the dispersion of CdTe QDs loaded hydrogel MPs in water immiscible organic solvents such as hexane causes a slight collapse of the agarose gel network, which is evidenced by disappearance of the non-fluorescent peripheral shells.

In addition to large and polydisperse agarose MPs, smaller PNIPAM MPs with sizes of 600 nm and 1.5 μm can be used for phase transfer and encapsulation of hydrophilic CdTe QDs in water immiscible organic solvents (Figure 3.5). The advantage of using small monodisperse PNIPAM MPs is that one can quantitatively analyze the concentration of CdTe QDs loaded in each hydrogel MPs with the aid of absorption spectroscopy and static light scattering.[105] The concentration of QDs in PNIPAM MPs was almost identical to that in original aqueous solution; each MP contained about 650 QDs (Figure 3.6 and calculations according to formula 3.1). This further confirms that loading of CdTe QDs in hydrogel MPs is a diffusion-driven process, no size matching and attractive affinity are needed between the QDs and the hydrogel networks. Otherwise, the concentrations of CdTe QDs loaded in hydrogel MPs would be different from those of their aqueous solutions.

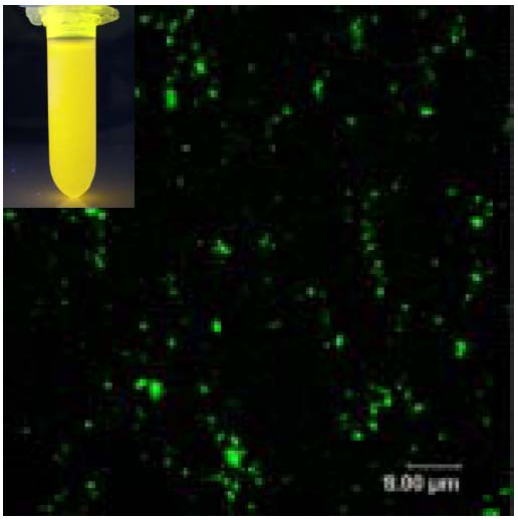


Figure 3.5. CLSM fluorescence image of hydrophilic CdTe QDs-loaded PNIPAM MPs, dispersed in hexane after solvent exchange. The size of the PNIPAM MPs is about 1.5 μm. The scale bar is 8 μm. The inset is the photograph of a hexane dispersion of CdTe QD-loaded PNIPAM MPs.

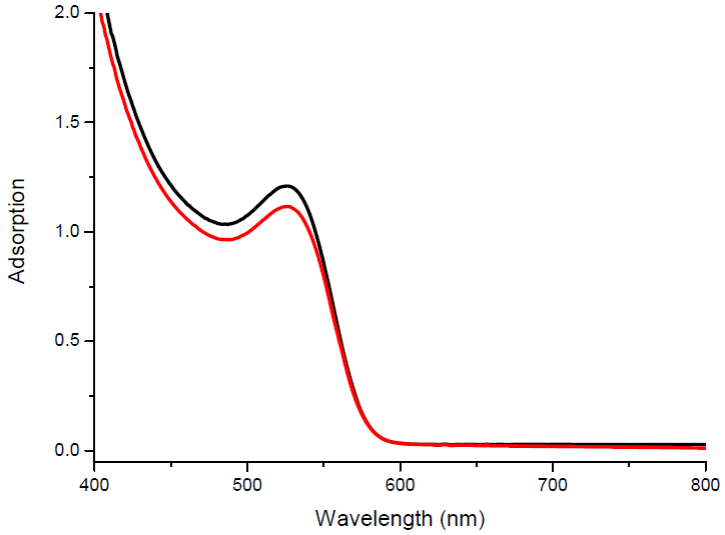


Figure 3.6. Absorption spectra of the aqueous dispersion of 3.3 nm CdTe QDs before (black curve) and after uptake of PNIPAM MPs (red curve). The number of PNIPAM MPS in aqueous media is counted by single particle light scattering. The number of CdTe QDs per PNIPAM MP is calculated according to the following equation:

$$N = [C_o \times (1 - \frac{I_a}{I_b}) \times V_{CdTe} \times N_A] / N_{mp} \qquad (3.1)$$

Where I_a and I_b are the intensities of the maximal absorbance bands of the CdTe QDs before and after uptake of PNIPAM MPs with a defined volume, C_o is the concentration of original QDs, V_{CdTe} is the volume of the original QD solution, N and N_{mp} are the numbers of CdTe QDs loaded in one PNIPAM MPs and PNIPAM MPs, and N_A is the Avogadro constant.

CdTe QDs-loaded hydrogel MPs remain fluorescent in water immiscible organic solvents such as hexane, but the fluorescence intensity is much weaker than that of aqueous dispersions of CdTe QDs; it is about 15% of that of the aqueous dispersions (Figure 3.7). This suggests that a considerable amount of the stabilizing ligands of MPA are removed from the surfaces of CdTe QDs during the solvent exchange, thus leading to a poor surface passivation and in turn a weaker fluorescence. When the solutions of MPA in IPA or THF with the same concentration as that of aqueous dispersions of CdTe QDs were used for the QDs transfer from water to water immiscible organic solvents, the fluorescence intensity of CdTe QDs-loaded hydrogel MPs remains almost identical to that of the QDs in original aqueous dispersions (Figure 3.7). This also proves that there is no QD loss during solvent exchange.

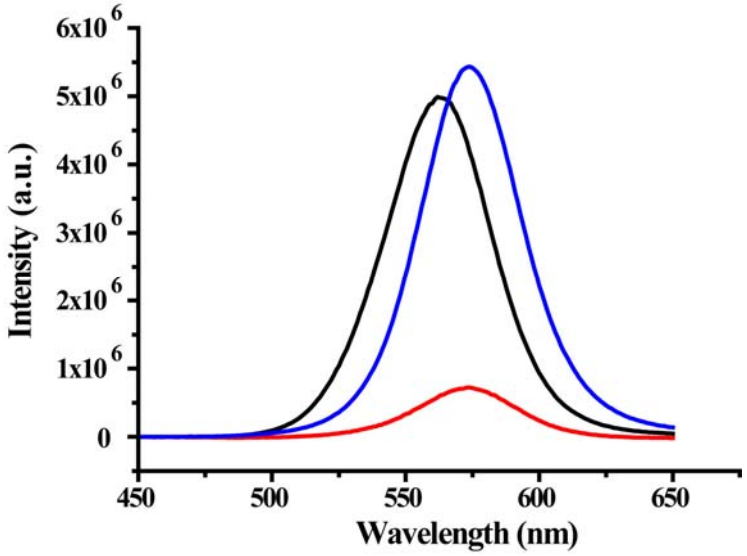


Figure 3.7. Fluorescence spectra of original CdTe QDs in water (black curve) and CdTe QD-loaded agarose MPs in hexane via solvent exchange by using pure IPA (red curve) and the IPA solution of MPA (blue curve). The MPA concentration in the IPA solution is identical to that in the aqueous dispersion of CdTe QDs. The CdTe QDs are about 3.3 nm in size.

The solvent exchange procedure described above can be reversed from water immiscible organic solvents to water miscible solvents and eventually back to water or aqueous solution of MPA. When CdTe QDs-loaded hydrogel MPs were redispersed back into water, CdTe QDs were released from the hydrogel MPs and redispersed in aqueous media, for instance MPA aqueous solution. As shown in Figure 3.8, the profiles of the fluorescence spectra of CdTe QDs are little changed before and after phase transfer from water into different water immiscible organic solvents via solvent exchange and transfer back to MPA aqueous solutions via reversed phase transfer. Nevertheless, an about 5 nm red shift of the fluorescence band of CdTe QDs is clearly visible in particular by comparison between original QDs in water and the QDs redispersed in water after reserved solvent exchange. The reason accounting for this small red shift could be threefold: 1) the rearrangement of MPA ligands on the QDs and/or the formation of CdS shells due to decomposition of the thiol-groups of the MPA lead to a better passivation;[142] 2) the growth of CdTe QDs in hydrogel MPs due to the removal and regeneration of the surface passivation shells of MPA; and 3) slight aggregation of CdTe QDs because all water-miscible organic polar solvents can be regarded as precipitating agents for CdTe QDs. Elucidation of the red shift of the fluorescence of CdTe QDs obviously requires a

meticulous study of the change of the structures and especially surface chemistry of the QDs during solvent exchange, which is currently underway.

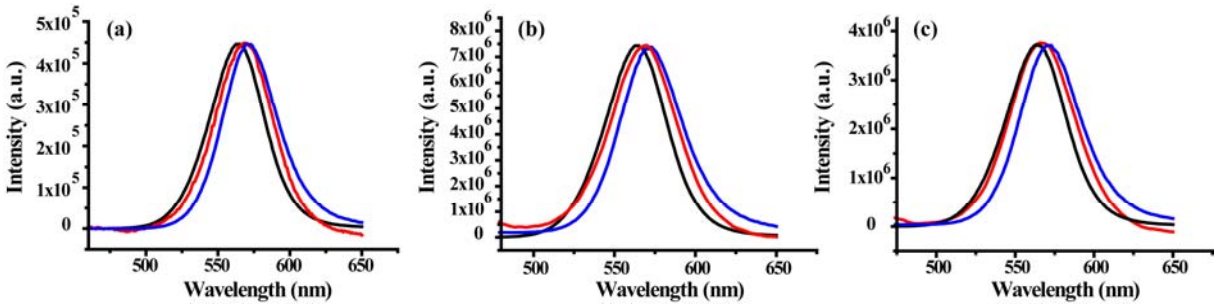


Figure 3.8. Fluorescence spectra of original CdTe QDs in water (black curve), CdTe QD-loaded agarose MPs in different water-immiscible organic solvents via solvent exchange (red curve), and CdTe QDs redispersed in water after release out of the agarose MPs via reversed solvent exchange (blue curve). The water-immiscible organic solvents used are hexane (a), toluene (b), and chloroform (c). The CdTe QDs are about 3.3 nm in size.

3.2.2. Hydrogel MPs for Phase Transfer and Encapsulation of Enzymes

Enzymes, as peculiar and environmentally friendly catalysts for organic reactions, are increasingly used in chemical synthesis because of their high activity and stereoselectivity. Lipases are frequently employed to catalyze the chemo-, regio- and/or stereoselective hydrolysis of esters and amides.[143, 144] However, the technical application of many hydrophilic and vulnerable enzymes requires immobilization on or in appropriate carriers to improve the dispersibility in organic media, the long-term operational stability, and the efficiency of recovery and reuse.[32, 74] Here the solvent exchange process was extended to the encapsulation of CalB in hydrogel MPs and transfer them to water immiscible organic solvents in order to provide a simple and efficient immobilization technique. For the benefit of easy visualization of enzyme loading, CalB was labeled with the fluorescence marker FITC (Experimental section).

Similar to CdTe QDs, CalB can be transferred to water immiscible organic solvents such as hexane and in turn encapsulated in MPs of agarose (Figure 3.9) or PNIPAM (Figure 3.10) via solvent exchange by using IPA as the intermediate solvent. Furthermore, the reversed solvent exchange allows release of CalB from the hydrogel MP hosts and redispersion in aqueous media. The CD spectra of CalB, redispersed in PBS buffer after release out of hydrogel MPs via reversed solvent exchange, are rather similar to those of native CalB; the bands in the

wavelength range of 190 nm - 240 nm, characteristic to the α-helix secondary structure,[145] are clearly observed in Figure 3.11.

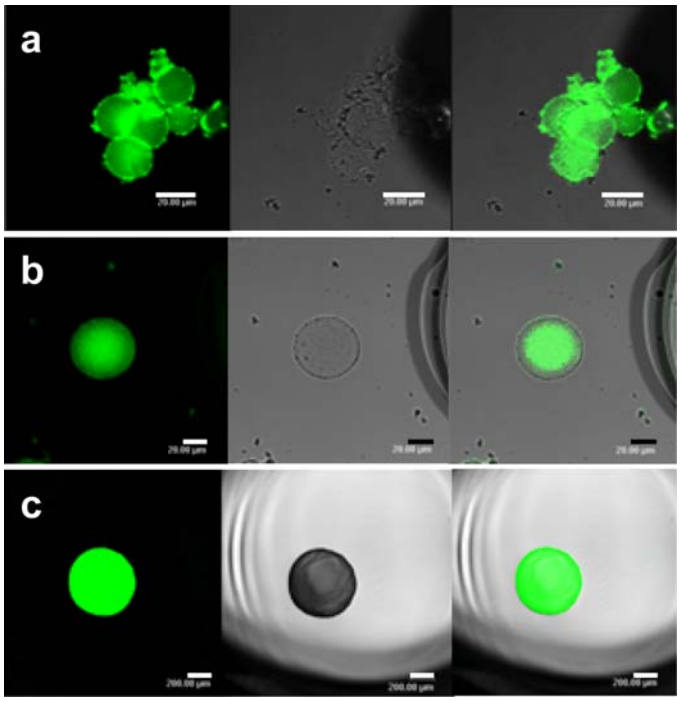


Figure 3.9. CLSM images of agarose MPs loaded with FITC labeled CalB dispersed in PBS (a), IPA (b), and hexane (c) in the course of solvent exchange. The fluorescence, transmission, and their overlay images are shown in the left, middle, and right panel. Part a was shot immediately after separation of the gel MPs from the aqueous dispersion of the CalB. The scale bar is 200 μm.

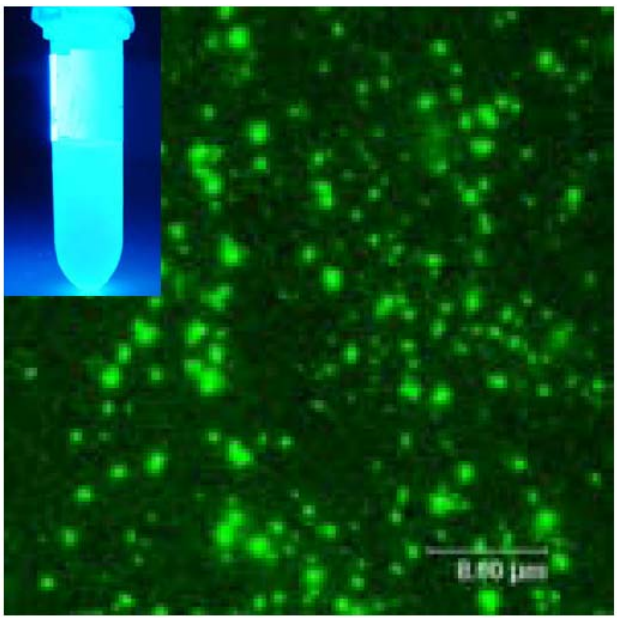


Figure 3.10. CLSM fluorescence images of FITC labeled CalB-loaded PNIPAM MPs, dispersed in hexane after solvent exchange. The size of the PNIPAM MPs is about 1.5 μm. the scale bar is 8 μm. The inset is the photograph of a hexane dispersion of FITC labeled CalB-loaded PNIPAM MPs.

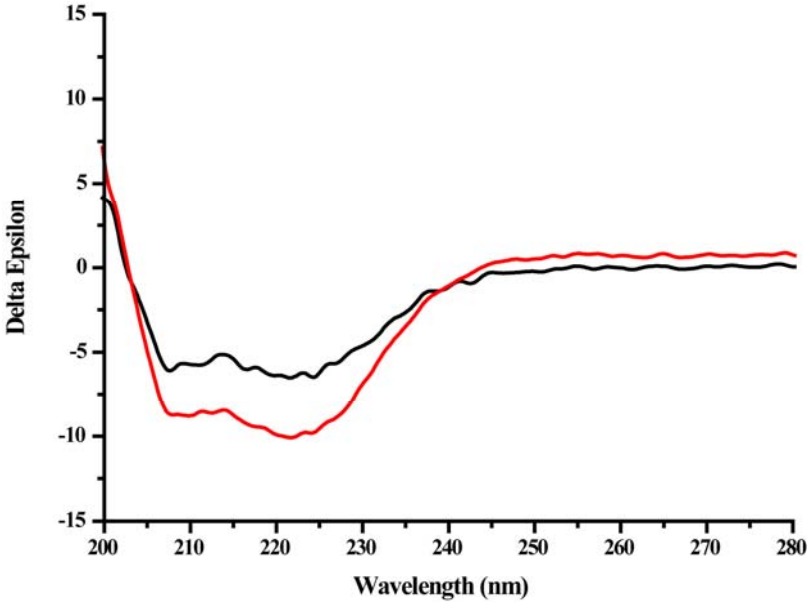


Figure 3.11. CD spectra of native CalB (black curve) in PBS and CalB redispersed in PBS after being released from agarose MPs via reversed solvent exchange (red curve).

In the case of native CalB, the enzymatic catalysis occurred only when substrates slowly diffused from heptane to the aqueous droplets of the CalB across the interface.[32] On the other hand, the aqueous droplets of the native CalB were not stable in heptane and tended to coalesce, thus leading to rather small interface area for enzymatic catalysis. In contrast, immobilization of enzymes in hydrogel MPs drastically increased the interfacial area of the hydrophobic substrates in contact with hydrophilic CalB. Figure 3.12a therefore shows that the specific catalytic activity of CalB-loaded hydrogel MPs, dispersed in heptane, is significantly higher than that of native CalB. Intriguingly, the specific activity of CalB in hydrogel MPs drastically increases from 2.7 U/mg in the first catalysis trial to 13.4 U/mg in the second trial and then slightly increases with every reuse (7 times). In contrast, the specific activity of native CalB just slightly increases in the second catalysis trial while afterward it slightly decreases with every reuse (Figure 3.12a). The excellent reusability of CalB-loaded hydrogel MPs could be of great interest for the practical use in chemical synthesis. Figure 3.12b shows that the residual activity of CalB in hydrogel MPs is little changed during storage

in hexane for at least 10 days. In contrast, the residual activity of native CalB greatly decreased with time when being brought in contact with hexane. This stabilization of CalB should be due to the immobilization effect of the gel network of agarose MPs on the enzymes.

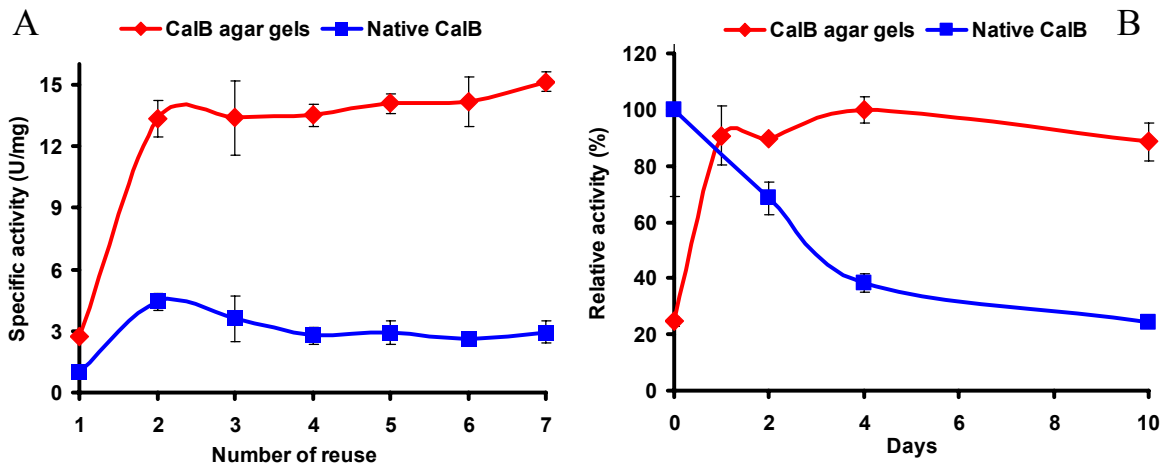


Figure 3.12. (a) Plot of the specific activity of native CalB (red curve) and CalB-loaded agarose MPs (black curve) versus the number of reuses. (b) Plot of the stability of native (red curve) and immobilized CalB (black curve) versus the time of storage in hexane; the data of the specific activity were normalized by taking the highest value as 100%.

In order to determine the overall applicability of the method for protein entrapment and transfer to organic solvents, the solvent exchange strategy was applied to fairly inert and stable BSA with a size of 4 nm × 4 nm × 14 nm and fairly active and vulnerable BAL with a size of 10.4 nm × 12.1 nm × 16.2 nm.[146] It was shown that both BSA and BAL can be successfully transferred from PBS buffer to water-immiscible organic solvents and encapsulated in agarose or PNIPAM MPs (Figures 3.13 and 3.14). Combined with the results obtained with CdTe QDs, this demonstrates that the presented method of solvent exchange is suitable for handling of different sized hydrophilic NPs. However, no residual catalytic activity was detected for BAL even when DMSO, the most commonly used organic solvent for BAL catalysis,[147] was used as the intermediate water-miscible organic solvent. This could be explained by the high sensitivity of BAL toward organic solvents at high concentrations and its overall low stability even in PBS buffer[148] and suggests that a fairly basic stability of enzyme catalysts is required before entrapment in hydrogel MPs by solvent exchange is beneficial.

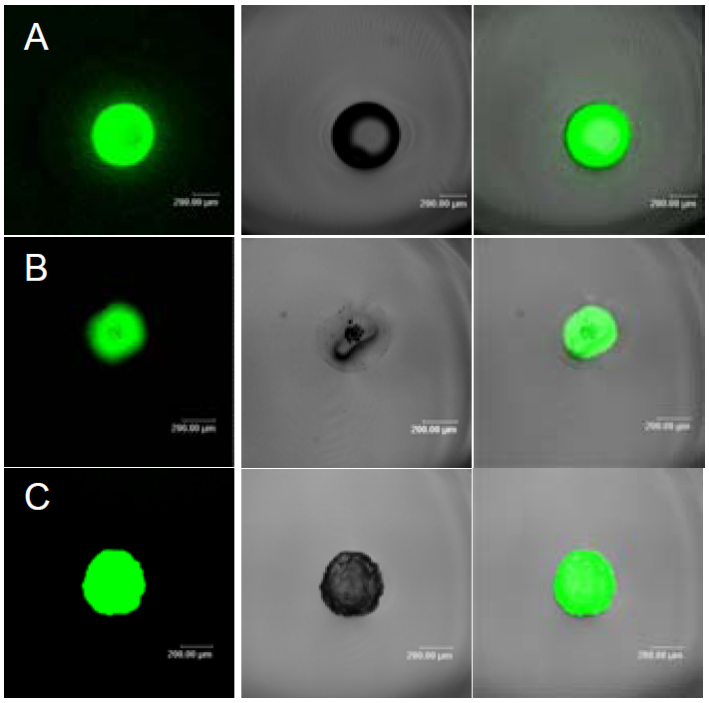


Figure 3.13. CLSM images of agarose MPs loaded with FITC labeled BSA dispersed in PBS (a), isopropanol (b) and hexane (c), in the course of solvent exchange. The fluorescence, transmission and their overlay images are shown in the left, middle and right panel. Part a was shot immediately after separation of the gel MPs from the aqueous dispersion of BSA. The scale bar is 200 μm.

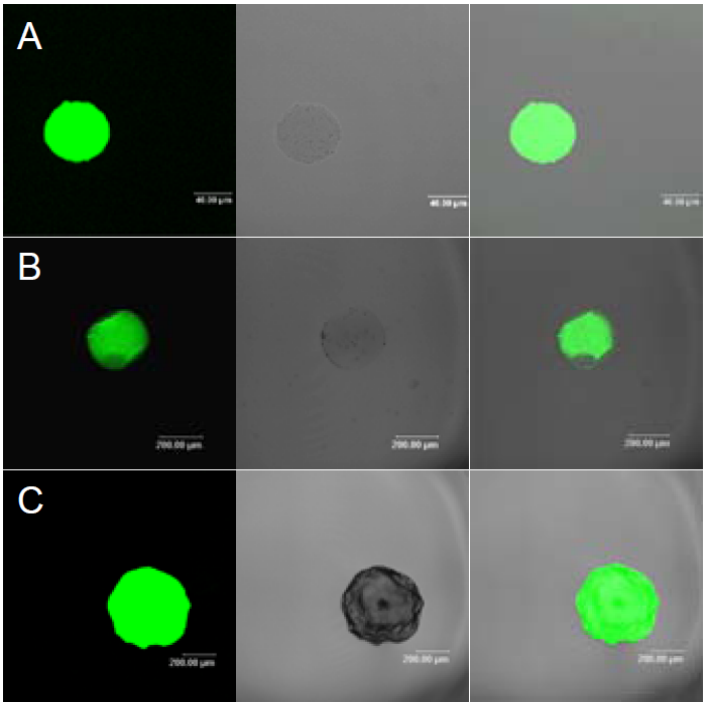


Figure 3.14. CLSM images of agarose MPs loaded with FITC labeled BAL dispersed in PBS (a), isopropanol (b) and hexane (c), in the course of solvent exchange. The fluorescence, transmission and their overlay images are shown in the left, middle and right panel. Part a was shot immediately after separation of the gel MPs from the aqueous dispersion of the BAL. The scale bar is 200 μm.

3.3. Conclusion

This study has demonstrated a simple and versatile approach to use hydrogel MPs as generic carriers and hosts for phase transfer of hydrophilic NPs into organic media via solvent exchange. Hydrophilic NPs have been encapsulated within hydrogel MPs in the course of phase transfer via solvent exchange from water to water miscible organic solvents and eventually to water immiscible organic solvents. The entrapment of the NPs results from their incompatibility with water immiscible organic solvents soaked in the gel matrices and in the surrounding environment. As a consequence, no chemical modification of the surfaces of hydrophilic NPs and/or of the networks of hydrogels is needed and, furthermore, any size matching or chemical affinity of the hydrophilic NPs for the hydrogel networks is unnecessary. The present approach is therefore independent on the chemical nature of hydrophilic NPs and hydrogels MPs. The concentration of hydrophilic NPs loaded in hydrogels after phase transfer to organic solvents such as hexane has been demonstrated to be comparable to that in their original aqueous dispersion. It has also been demonstrated that the surface stabilizing shells of CdTe QDs can be removed and regenerated during solvent exchange in a controlled fashion, which is exemplified by their photoluminescence quenching and recovery. This suggests the capability and flexibility of tuning the surface chemistry of hydrophilic inorganic NPs loaded in hydrogel MPs, which should be of importance for both fundamental research, for instance study of the surface chemistry effect, and technical applications such as catalysis. Of importance is that the phase transfer and encapsulation of hydrophilic NPs are reversible; the reversed solvent exchange of hydrogel MPs loaded with hydrophilic NPs from organic media lead to release and recovery of the hydrophilic NPs in aqueous media. The presented approach has been successfully extended to the encapsulation of the enzyme catalyst CalB, and its phase transfer to organic media with little change of catalytic activity, good reusability and stability, thus showing a promising potential for use in biocatalysis.

3.4. Research Collaboration

To improve the interface of hydrophilic immobilisates, two types of classical hydrogels, agarose gels and poly(N-isopropylacrylamide) (PNIPAM), were targeted. A solvent exchange method (Scheme 3.1) was used to load different size enzymes and nanoparticles into these hydrogels. This was done in a collaboration research within UniCat among three groups of Professor Ansorge-Schumacher (TU Berlin), Professor Möhwald (MPI Potsdam), and Professor Klitzing (TU Berlin). In this project, I implemented experiments on preparing and labeling three enzymes (CalB, BSA, and BAL), partially preparing agarose microparticles (MPs), and all catalytic assays of immobilized and free enzymes. Shuo Bai (MPI Potsdam) conducted preparation of CdTe nanoparticles and partial agarose MPs, confocal microscopy characterization on agarose gels, fluorescence spectra, and circular dichroism (CD) spectroscopy. Kornelia Gawlitza (TU Berlin) prepared the PNIPAM MPs and characterized them by confocal microscopy.

4. Optimized Biocatalytic Active Static Emulsions (BASE) for Organic Synthesis in Non-aqueous Media

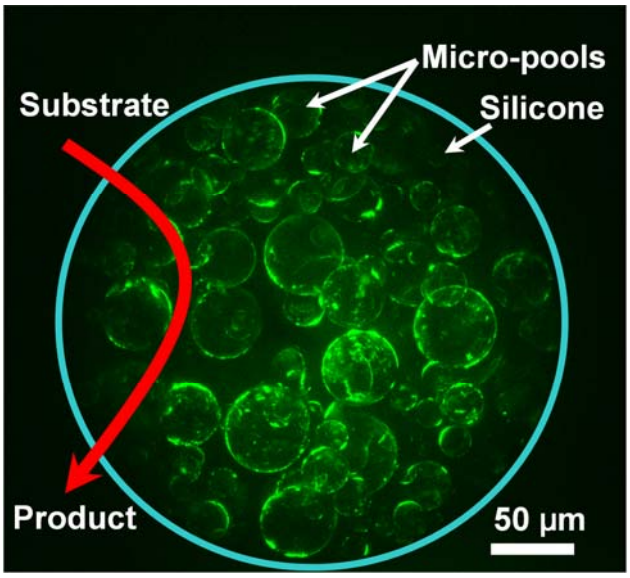
4.1. Introduction

The application of biocatalysts in the presence of non-aqueous reaction media, predominantly organic solvents, has become a well established technique in organic synthesis, especially for synthesis of fine chemicals as building blocks for pharmaceuticals and agrochemicals.[1, 74] Use of these media enables many chemical reactions that are not feasible in aqueous solution, diminish undesirable side reactions, reduce hydrolytic reactions in favor of synthesis, and facilitate product recovery.[149] Three major principles are frequently applied in biocatalysis: Monophasic mixtures of aqueous enzyme solutions and varying fractions of fully miscible organic solvents,[35, 150] pure hydrophilic or hydrophobic organic solvents containing dispersed dried enzyme,[74, 151] and biphasic aqueous-organic media with the enzyme dissolved in an aqueous solution and reactants partitioning between the phases.[148] All systems have their advantages and pitfalls,[32, 59] and case studies of successful synthetic application have been reported for each of them.[148, 152, 153]

Aqueous-organic biphasic systems are usually selected when conversion of hydrophobic compounds shall be achieved with biocatalysts requiring bulk water or hydrophilic-hydrophobic interfaces for activity.[32, 154, 155] A crucial parameter for the synthetic performance of these systems is the extent of interfacial area for transfer of reactants or/and enzyme activation.[156] This is considerably increased when emulsions are formed.[157] Widely explored have been so called microemulsions or reverse micelles, which are aqueous enzyme solutions thermodynamically dispersed in a non-miscible solvent and stabilized by addition of a surfactant.[158, 159] Recyclability of and product separation from these systems were improved by solidification with gelatin into microemulsion-based organogels.[160] The mechanical strength of these gels, however, is very low, and as in all microemulsion systems the presence of surfactants frequently causes enzyme deactivation.[161, 162] Thus, synthetic application is considerably restricted.

In contrast, biocatalytic active static emulsions, from now on designated as BASE, hold considerable potential in terms of use on a technical scale.[33, 163, 164] Consisting of a large number of aqueous micro-pools stably entrapped in macroscopically large silicone spheres

(Scheme 4.1),[33] these particles combine provision of a distinct aqueous environment and large interfacial exchange area with high biocompatibility and appropriate mechanical strength. This has been demonstrated for BASE mediated direct esterification of biodiesel by-products,[164] and recently in the synthesis of chiral hydroxy nitrils. Additional advantages arise from the use of low-cost materials and simple preparation.



Scheme 4.1. Typical BASE dispersed in an organic solvent.

In this study, the prototype BASE were advanced in terms of catalytic performance towards esterification. In this context the preparation protocol towards a better reproducibility of size and catalytic activity was optimised, and a systematic study of morphology, structure, and mass transfer characteristics was performed. As model biocatalyst lipase A from *Candida antarctica* (CalA) was selected due to its reported performance in prototype BASE.[33]

4.2. Results and Discussion

4.2.1 Improved Preparation of BASE

Prototype BASE were prepared by emulsifying aqueous enzyme solutions in siloxane precursor and subsequent solidification in polyvinyl alcohol (PVA).[33] For bead formation this immiscible biphasic solution was stirred in a plain beaker glass using a simple magnetic stir bar (Figure 2.1). A typical size distribution of particles resulting from this procedure is illustrated in Figure 4.1. It covers a broad range from 150 µm in diameter to 1400 µm and is widely at random.

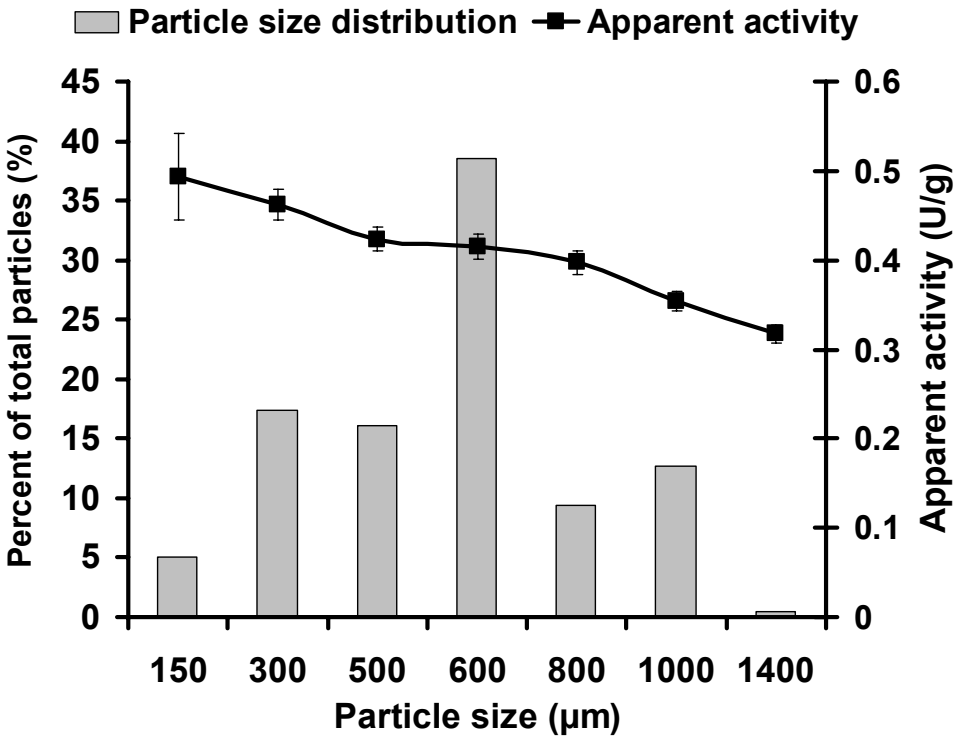


Figure 4.1. Particle size distribution and catalytic activity of prototype BASE prepared in a plain beaker glass with a magnetic stir bar for mixing.

This can be explained from the tangential velocities predominant in the chosen mixing system inducing the entire fluid to rotate, creating a swirl. Thus, only low shear forces are exerted and as a consequence of this the size of emulsion droplets can hardly be controlled. The bead size, however, greatly influences the apparent catalytic activity. Figure 4.1 illustrates an increase in catalytic activity with decreasing particle size. The activity of the smallest and biggest investigated particles differs by about 30 %, which can be explained by the larger surface area exposed to the surrounding reaction medium by smaller beads facilitating the transfer of reactants over the matrix. Consequently, a reproducible catalytic activity of different batches of BASE can hardly be achieved with this preparation method. Thus, a new preparation method to better control the size of BASE beads is required.

A considerable improvement of bead size distribution was achieved by using a baffled beaker glass as vessel and a motor-controlled vertical impeller as mixing device to keep agitation speeds constant (Figure 2.2). This is illustrated in Figure 4.2. Bead size distribution is narrowed to 150 µm to about 1000 µm in diameter with over 60 % (w/w) pooled at a size of 600 µm. In contrast to the results from the original preparation method this distribution is

highly reproducible as was demonstrated by preparation of different batches (Figure 4.2). The improvement can be explained by the effect of baffles in the beaker glass on agitation. The baffles (four in this case) prevent swirling and lead to an improved velocity field, in which BASE particles are formed and stabilized until solidification.[165, 166]

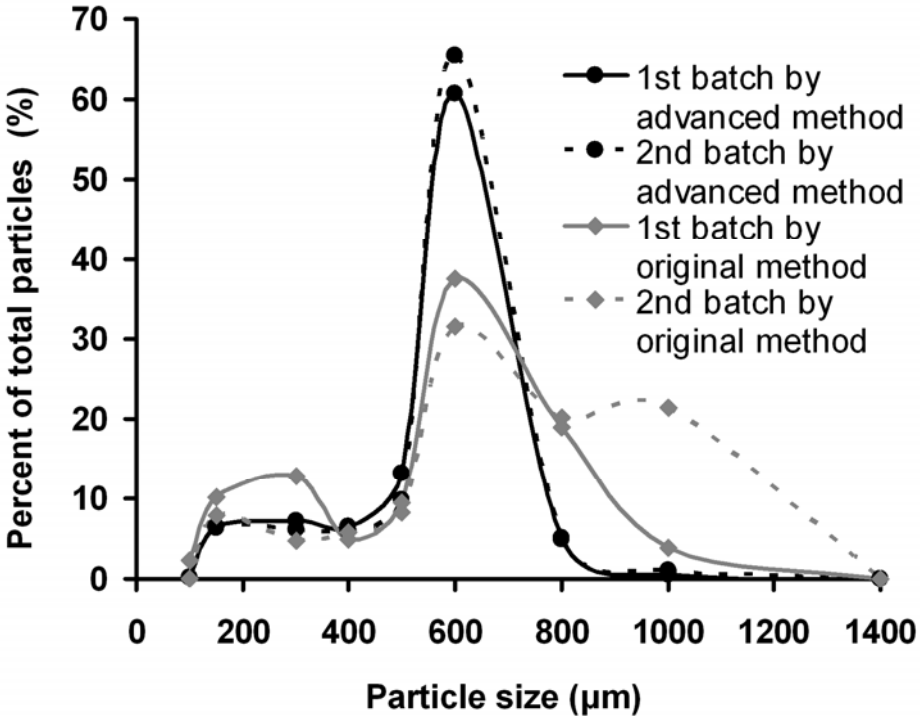


Figure 4.2. Particle size distribution of different batches of prototype BASE prepared by the original and advanced method, respectively.

An endoscope camera was inserted into both original and advanced mixing systems to observe the initial status of the liquid emulsion from which BASE gradually solidified. The obtained pictures (Figure 4.3) confirm that the size distribution of siloxane droplets prepared with the advanced method is much narrower than with the original method and thus illustrates the previously observed improved particle size distribution after polymerization.[165]

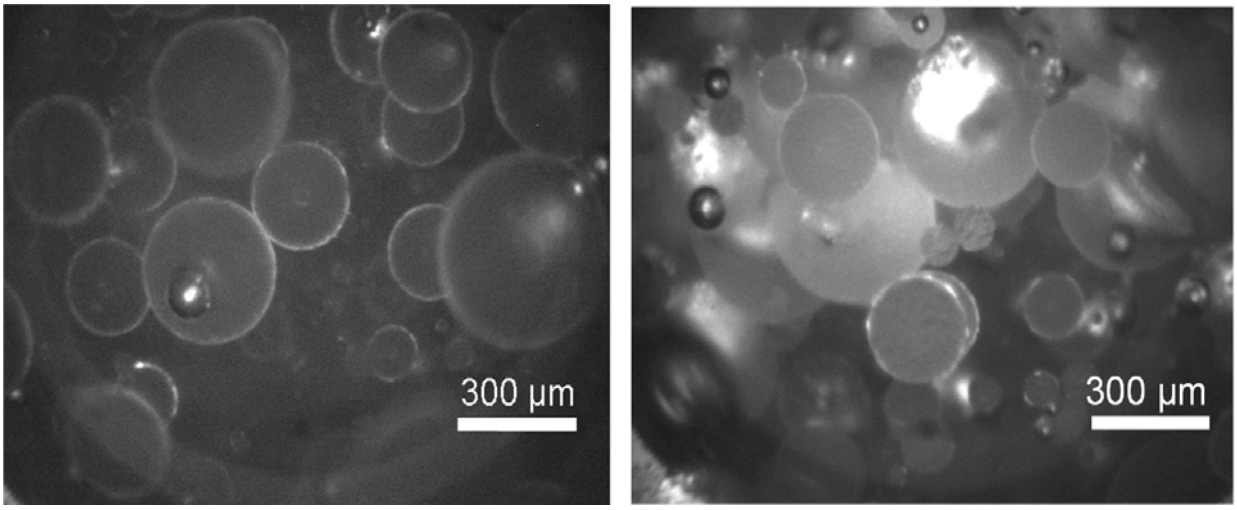


Figure 4.3. Endoscope pictures taken after 5 minutes mixing of water-in-siloxane emulsion in PVA solution with the original (right) and advanced (left) mixing system.

In accordance with the excellent reproducibility of BASE formation with the advanced preparation method the catalytic activity of particles from different batches deviated by less than 10 % (Figure 4.4). Considering the many separate steps involved in the preparation of BASE and the usual error connected with the activity assay this is a very good result. A systematic assessment and improvement of bead properties was now possible.

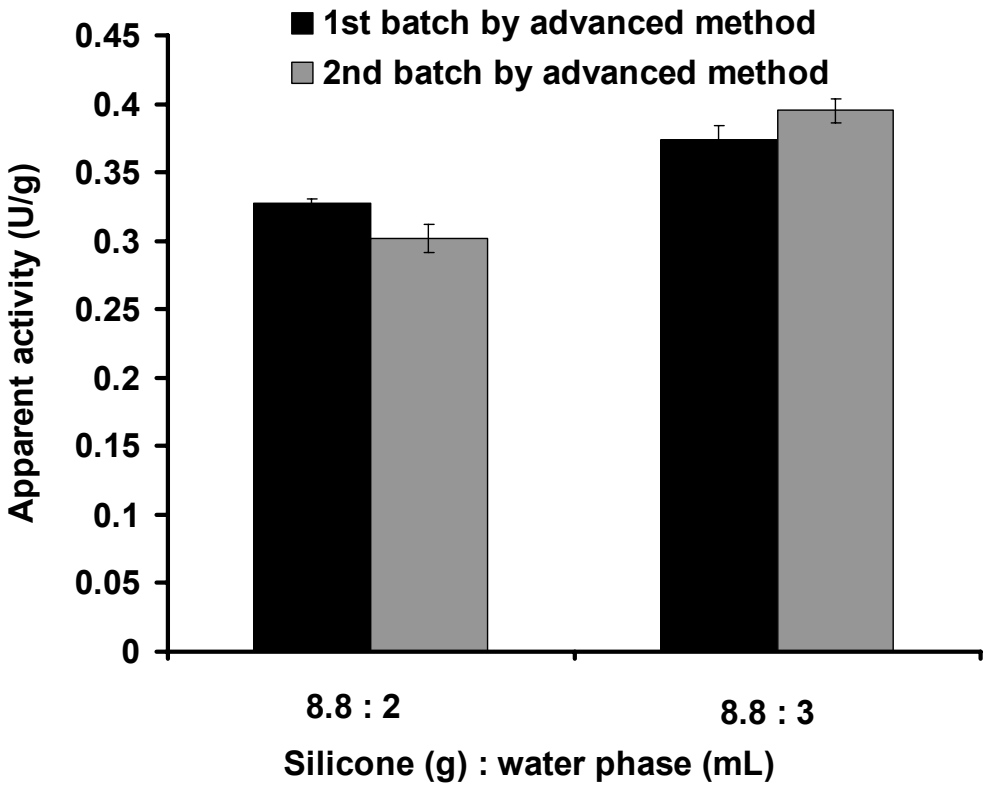


Figure 4.4. Catalytic reproducibility for beads prepared in the advanced mixing system under different bead composition.

4.2.2 Morphology and Structure of BASE

The aqueous micro-pools forming the interior of BASE were investigated via scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). The cross section micrograph from SEM in Figure 4.5 illustrates that numerous spherical droplets are separately embedded in the continuous silicone phase. This is in accordance with the assumptions of Buthe *et al*,[33] which were derived from a crude photograph of prototype BASE including a dyed water phase. The droplets are 15 µm to 60 µm in diameter and are randomly distributed over the complete matrix. These results confirm that the catalytic interfacial area provided by BASE is predominantly determined by the number and size of micro-pools within the matrix.

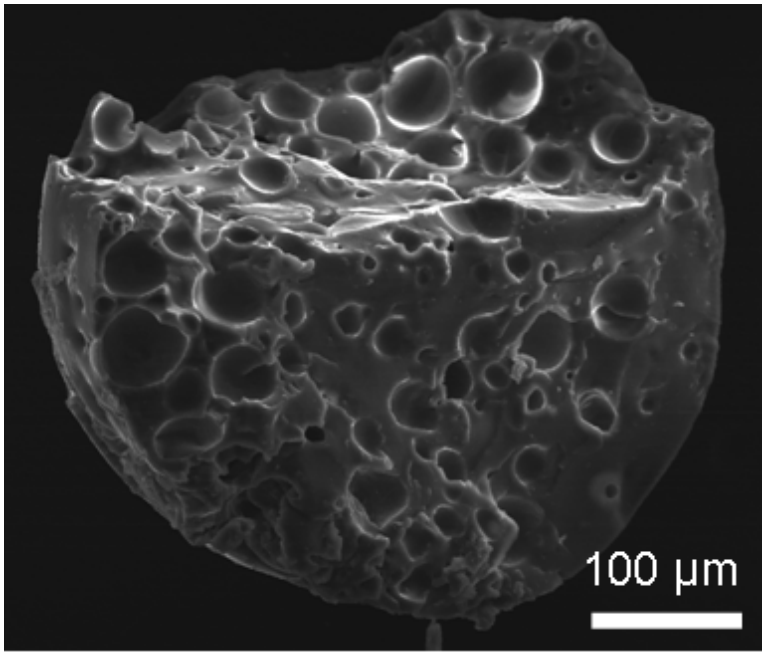


Figure 4.5. SEM micrograph of a cross section of prototype BASE.

CLSM performed on a BASE particle (~ 500 µm in diameter) entrapping fluorescence labeled lipase A from *Candida antarctica* (CalA) demonstrated that the catalytic active part of BASE is solely located within the aqueous micro-pools and seems to be distributed over the complete volume of the particle (Figure 4.6a).[167] The slightly concentrated green color on the surface of micro-pools may be due to the good affinity of lipases to the hydrophobic silicone

surface. The observed shape and overall size of the micro-pools is in accordance with the previously described findings from SEM (Figure 4.5). It can be assumed that sphericity results from the dominant role of surface tension in this kind of system. In the investigated bead, 40 aqueous micro-pools with diameters ranging between 18 µm and 64 µm and a majority of 60.2 % of the total number of micro-pools at diameters between 20 µm to 35 µm were present (Figure 4.6b). With this information an interfacial area of 0.25 m^2 in a typical batch of BASE particles was calculated (calculation in the next paragraph), which is 337 times larger than in a single water droplet with a volume equal to the added volume of all micro-pools.

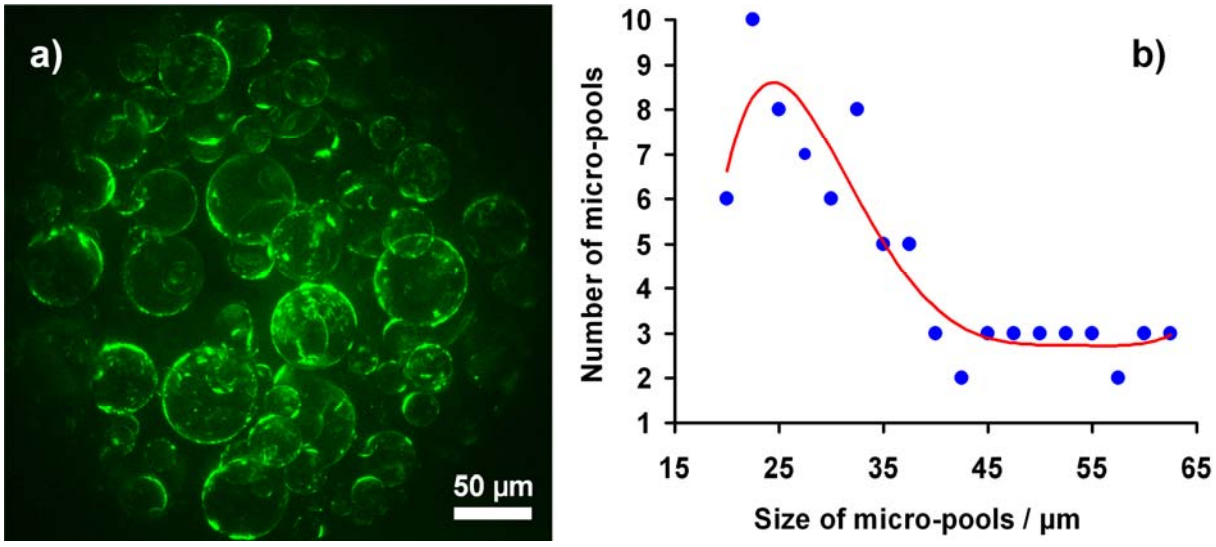


Figure 4.6. a) CLSM photograph of a BASE bead at a silicone to water ratio of 8.8 g : 2 mL. Measurements were performed at an excitation wavelength of 488 nm and an emission wavelength of 512 ~ 582 nm. b) Size distribution of all micro-pools contained in the sample BASE bead (40 in total).

The surface area of micro-pools in a typical batch of BASE (2 mL enzyme solutions in 8.8 g silicone) was roughly calculated in formula 4.1, based on two assumptions: 1) enzyme solutions were emulsified into the micro-pools in the same degree as the case in Figure 4.6a, 2) entrapment efficiency of aqueous phase into silicone phase was the same to loading efficiency of enzymes in BASE. The minimal surface area of 1.9 mL (= 2 mL × 0.95) coalescing as a big aqueous droplet in a hydrophobic solvent is a spherical shape. Its surface area was calculated according to formula 4.2.

$$A_{\text{total micro-pools}} = \frac{\sum_{d_i=18\,\mu m}^{64\,\mu m} \pi \times d_i^2}{\sum_{d_i=18\,\mu m}^{64\,\mu m} \frac{\pi \times d_i^3}{6}} \times V_{\text{aqueous phase}} \times E_{CalA} \qquad (4.1)$$

$$= 0.25\ \text{m}^2$$

$$A_{\text{one spherical droplet}} = \pi \times \left(\frac{6 \times V_{\text{aqueous phase}} \times E_{CalA}}{\pi} \right)^{\frac{2}{3}} \qquad (4.2)$$

$$= 7.42 \times 10^{-4}\ \text{m}^2$$

Where $A_{\text{total micro-pools}}$ is the total surface area of micro-pools in a typical batch of prototype BASE (2 mL enzyme solutions in 8.8 g silicone), d_i is the diameter of each micro-pool in Figure 6a, $V_{\text{aqueous phase}}$ is the volume of 2 ml aqueous solutions, and E_{CalA} is the CalA loading efficiency (95 %), π = 3.14, and $A_{\text{one spherical droplet}}$ is the total surface area when the same volume of micro-pools (= 2 mL $\times$ 0.95) coalesces as one liquid sphere.

4.2.3 Enhancement of Interfacial Area

The effect of variations in water content on the interfacial area provided by BASE was determined by keeping the amount of silicone constant at 8.8 g and increasing the volume of the aqueous phase from 2 mL (standard) to 3 mL and 4 mL. This had almost no effect on the efficiency for enzyme loading (> 95 %) (Figure 4.7). Interestingly, Figure 4.8 illustrates that with the increase in water content not only the number of water-pools in BASE increased as expected, but concomitantly the overall size of the pools decreased. While at the typical ratio of 8.8 g silicone to 2 mL aqueous solution a size range of 15 µm to 60 µm in diameter could be estimated (Figure 4.8a), the size decreased to 6.5 µm to 40 µm at a ratio of 8.8 g silicone to 3 mL aqueous solution (Figure 4.8b), and further to 4.4 µm to 34 µm at a ratio of 8.8 g silicone to 4 mL aqueous solution (Figure 4.8c). Accordingly, a considerable increase in interfacial area with increasing water content can be expected.

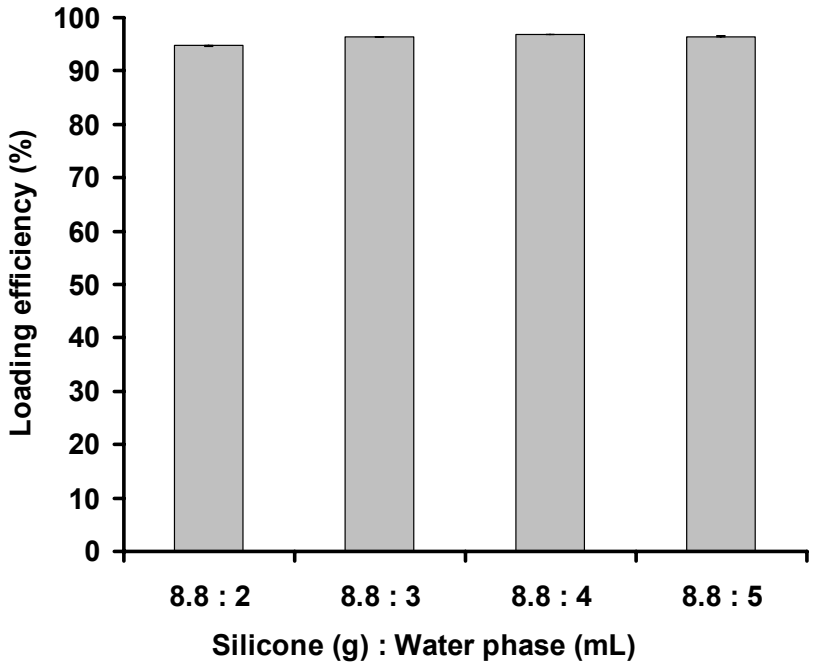


Figure 4.7. CalA entrapment efficiency for BASE prepared with different composition.

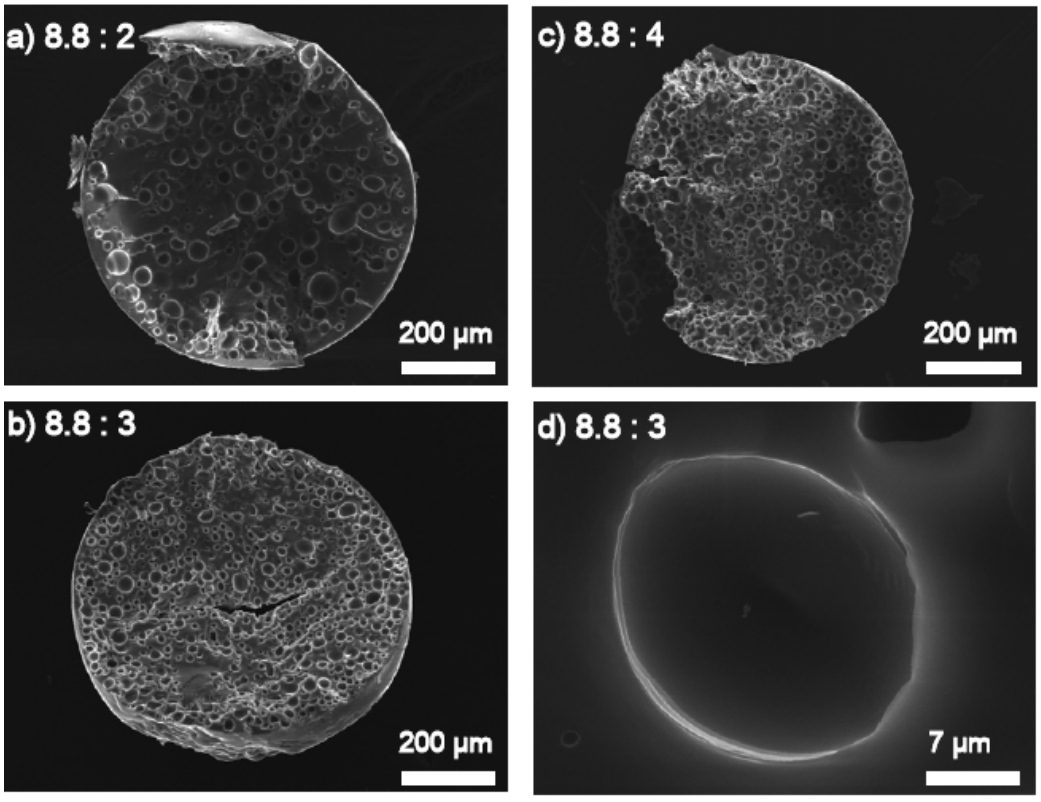


Figure 4.8. SEM cross section structure of BASE with varying water content. a) Silicone to water ratio 8.8 g : 2 mL. b) Silicone to water ratio 8.8 g : 3 mL. c) Silicone to water ratio

8.8 g: 4 mL. d) Typical and amplified micro-pool in a BASE particle at a silicone to water ratio of 8.8 g : 3 mL.

In addition, it was also observed that the water content within a BASE particle influences its overall surface. With increasing water content enhanced ruffling was observed (Figure 4.9). Literature reported the great difficulty of producing pores on the surface of these engaged silicone beads (polydimethylsiloxane).[168, 169] Figure 4.9d and very small surface area with BET measurement (data not shown) indicate that there are not large quantities of pores forming on the outer surface of BASE. The existence of ruffling may come from the presence of water pools close to the surface during BASE solidification. In any case, the small external surface area of BASE with dense silicone layer (Figure 4.5) indicates it catalytically important to investigate diffusion efficiency of reactants and products cross the particles.

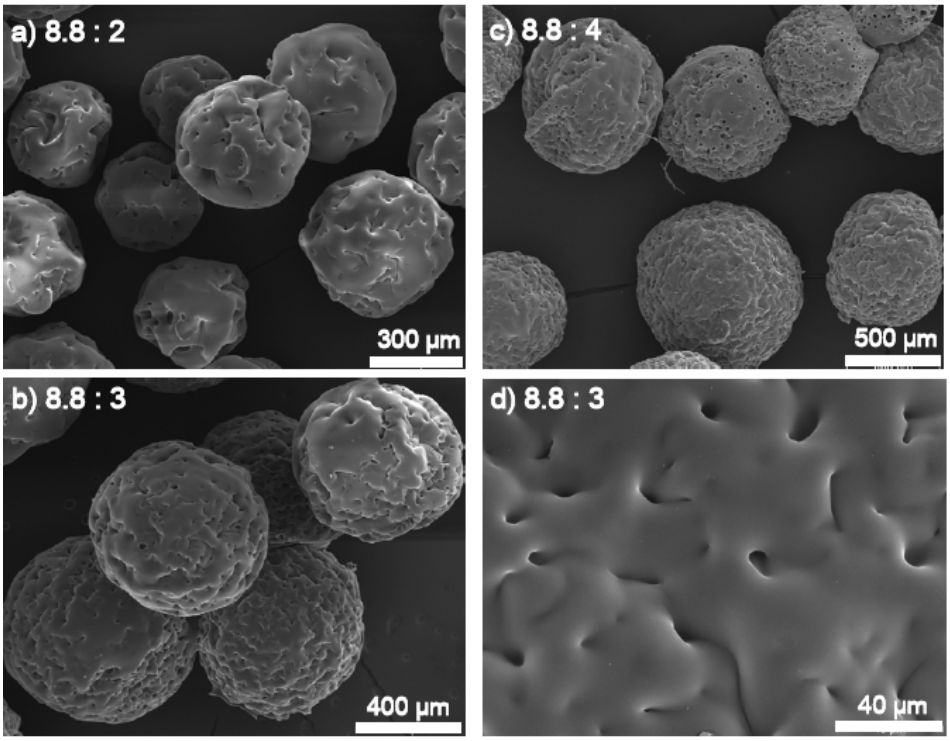


Figure 4.9. SEM morphology of BASE with varying water content. a) Silicone to water ratio 8.8 g : 2 mL. b) Silicone to water ratio 8.8 g : 3 mL. c) Silicone to water ratio 8.8 g: 4 mL. d) Typical and amplified bead surface at a silicone to water ratio of 8.8 g : 3 mL.

The influence of water content on the catalytic activity of BASE was determined for a range of particles with a content of up to 5 mL aqueous phase at a fixed amount of silicone (8.8 g) and constant enzyme concentration (6.4 mg•mL^{-1}). Above this ratio formation of BASE was

no longer possible. With the increase in water content a shift of the size distribution of BASE towards a majority of beads with a diameter of 800 µm was observed (data not shown). However, for better comparability of catalytic performance beads with a mean diameter of 600 µm were selected for investigation from all preparations. As expected from the increased total amount of enzyme per bead and the findings described in the previous paragraphs, the apparent catalytic activity of BASE particles (activity per total weight of beads) increased with increasing content of aqueous phase (Figure 4.10). However, the specific catalytic activity (activity per total content of protein) decreased, that is the contribution of single enzymes to the total catalytic activity decreased. This can be explained by a limitation of mass transfer independent of the improved interfacial area, most probably resulting from the increased mass transfer limitation of the continuous polymer phase.

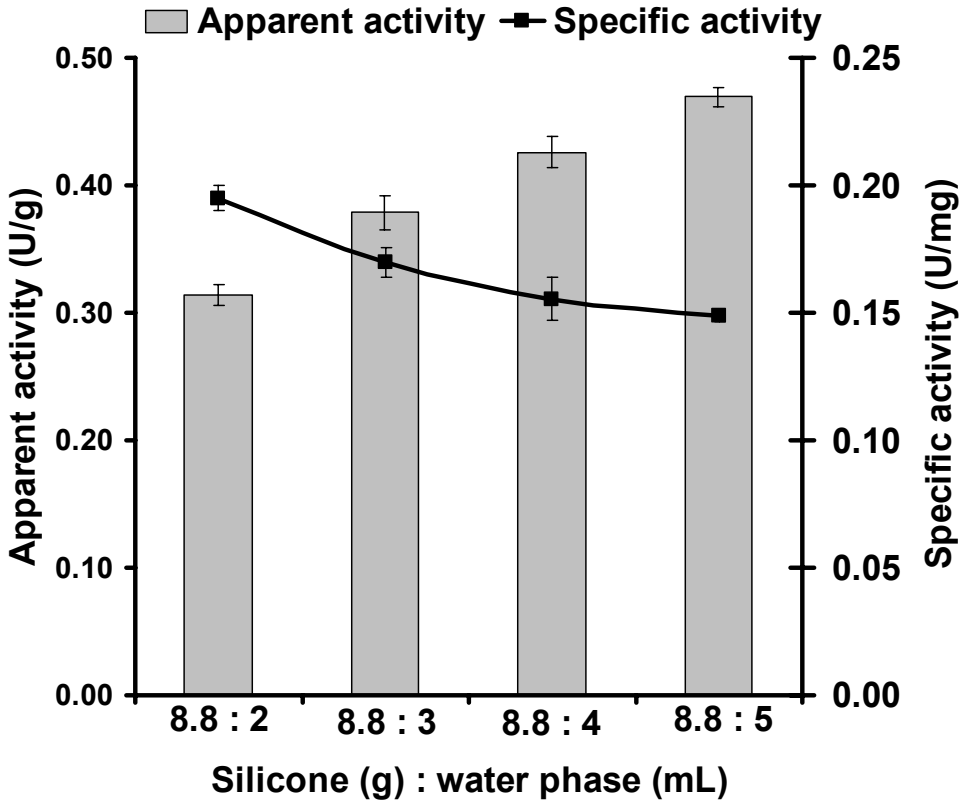


Figure 4.10. Apparent catalytic activity ($U \bullet g^{-1}_{BASE}$; gray columns) and specific catalytic activity ($U \bullet mg^{-1}_{protein}$; black line) of BASE with varying water content.

4.2.4 Assessment of Mass Transfer

A qualitative assessment of mass transfer in BASE particles was attempted by observation of matrix penetration with dyed solutions. Fluorol yellow, a fluorescence dye, dissolved in heptane penetrated easily into the silicone polymer and was distributed evenly over the complete matrix after 30 minutes (Figure 4.11). Comparable results were achieved with a solution of Sudan red in heptane. However, progression studies with this dye demonstrated that the penetration process is slow (Figure 4.12). After three minutes, the dye covers only a thin layer below the surface of the BASE particle. Therefore enzyme molecules towards the centre of the BASE particle are likely to experience significant mass transfer limitation.

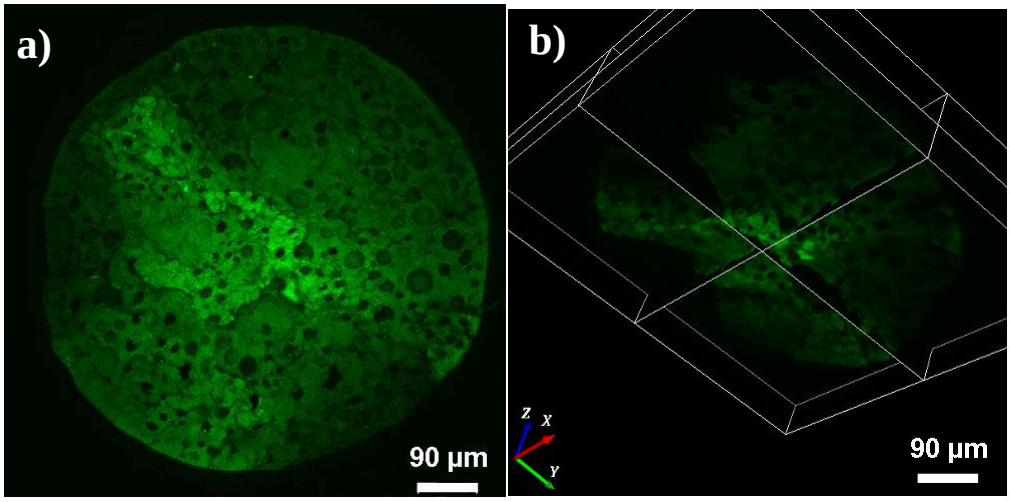


Figure 4.11. CLSM photo of penetration of fluorol yellow in heptane: a) after 30 min penetration, the sample was characterized at excitation of 476 nm and emission of 490 ~ 518 nm; b) the 3D image for the scanned layers in Figure 4.11a.The photos were edited by the software of BioImageXD.[170]

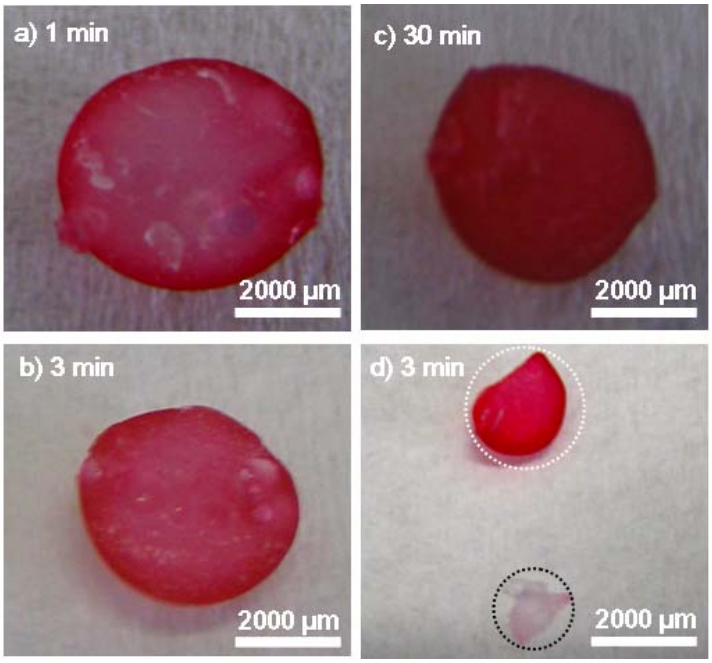


Figure 4.12. Penetration progression in BASE by Sudan red in heptane with different time: a) cross section photo of 1 min penetration of Sudan red in heptane; b) cross section photo of 3 min penetration; c) cross section photo of 30 min penetration; d) the core of the bead (in black dash circle) after 3 min penetration, and the surface of the bead (in white dash circle) after 3 min penetration.

As indicated by Klibanov,[37] the reduction of diffusional limitations can be achieved to increase activity by the increase of substrate concentration providing a steeper concentration gradient between the reaction medium and the interior of BASE. This is illustrated in Figure 13, where an increase of substrate concentration from 50 mmol•L^{-1} to 250 mmol•L^{-1} achieved an almost doubled apparent catalytic activity (increase from 0.54 U•g^{-1} to 1.1 U•g^{-1}). Additional effects can probably be expected from other variations in reaction conditions, e.g. an increase in stirrer speed.[37]

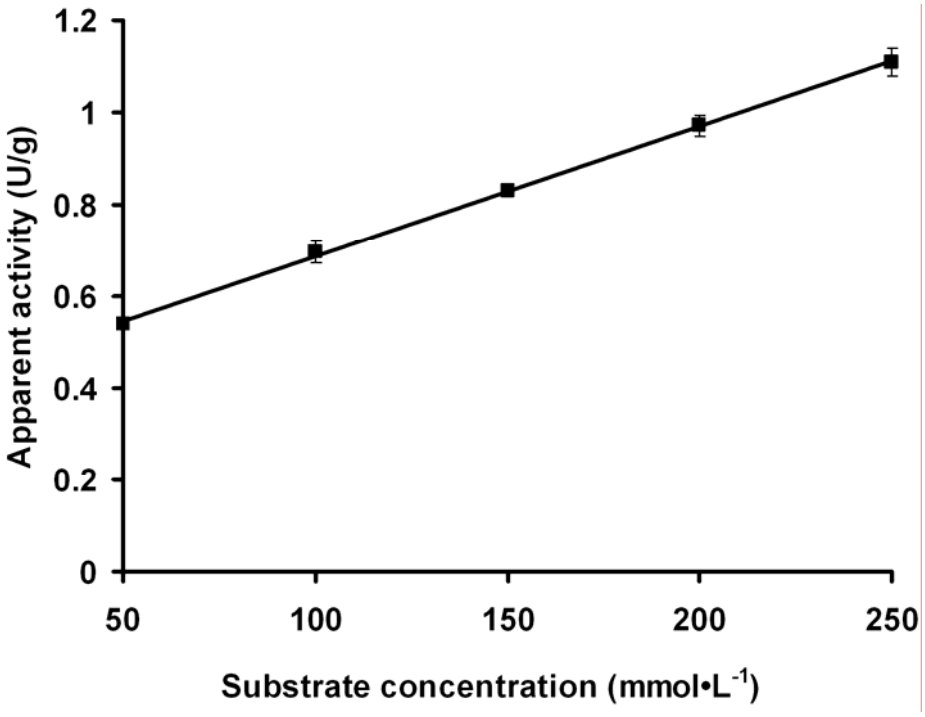


Figure 4.13. Apparent catalytic activity of BASE ($U \bullet g^{-1}$) at different substrate concentrations ($mmol \bullet L^{-1}$).

4.2.5 Optimization of Catalytic Activity

For technical application of BASE, a high apparent catalytic activity rather than a high specific activity is desirable since higher product yields per batch can be obtained and the cost of CalA is not expensive. Therefore, apparent activity was chosen as the target for further optimization.

At a maximum ratio of aqueous enzyme solution to silicone (5 mL in 8.8 g), the effect of enzyme concentration on catalytic activity was investigated by a stepwise increase of lipase content in the enzyme solution. A maximum apparent activity of 0.71 $U \bullet g^{-1}$ and specific activity of 0.21 $U \bullet mg^{-1}$ was achieved when the enzyme solution consisted of 4 mL commercial lipase preparation and 1 mL water. These decreased slightly, when the enzyme content was further increased to 4.5 mL (Figure 4.14), and dropped significantly when the enzyme preparation was used with no additional water. As commercial preparations frequently display very low water content this might indicate a strong influence of water activity, but might also reflect influences of interfacial activation or mass transfer restriction.

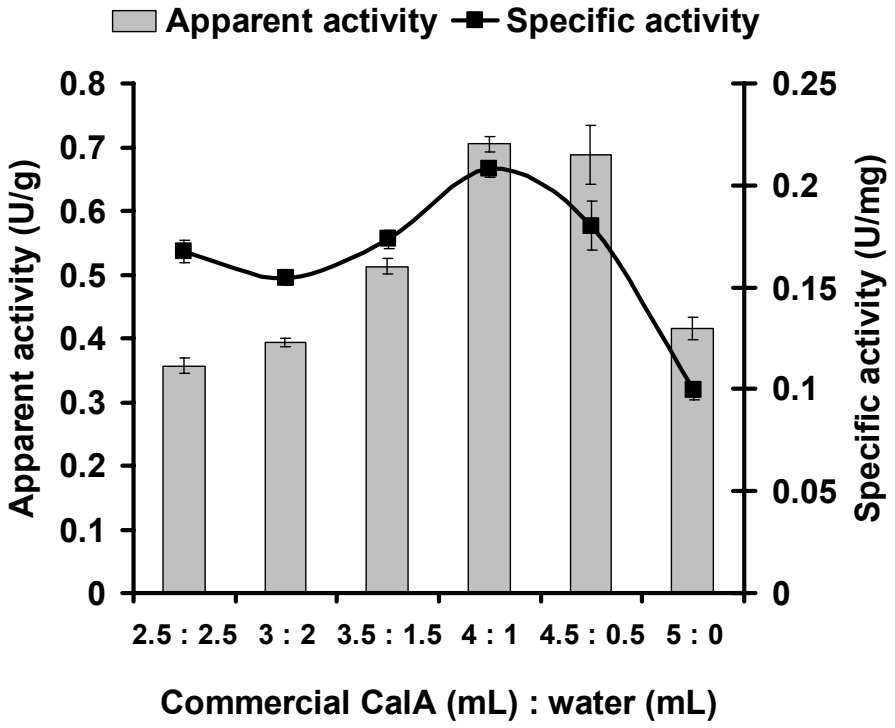


Figure 4.14. Apparent catalytic activity ($U \cdot g^{-1}_{BASE}$; gray columns) and specific catalytic activity ($U \cdot mg^{-1}_{protein}$; black line) of BASE at fixed water content.(5 mL) in silicone (8.8 g), but varying volume ratios of commercial CalA preparation to added water.

In total the apparent activity of BASE (0.71 $U \cdot g^{-1}$) by measures described in this study was improved by a factor of almost two (91 %) compared to prototype BASE (0.37 $U \cdot g^{-1}$). The specific activity (0.21 $U \cdot mg^{-1}$) of CalA in BASE here is 53 times higher than that data (0.004 $U \cdot mg^{-1}$), reported by literature,[33] of CalA entrapped in classic sol-gel silica.

4.3. Conclusion

The systematic study of morphology and composition of BASE enabled a considerable improvement of their catalytic performance towards esterification. Thus, a great leap towards the immobilization of biocatalysts requiring interfaces or distinct water pools for activity in organic solvents has been made.

Reproducibility of results was greatly enhanced by advancing the preparation method towards defined size distributions of both complete BASE particles and aqueous micro-pools within the continuous silicone matrix. Combined to the endoscopic analysis of emulsions forming the

basis of final particles this approach could provide a general possibility to improve the preparation of immobilized biocatalysts via suspension polymerization.

4.4. Research Collaboration

This research project aims at improving the interface of hydrophobic immobilisates for biocatalysis in non-aqueous media. For this, the hydrophobic BASE were selected as research object because BASE as typical hydrophobic immobilisates have high potential for industrial applications. However, preparing reliable and size-controllable BASE to improve BASE catalytic reproducibility requires a well-established knowledge in chemical engineering. Within UniCat, we collaborated with Professor Kraume (TU Berlin) to improve the BASE preparation process. I conducted all experiments in this project, except CLSM characterization which was carried out by Andreas Klee (TU Berlin) and endoscope measurement by Stephanie Hermann (TU Berlin).

5. Optimizing Water Activity in Lipase-containing Biocatalytic Active Static Emulsions (BASE)

5.1. Introduction

Biocatalysis in organic solvents has achieved tremendous success for chemical and pharmaceutical synthesis in decades, with high chemo-, regio-, and enantioselectivity. But owing to biological fragility, enzymes are readily inactivated in the hostile environment of organic solvents. For this, a proper immobilization method is often required to improve enzyme stability, protecting them from solvent invasion, and increasing their rigidity against conformational change.[72] Among all immobilized enzymes, lipases are most used enzymes for synthetic organic chemistry due to their broad spectrum of substrates and good stability in organic solvents. A critical characteristic of lipase catalyzed reaction is the interfacial activation, namely, lipases are greatly activated in presence of a lipid-water interface. Thus, hydrophobic carriers are preferably adopted to immobilize lipases for providing a hydrophobic surface. An outstanding example is the lipase entrapment in sol-gel, where hydrophobic silica enhances lipase activity up to 80 times while the activity deceases in hydrophilic silica.[29-31, 143] This hydrophobic effect explicitly illustrates the importance of carrier microenvironment to enzyme catalytic performance.

However, sol-gel lipase immobilization only take advantage of hydrophobic effect but neglect the importance of water, which is believed to acts as a lubricant or plasticizer to offer enzymes sufficient conformational flexibility needed for catalysis.[62] The combined effects of hydrophobic interface and water activation to lipases were recently realized in a so called “biocatalytic active static emulsions” (BASE). In this system, aqueous enzyme solutions are emulsified into numerous micron-sized water pools in the super hydrophobic polydimethylsiloxane (PDMS). These emulsified aqueous micro-pools contribute to a large water-hydrophobic interface for lipase catalysis, resulting in even better lipase activity than sol-gel.[33] More recently, the BASE composition was optimized to further increase interfacial area of micro-pools in silicone, with almost double activity increased than the prototype BASE.[20] Interestingly, BASE activity was found to substantially decrease in absence of water. But excessive water presented led to poorer catalytic performance. These findings

indicate the importance of water presence for BASE catalytic performance, and encourage next optimization of BASE in tem of water content.

In this study, BASE-catalyzed organic synthetic system was further advanced in term of optimizing water phase in BASE. Success of the optimizations can drive BASE as a robust biocatalyst towards industrial applications. As model system, lipase A from *Candida antarctica* (CalA) entrapped in BASE is used to catalyze esterification reaction.

5.2. Results and Discussion

5.2.1. Characterization and Catalytic Assessment of Dried BASE

The previous findings indicate that altering water content in BASE resulted in significant change of BASE activity for synthesis. These results are obtained from the freshly prepared BASE, where enzymes are distributed in remarkable aqueous phase of micro-pools in silicone. But the recent experiments indicate that some outer water layers present on these fresh BASE surfaces, leading to serious diffusion resistance to substrates and products. Slightly drying of the BASE surface can obviously increase activity. Moreover, lipase-catalyzed synthesis only requires a small quantity of water to activate enzymes, and excessive water in BASE system is unfavorable to reaction equilibrium. Thus the freshly prepared BASE, which usually possess bulk water phase in system, is impossibly optimized for a low-water system. To resolve the problems, a process, pre-evaporating and post-recruiting water, was employed to exquisitely control low-water in BASE for synthesis. In this process, the fresh BASE were firstly dried in air for two months (seen experimental section). Then they were incubated under low-water conditions with different water activity (a_w). a_w is a thermodynamic index to describe the continuum of energy states of the water in a system, thus is more suitably used to control low-water system for biocatalytic synthesis.[63]

The drying status of BASE was visualized by confocal laser scanning microscopy (CLSM). From Figure 5.1a, CalA labeled with FITC forms numerous green circles in BASE, which indicates that drying BASE can relocate enzymes onto inner surfaces of micro-pools. A 3D model of enzymes in dried BASE further proves that drying BASE eventually concentrates enzymes onto micro-pool surface (Figure 5.1b), which renders more enzymes ready for catalysis. Though aqueous phase is removed in dried BASE, the immobilization system still belongs to the unique BASE. This is because the major characteristic of BASE is to entrap

enzymes inside the micro-pools of hydrophobic silicone with proper water amount, which is later introduced with low-water system. To distinguish dried BASE from conventional adsorption immobilization, Novozym 435 and dried BASE were stirred in reaction media (heptane) for one day. Obvious leaching of enzymes from Novozym 435 is found, while no leaching can be detected from dried BASE (Figure 5.2). The serious leaching from Novozym 435 is explained due to the weak mechanic strength of carriers (Figure 5.3), and weak adsorption of enzymes on the outer surface of macroporous carriers.[80] In contrast, BASE are composed by hydrophobic silicone (PDMS) which possesses much stronger mechanic resistance to organic solvents. After one day string in heptane, BASE remain almost same but most Novozym 435 are almost destroyed (Figure 5.3). In addition, BASE entrap enzymes inside of mcrio-pools of silicone, which have no pores to allow enzymes leaching.[20] Therefore drying BASE has advantages to normalize all BASE as very low-water system ready for BASE optimization with low-water content, and to condense more enzymes on surface of micro-pools for catalysis.

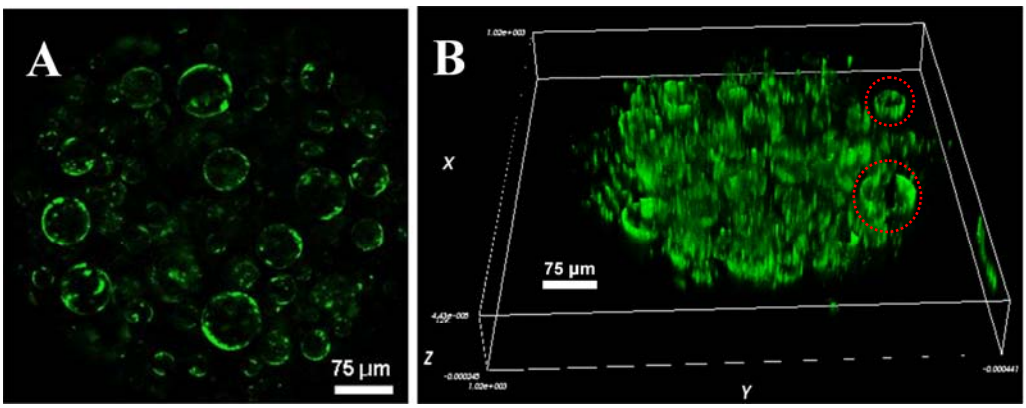


Figure 5.1. a) CLSM photograph of a prototype dried BASE bead. b) 3D model of CalA in dried BASE (experimental details in the section of materials and methods).

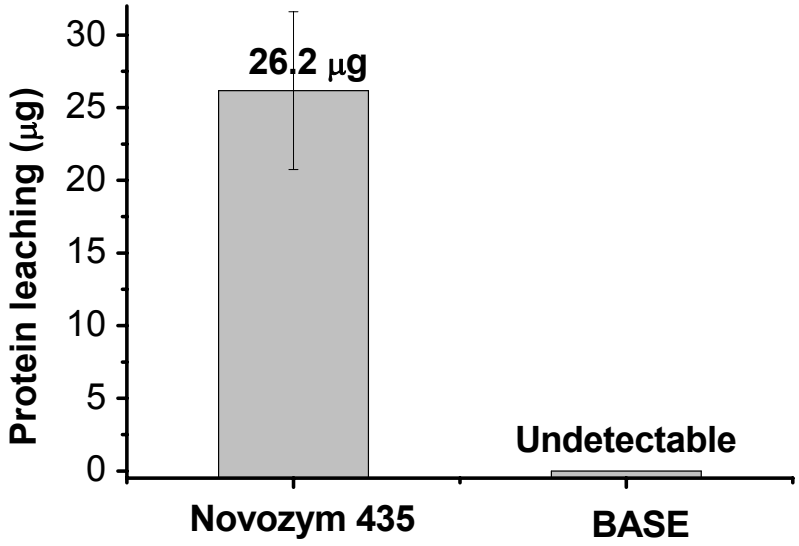


Figure 5.2. Protein leaching between Novozym 435 and BASE.

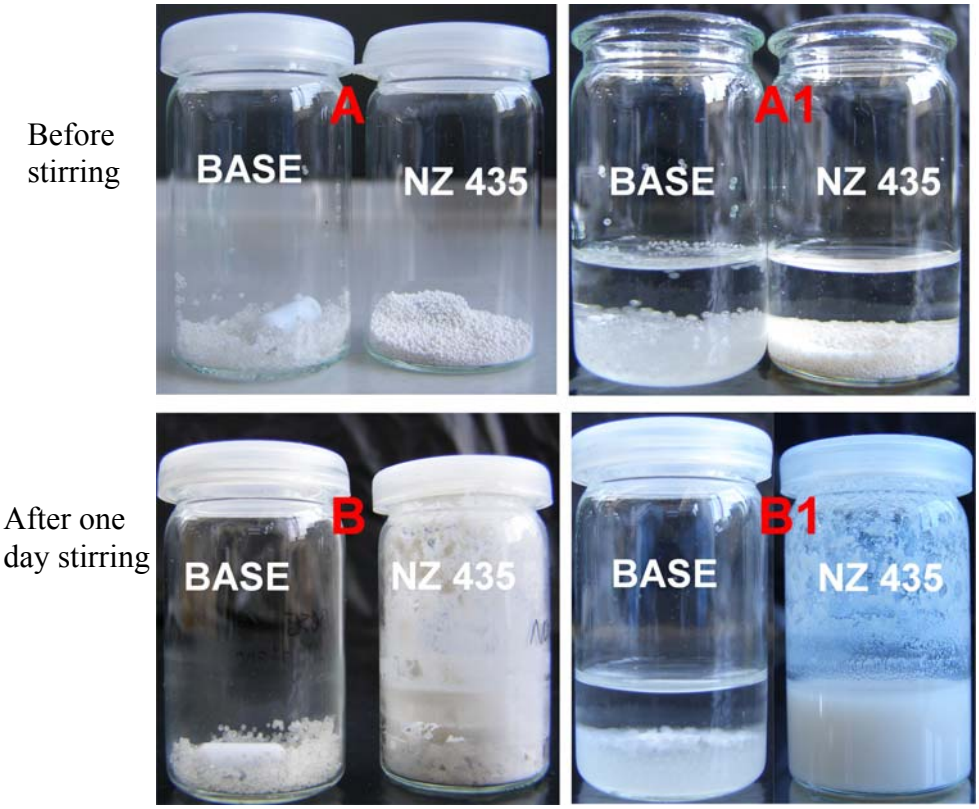


Figure 5.3. Mechanic stability of BASE and Novozym 435 (NZ435).

Catalytic performance of dried BASE was compared with that of freshly prepared BASE. Interestingly, in bigger particle size BASE (diameters in: 500, 600, 800, 1000, and 1400 μm), drying BASE decreases apparent activity (Figure 5.4). For example, in a typical size of 600

μm BASE, apparent activity decreases from 0.41 μmol•min^{-1}•g$^{-1}_{BASE}$ in fresh BASE to 0.15 μmol•min^{-1}•g$^{-1}_{BASE}$ in dried BASE (Insert in Figure 5.4). These results demonstrate substantial removal of water from BASE indeed depresses enzymatic synthesis. But in these smaller particles of BASE with diameters of 150 and 300 μm, apparent activity of dried BASE tremendously increase up to 48 and 5 times folder than fresh BASE, respectively (Figure 5.4). This probably indicates that interfacial area benefits of smaller BASE can not take effects in fresh BASE due to deleterious outer water layers resulting in serious mass transfer limitation. Drying these smaller BASE can effectively eliminate these adverse water layers for catalysis. Accordingly, after 17 hour reaction, smaller particle size of dried BASE converted more substrates into products (Figure 5.5). For instance, the conversion of 150 μm dried BASE is close to 100 % after 17 h, while at the same conditions it is only 15.78 % conversion for 500 μm dried BASE. As a consequence, drying BASE greatly changes BASE catalytic microenvironment, which eliminates the adverse outer water layer, benefiting for smaller BASE, but lacks of water activation needed to be optimized latter.

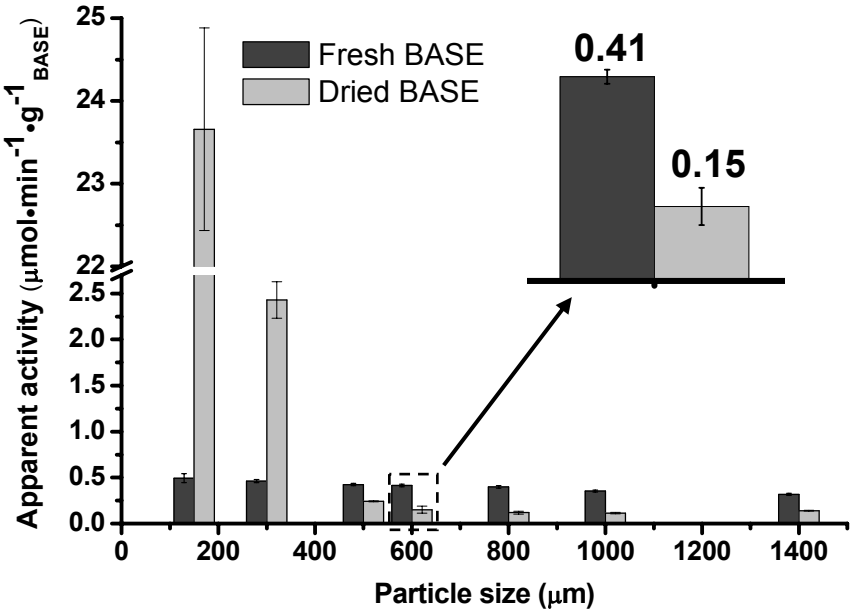


Figure 5.4. Apparent activity of dried BASE and freshly prepared BASE.

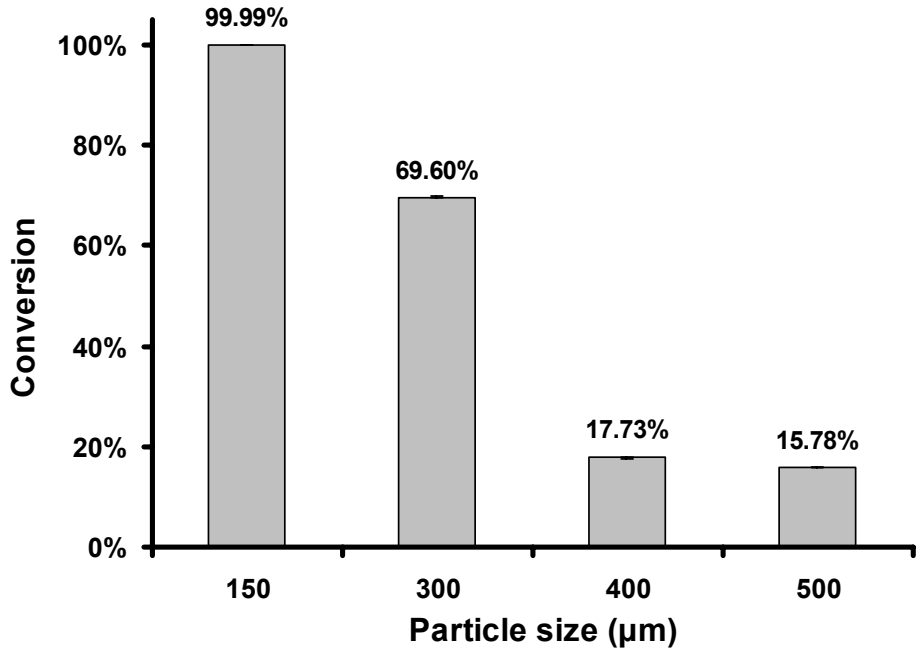


Figure 5.5. Conversion of dried BASE with different particle size.

5.2.2. Optimization of BASE with Low-water System for Synthesis

Adding low-water to dried BASE for catalysis is realized through incubating BASE beads (600 μm) in a series of closed desiccators where different saturated salt solutions were placed to control equilibrium relative humidity (ERH) (Table 5.1). As a_w = ERH (%) / 100, incubating BASE there provides them with different thermodynamic water activity according to Table 5.1.

Table 5.1. Saturated salt solutions for different water activity.

Saturated salt solutions	$CaCl_2$	NaCl	Na_2CO_3	Distilled water
Equilibrium relative humidity (ERH) (%)	32	76	92	100
Water activity (a_w)	0.32	0.76	0.92	1.00

Incubating dried BASE under a_w = 1 condition, it is found that, after 1 day, the apparent activity suddenly increases from 0.15 to 0.85 μmol•min^{-1}•g$^{-1}_{BASE}$ (Figure 5.6). This indicates that a short-term incubation in low-water conditions can greatly provide enzymes with some "essential" water for catalysis.[62] Further incubating them for 3 days results in a maximum apparent activity (0.97 μmol•min^{-1}•g$^{-1}_{BASE}$), while activity slightly decreases when extending

incubation time up to 4 or 5 days. This activity decrease might be due to experimental fluctuations. Thus one day incubation at $a_w = 1$ is required to activiate BASE with necessary water activity in organic media. But to guarantee all sample sufficiently incubated with optimal water activity, all the samples used later were incubated for three days at $a_w = 1$.

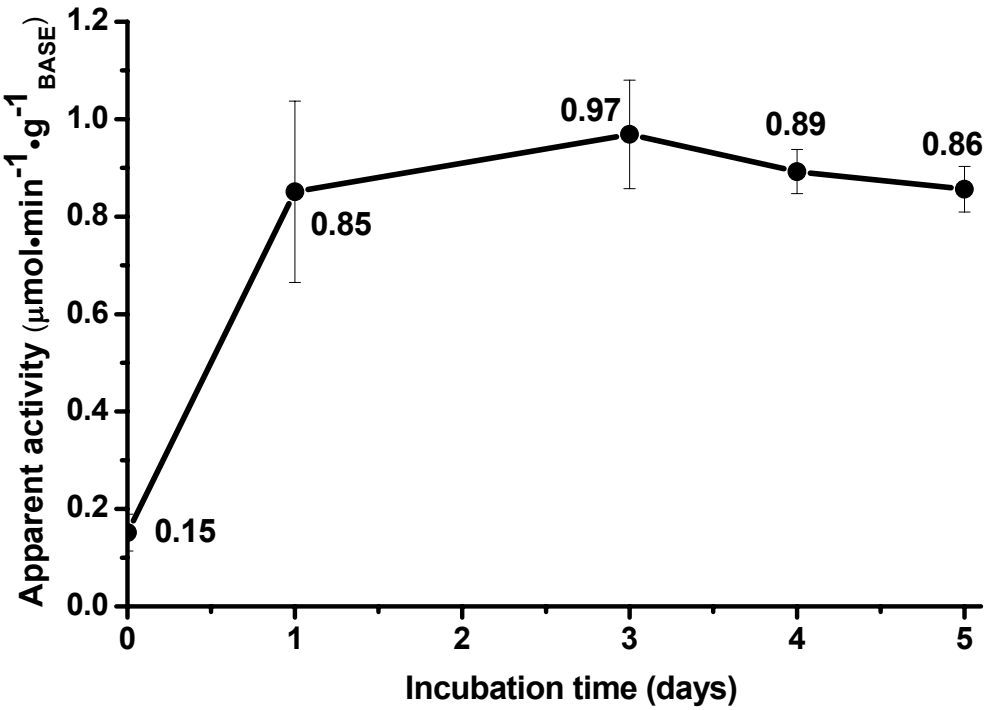


Figure 5.6. Incubating BASE at $a_w = 1$ in different time.

In addition, dried BASE were optimized under various low-water conditions with 3 day incubation in closed desiccators. From Figure 5.7, apparent activity of BASE enhances with the value of a_w increased. At $a_w = 1$, BASE have an optimal apparent activity of 1.12 $\mu mol \bullet min^{-1} \bullet g^{-1}_{BASE}$, which is slightly higher than the value in Figure 5.6 due to experimental fluctuations. The increased BASE activity at higher a_w indicates that even the case of $a_w = 1$ is not sufficient to offer enzymes with all "essential" water. This is because water capillary condensation even at $a_w = 1$ can not take place in these micron-sized pores in BASE.[171] However, supplying dried BASE with $a_w = 1$ is the optimized condition under the current employed method. Moreover, comparing with fresh BASE, this optimized low-water condition increases BASE apparent activity with 3 times (Figure 5.8). This result is attributed to two factors: 1) pre-evaporating water from BASE concentrates enzymes onto surface of micro-pools; 2) incubation of enzymes under various low-water conditions offers them most flexibility for catalysis. On other hand, controlling BASE under constant low-water system

can obviously improves BASE catalytic reproducibility as shown in Figure 5.9. Therefore, optimizing dried BASE under low-water conditions is good way which not only enhances BASE catalytic performance but improves their reproducibility.

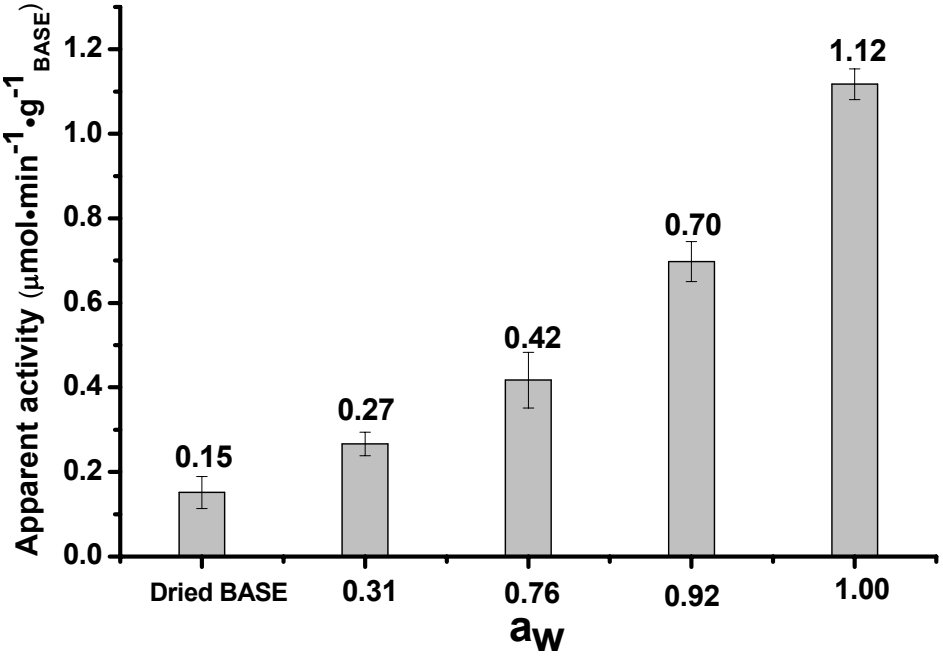


Figure 5.7. Incubating BASE under low-water system with different a_w.

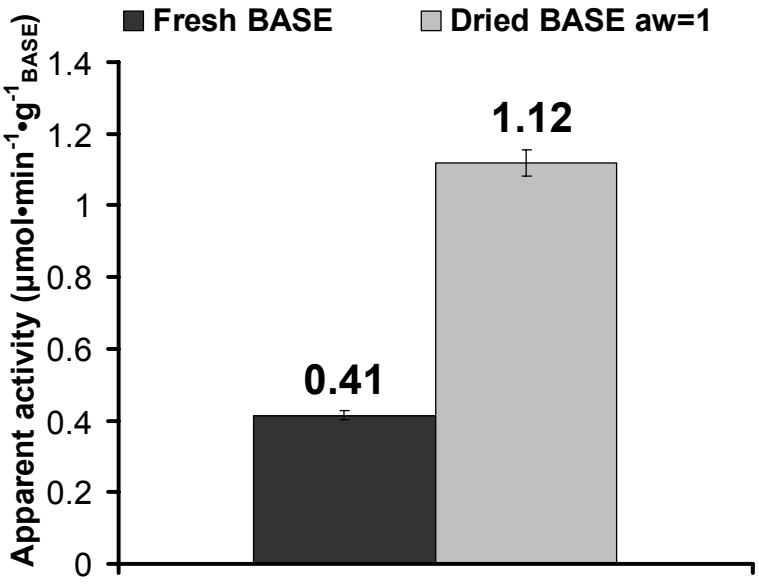


Figure 5.8. Comparison between optimized BASE with $a_w = 1$ and fresh BASE.

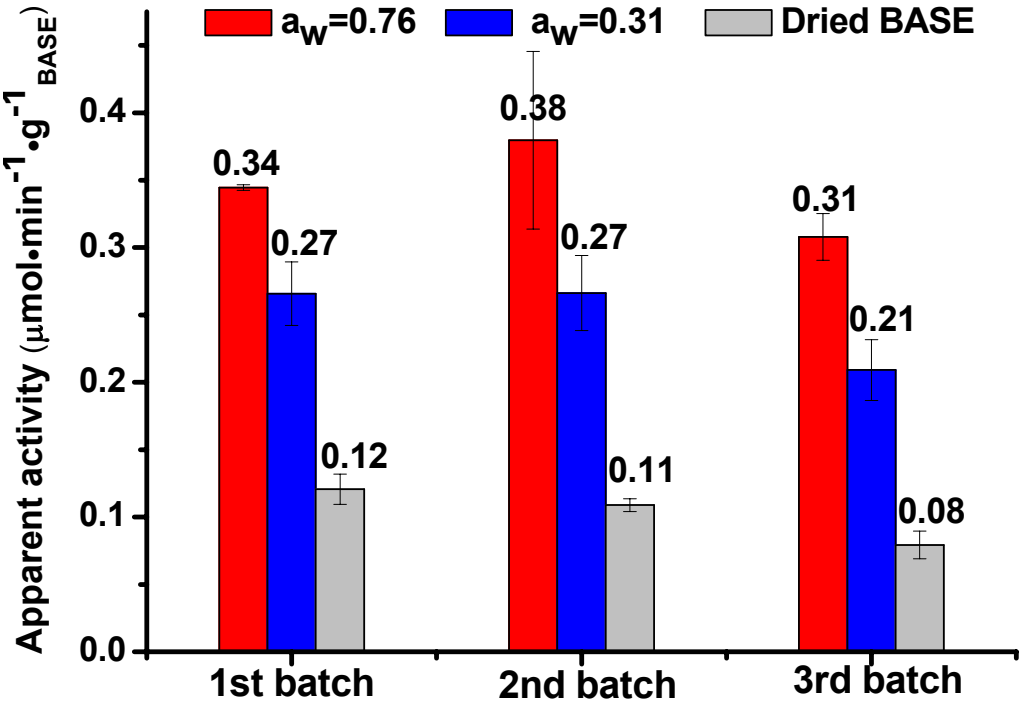


Figure 5.9. Reproducibility of three batches BASE incubated in three different a_w.

5.3. Conclusion

In this study, BASE were further optimized for synthetic applications under low-water system. Dried BASE were incubated under different water activity (a_w) conditions to obtain various low-water compositions. It is found that 3 days incubation of BASE under $a_w = 1$ results in optimal apparent activity, approximately 3 times higher than fresh BASE. Therefore, the study successfully optimizes BASE with the low-water system, which greatly advances BASE as robust heterogeneous biocatalysts for synthetic applications.

6. Nanoparticle Cages of Enzyme for Biocatalysis in Organic Media

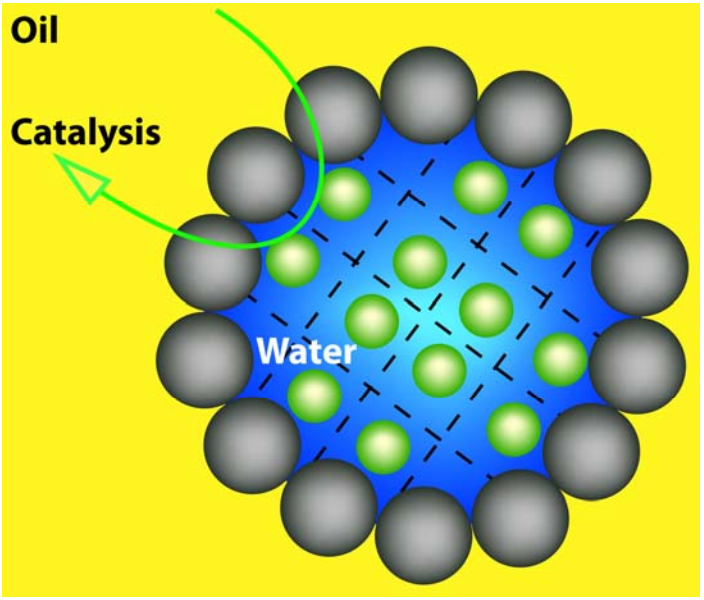
6.1. Introduction

It has been well-known for a long time that colloidal nanoparticles (NPs) can behave similar to amphiphilic molecules; they readily adsorb to oil/water interfaces and efficiently stabilize emulsions – Pickering emulsions.[96, 172] Distinct from amphiphilic molecules that adsorb and desorb in a rather dynamic manner, however, NPs irreversibly adsorb at oil/water interfaces as a result of the large energy required to detach the NPs from such an interface. Accordingly, Pickering emulsions are more stable than emulsions stabilized by amphiphilic molecules. Nowadays, NPs have been implemented as better and more efficient stabilizers to partially or completely replace amphiphilic molecules in various emulsified products (e.g. food, pigments, cosmetics, pharmaceutics) in order to avoid the technical problems associated with the use of amphiphilic molecules, such as foam formation. In the context of nanotechnology, the interest in studying NPs at interfaces has recently been resurged.[173-177] Locking the NPs adsorbed on emulsion droplets has led to hollow, micron-sized, capsules enclosed by closely-packed NP monolayers, denominated as colloidosomes.[178-181] Herein, the study report the first successful application of Pickering emulsions and the colloidosomes derived thereof to the reversible immobilization of enzymes for biocatalysis.

Enzymes are powerful biological catalysts for a broad variety of organic reactions, providing industrially important molecules with an overall high chemo-, regio-, and stereoselectivity.[1, 39, 59, 74] However, in order to adapt to organic reaction media and improve the recovery and reuse efficiency, the usually hydrophilic enzymes need to be immobilized on or in specific carriers via physical adsorption or chemical binding.[2, 32, 35-37] A simple and versatile way to do this, is the emulsification of an aqueous enzyme solution in an organic medium using amphiphilic molecules such as small surfactants and block polymers for stabilization.[2, 32, 35] The surface active properties of these amphiphilic molecules, however, have a devastating effect on many enzymes. Therefore, only a small amount of fairly stable enzymes, such as lipases, can be successfully immobilized by emulsification to date, while the vast majority of much more vulnerable enzymes is rapidly deactivated.[182, 183] Another disadvantage of using amphiphilic molecules for technical synthesis, is their dynamic interfacial attachment, which

makes a removal from organic reaction media and final products rather difficult.[101] Both serious drawbacks can easily be circumvented by using NPs for emulsification, but no attempt to apply Pickering emulsions to the immobilization of catalytically active enzymes as been reported so far.

In this study, hydrophobic SiO_2 NPs have been used to emulsify aqueous solutions of selected enzymes in organic reaction media (Scheme 6.1) and evaluated the catalytic activity of the Pickering emulsions obtained thereby. For benchmarking, the two considerably distinct enzymes, lipase B from *Candida antarctica* (CalB), which offers a very high intrinsic stability,[144] and benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I (BAL), which is a much more vulnerable biocatalyst, were chosen; both enzymes show significantly enhanced catalytic performance after immobilization. To my best knowledge, this is the first report on such an immobilization-induced activation of a delicate enzyme like BAL.[184]



Scheme 6.1. Cartoon of an aqueous droplet (highlighted by blue) of enzymes (green particles), stabilized by SiO_2 NPs (grey particles), for catalysis of organic reactions in organic media (highlighted by yellow). The agarose network is highlighted by dashed grids.

6.2. Results and Discussion

6.2.1. Encapsulating CalB in Pickering Emulsions

SiO_2 NPs with a diameter of 140 nm were hydrophobized with TMODS, and then used to stabilize water-in-oil Pickering emulsions. For the first proof-of-concept, the resulting

hydrophobic SiO_2 NPs were utilized to stabilize droplets of an aqueous solution containing CalB in heptane. For easy processing and characterization, the aqueous core of the Pickering emulsions was solidified by addition of agarose (1.5 % (w/w)) to the CalB solution prior to emulsification. The jellified droplets containing CalB were visualized via CLSM fluorescence imaging using enzyme labeled with fluorescein isothiocyanate (FITC) (Figure 6.1a). The droplet size was in the range of 5 – 15 µm. SEM imaging clearly reveals the enclosing of the jellified CalB droplets by random, closely-packed, 140 nm SiO_2 NP shells (Figure 6.1b). After separation from heptane via gentle centrifugation, the jellified droplets of FITC-labeled CalB were dispersed in buffer solutions. The appearance of FITC fluorescence in the buffer solutions indicates the release of CalB from the jellified droplets, thus suggesting the reversibility of the immobilization process (inset in Figure 6.1c). The secondary structure of the enzyme before emulsification and after release from the emulsion was investigated via circular dichroism (CD). The resulting spectra (Figure 6.1c) show almost no difference, suggesting little impact of the emulsification and jellification process on CalB.

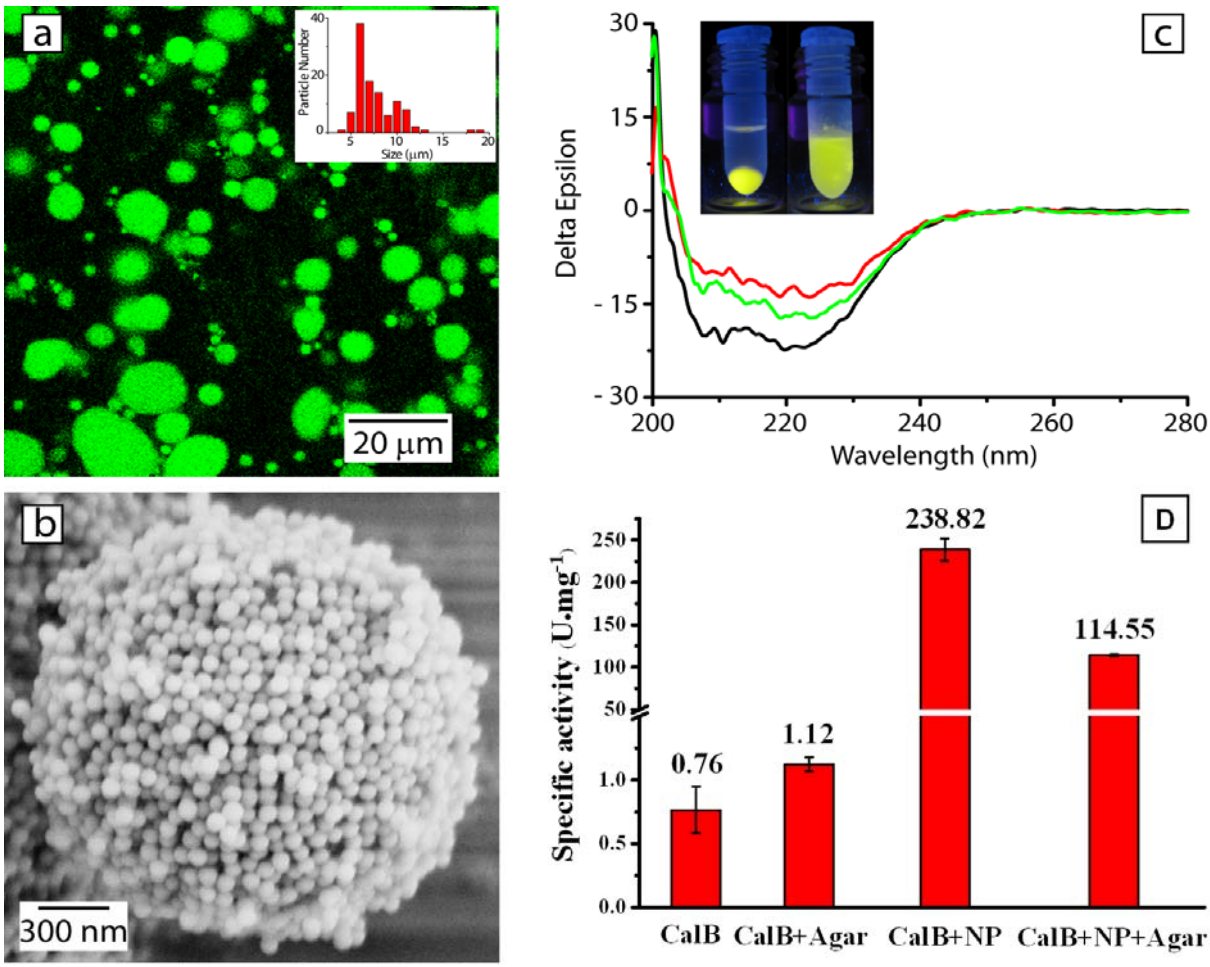


Figure 6.1. (a) Fluorescence micrograph of aqueous solution droplets of FITC-CalB, stabilized by 140 nm SiO_2 NPs, in heptane. The concentration of agarose in the aqueous phase is 1.5 % (w/w) and the concentration of the NPs used for Pickering emulsification 1.0 % (w/w). The inset is a histogram of the size distribution of the resulting jellified FITC-CalB droplets, counted from the fluorescence micrograph. (b) SEM micrograph of a jellified aqueous droplet of FITC-CalB, stabilized by 140 nm SiO_2 NPs. (c) CD spectra of native CalB (black curve) and CalB released from fluidic (red curve) and jellified (green curve) Pickering emulsion droplets, stabilized by 140 nm SiO_2 NPs. The inset shows jellified Pickering emulsion droplets, loaded with FITC-CalB, separated as a fluorescent sediment at the bottom of a plastic eppendorf tube from heptane (Left photo) and FITC-CalB is released into water and makes the whole phase fluorescent when the jellified emulsion droplets are brought in contact with water after heptance decanting (Right photo). (d) Chart of the specific activities of native CalB, CalB immobilized in bare agarose (CalB+Agar), and CalB immobilized in fluidic (CalB+NP) and jellified (CalB+NP+Agar) Pickering emulsion droplets, stabilized by 140 nm SiO_2 NPs. The specific activity values are given in the chart.

The catalytic performance of CalB was investigated at the esterification of 1-octanol and octanoic acid. Aqueous solutions of CalB were emulsified directly in heptane containing a mixture of 1-octanol and octanoic acid using the 140 nm hydrophobic SiO_2 NPs described in the previous paragraph. It was found that the specific activity of CalB immobilized in these liquid Pickering emulsion droplets (238.82 $U \bullet mg^{-1}$) is more than 300 times higher than that of native CalB (0.76 $U \bullet mg^{-1}$) (Figure 6.1d). This significant enhancement can be explained by the considerably increased interfacial area provided by the emulsification of the aqueous enzyme solution in heptane, since this improves mass transfer and accessibility of the enzyme catalyst in the reaction system. When the emulsion droplets were jellified with agarose, the specific activity of the immobilized CalB decreased to 114.55 $U \bullet mg^{-1}$, but was still about 150 times higher than that of native CalB (0.76 $U \bullet mg^{-1}$) and about 100 times higher than that of CalB entrapped in bare agarose (1.12 $U \bullet mg^{-1}$). These observations are in accordance with the exertion of diffusional limitations by the agarose gel network.[37]

6.2.2. Encapsulating BAL in Pickering Emulsions

Distinct from CalB, which is characterized by a high intrinsic stability at various reaction conditions, many enzymes with promising catalytic properties are much more vulnerable to

environmental changes and therefore easily deactivate during immobilization. A proper representative of such a biocatalyst is BAL from *Pseudomonas fluorescens* Biovar I, which catalyzes the industrially relevant stereoselective condensation of benzoins. Accordingly, only few approaches were able to immobilize this enzyme without deactivation so far,[184, 185] and none succeeded in achieving an activation. Here, aqueous solutions of BAL containing all essential cofactors were emulsified in the organic solvent MTBE by using the previously described 140 nm hydrophobic SiO_2 NPs (0.5 – 5 % (w/w)). The emulsion droplets were jellified by 1.5 % (w/w) agarose yielding particles with a size range of 2 – 20 µm (Figure 6.2a). The CD spectrum in Figure 6.2b shows only a negligible change in the secondary structure of BAL after immobilization in either liquid or jellified Pickering emulsions, indicating that, like CalB, the enzyme was not affected by the immobilization process.

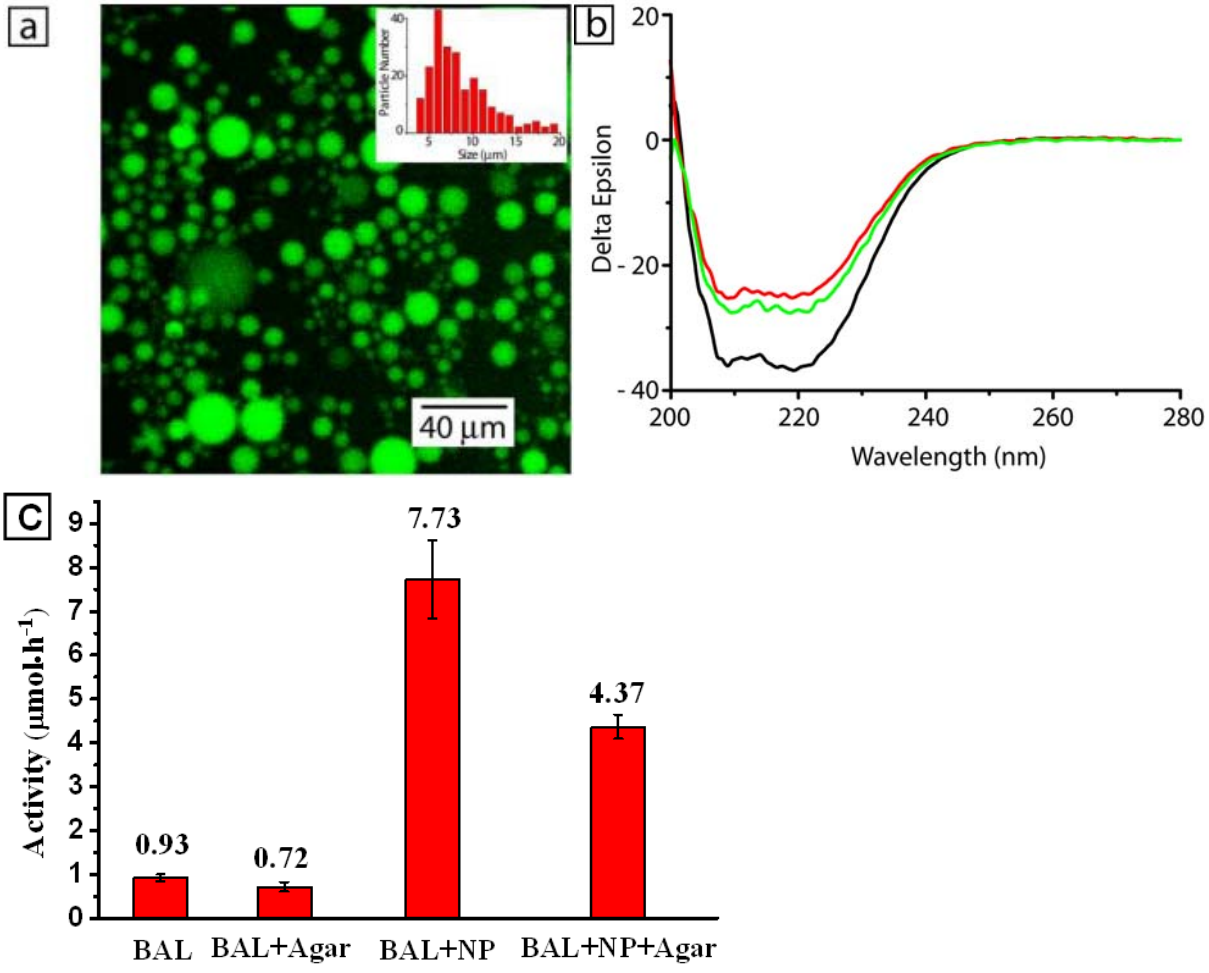


Figure 6.2. (a) Fluorescence micrograph of aqueous solution droplets of FITC-BAL, stabilized by 140 nm SiO_2 NPs, in MTBE. The concentration of agarose in the aqueous phase is 1.5 %

(w/w) and the concentration of the NPs used for Pickering emulsification 1.0 % (w/w). The inset is a histogram of the size distribution of the resulting jellified FITC-BAL droplets, derived from the fluorescence micrograph. (b) CD spectra of native BAL (black curve) and BAL released from fluidic (red curve) and jellified (green curve) Pickering emulsion droplets, stabilized by 140 nm SiO_2 NPs. (c) Chart of the activities of native BAL, BAL immobilized in bare agarose (BAL+Agar), and BAL immobilized in fluidic (BAL+NP) and jellified (BAL+NP+Agar) Pickering emulsion droplets, stabilized by 140 nm SiO_2 NPs. The activity values are given in the chart.

The catalytic performance of native and immobilized BAL was determined via the stereoselective formation of benzoin. Aqueous solutions of BAL and its cofactors were emulsified directly in MTBE containing benzaldehyde as substrate. As illustrated in Figure 6.2c, native BAL retained a rather low activity under these conditions (0.92 $\mu mol \bullet h^{-1}$) which is in accordance with the reported sensitivity of the enzyme against organic solvent molecules dissolved in the aqueous phase, the interfaces between aqueous and organic phases, and even the substrate molecules.[148] In contrast, in Pickering emulsion, the activity of BAL increased to 7.73 $\mu mol \bullet h^{-1}$, which is more than eight times higher than that of native BAL. This suggests that the drastic increase of the interfacial area by Pickering emulsification largely offsets the negative impact of the reaction system on the enzyme. A comparable enhancement of the catalytic performance of BAL has never been achieved by any other immobilization technique so far.[184, 185]

Jellification of the Pickering emulsion droplets with agar reduces the activity of BAL to 4.37 $\mu mol \bullet h^{-1}$(Figure 6.2c), which, however, is still more than 4.5 times higher than the activity of native BAL. A noticeable reduction of the BAL activity in jellified Pickering emulsion droplets was observed, when the temperature at which the aqueous solutions of agarose and BAL were mixed was increased (Figure 6.3a). In order to minimize this deactivation, an agarose with a fairly low gelling temperature ($\leqslant$ 17 ºC) allowing the mixing of aqueous solutions of BAL at room temperature and jellification at 0 ºC was applied.

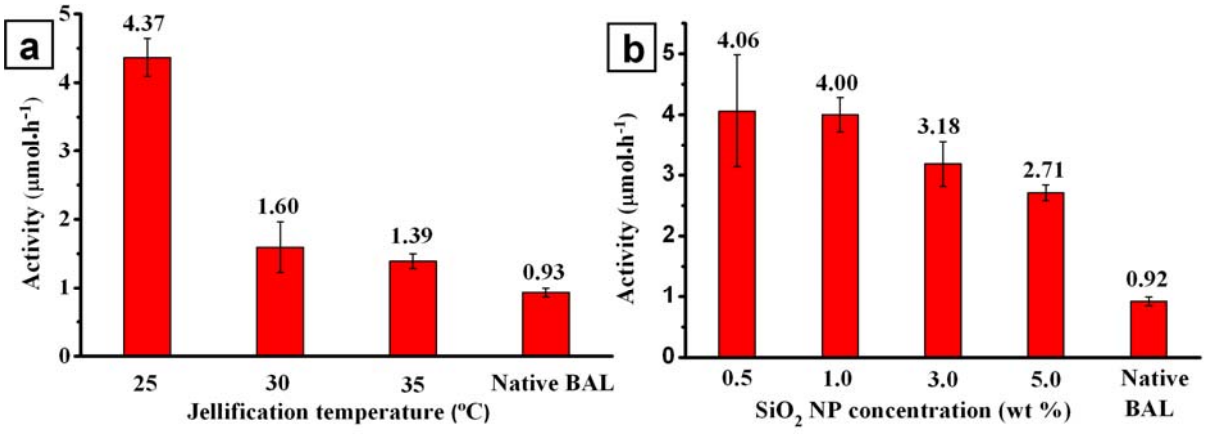


Figure 6.3. a) Chart of the activities of BAL immobilized in jellified Pickering emulsion droplets, stabilized by 140 nm SiO_2 NPs, versus the concentration of the NPs used to form Pickering emulsions. b) Chart of the activities of BAL immobilized in jellified Pickering emulsion droplets, stabilized by 140 nm SiO_2 NPs, versus the temperature at which the aqueous solutions of agarose and BAL are mixed. The activity values are given in the charts.

Figure 6.3b shows that the activity of BAL immobilized in Pickering emulsions increases with the decrease of the concentration of hydrophobic SiO_2 NPs used to create Pickering emulsions. This could be explained by the fact that lowering the concentration of hydrophobic SiO_2 NPs used for Pickering emulsification leads to thinner NP shells enclosing the aqueous BAL droplets, thus facilitating mass transfer and interaction with the enzyme. Taking into account that the decrease of the hydrophobic SiO_2 NP concentration may reduce the colloidal stability of the resulting Pickering emulsions, the optimal concentration of 140 nm hydrophobic SiO_2 NPs was found in the range of 0.5 – 1.0 % (w/w).

6.3. Conclusion

In conclusion, this study demonstrated the first successful use of NPs for the emulsification and immobilization of different enzymes in organic media for synthetic use. Thereby, the catalytic performance was considerably enhanced. The immobilization technique itself is fairly easy to process and obviates sophisticated modification of enzymes and carriers. It also allows reversible immobilization since the immobilized enzymes can be released into the aqueous phase when the NP-stabilized emulsion droplets, liquid or jellified, are separated from the organic media and re-dispersed in an aqueous solution. This is definitely beneficial to the spectroscopic study of the catalytic performance of enzymes at a molecular level and,

moreover, provides the prerequisite for an economic recycling of immobilized materials in technical use. Here, agarose was used to achieve a reasonable mechanical robustness of NP-stabilized enzyme droplets. In principle, however, a whole lot of alternative techniques described in literature could be applied for the same purpose. For example, click chemistry[181] could be used for locking the NPs stabilizing the enzyme droplets, which might minimize the impact on the catalytic performance of the immobilized enzymes. Furthermore, it is anticipated that tailoring the nature of NPs and their surface properties will provide additional functions to the enzymes immobilized in NP-stabilized emulsion droplets and thus might widen the spectrum of possible applications.

6.4. Research Collaboration

This study targets to improve interface of emulsified immobilisates for biocatalysis in organic solvent media. Enzymes immobilized in conventional microemulsions (or reverse micelle) often suffer from an unfavorable interface formed by surfactants, which not only denature enzymes but are hardly separated from the system. For this, Pickering emulsions are designed to encapsulate enzymes in small aqueous droplets stabilized by nano/micron-sized particles in organic solvents. This novel immobilization combines the advantages of large interfacial area, easy separation, and less inactivation to enzymes. This was a cooperation project between the groups of Professor Ansorge-Schumacher (TU Berlin) and Professor Möhwald (MPI Potsdam). I conducted all experiments except CLSM characterization of Pickering emulsions and CD spectra which were carried out by Shuo Bai (MPI Potsdam).

7. General Conclusion and Outlook

7.1. General Conclusion

The present study has systematically explored to improve the interface of immobilisates for biocatalysis in organic solvent media. Three typical immobilisates (enzymes immobilized in hydrophilic and hydrophobic carriers and in emulsions) were selected as research objects. For each of them, a novel method was presented to improve the interface in organic solvent media for catalysis. Specifically for hydrophilic immobilisates, a solvent exchange process was designed to wet hydrophilic surface of immobilisates, and to successfully transfer them into hydrophobic organic solvents. For hydrophobic immobilisates, highly applicable BASE were selected to extend the interfacial area of aqueous phase for esterification in heptane. For emulsion system, Pickering emulsions were created to encapsulate enzymes to obtain a large interfacial area for catalysis and to avoid adverse denaturation to enzymes by surfactants which are often used to stabilize emulsions. Due to the representative carriers and widely applicable methods used in this thesis study, it has contributed to present detailed experiments and general methodology for improving the interface of immobilisates for catalysis in organic media.

Solvent exchange approach was successfully demonstrated as a versatile method to wet the surface of hydrophilic carriers and to load enzymes into carriers for catalysis. Compared with other methods for phase transfer, solvent exchange avoided chemical modification of the surface of hydrophilic carriers and was the easy-to-use process to quickly wet and transfer enzymes, nanoparticles, and hydrophilic hydrogels into organic meida. Furthermore, enzymes could be concentrated in the core of hydrogels by solvent exchange, which increased enzyme stability by minimizing their contact with solvent meida. Therefore, this presented method showed very good practicibilty to immobilize enzymes in hydrophilic gels for reactions in hydrophobic organic meida. Improving the interface of hydrophobic BASE was realized by optimizing BASE composition in term of aqueous phase in solid silicone. Systematic study of stucture and morphology further proved the larger interface under optimized BASE composition. The optimization process relied on improving BASE catalytic reproducibility by producing BASE particles under an improved mixing system for suspension polymerization. All involved techniques during BASE optimization directed towards their successful preparation and applications in non-aqueous media. Specifically for lipase-containing BASE

for synthesis, the low-water content was required for reaction equilibrium. Improvinig interface for biocatalysis in this case was conducted through pre-evaporating and post-recruiting water under different water activity. Pre-evaporating water removed deterious out-layer water, which slowed down mass transfer, and standardized them in very low water content ready for optimization. Post-recruiting water for the dried BASE was controlled under different water activity, which finaly increased BASE activity and reproducibility. Thefore, the used method provided a general methodology to optimize hydrophobic immobilisates with low-water system. Pickering emulsions were used for the first time to immobilize enzymes for biocatalytic purpose. Similar to micro-emulsions, Pickering emulsions had large interfacial area of aqueous phase in organic meida, which greatly facilitated mass transfer when encapsulating enzymes for catalysis. But distinct from micro-emulsions, enzymes immobilized in Pickering emulsions were easily separated from reaction system and had better stability. The considerably increased activity of CalB and BAL in Pickering emulsions showed the high potential of their use as a novel immobilization technology for biocatlysis in organic media. In conclusion, this thesis has dedicated to improve the interface in three typical immobilisates for better catalytic performance in organic synthesis.

7.2. Outlook

The present research has successfully provided three different strategies to improve the interface of immobilisates from hydrophilic and hydrophobic carriers, and from Pickering emulsions, respectively. However, due to the complex nature of the research and the time limit of a PhD work, further work is encouraged to extend the current research more appeal for industrial applications.

Solvent exchange processes proved to effectively improve the solubility of hydrophilic immobilisates in non-polar solvents like heptane. This method was particularly successful to enhance catalytic performance of such stable enzymes as CalB. But some delicate enzymes like BAL was not suitable for this method because water miscible solvents used in this method were prone to denature them. Thus later on, combined immobilization techniques may be required to improve stability of these delicate enzymes. For example, stability of enzymes can be enhanced by chemical modification of enzymes prior to solvent exchange. In addition, hydrophobization of hydrophilic gels through surface modification before or after enzyme

immobilization can directly improve their solubility in non-polar solvents to avoid enzyme inactivation by water miscible solvents used in solvent exchange method.

Optimization of BASE to extend their interfacial area for catalysis is a really good method to increase BASE activity. The succeeding work should investigate more other hydrophobic immobilisates to improve their interface, especially study these which are currently applied in industry. Improving their interface should focus on increasing carrier porosity and decreasing particle size, both of which extend contact area of biocatalysts in organic media. Optimizing water content in immobilisates and solvents in reaction media is a wise idea to improve the interface of hydrophobic immobilisates for biocatalysis. The provided method to supply low-water system to BASE is a good way to remove deleterious outer water layer and thus to improve their interface for lipase esterification reactions. However, for reactions by other enzymes, substantive bulk water content may be important to activate enzymes, thus entrapping other enzymes in BASE should be optimized again in terms of water content. Selecting suitable solvents for BASE esterification reaction is essential work to enhance their catalytic performance. Therefore it will be interesting to investigate solvents to draw a general conclusion for selection of solvents in such BASE catalyzed reaction system.

Increasing the interfacial area of immiscible liquids dissolved with biocatalysts is successfully demonstrated by encapsulating enzymes in Pickering emulsions. Increased activity on both stable CalB and unstable BAL indicates the general practicability of this method. The next application of this method should encapsulate other enzymes with high market value, such as enniatin synthetase. Additionally, it will be more interesting to optimize the Pickering emulsion system for biocatalytic purpose in terms of different nanoparticles, ratio of water phase to solvent phase, enzyme concentration, etc.

Overall, the present research provides various novel strategies to improve the interface of immobilisates in hydrophilic and hydrophobic carriers, and in emulsions for biocatalysis in organic media. For hydrophilic immobilisates, a solvent exchange process was undertaken to improve their solubility with enhanced reusability and stability achieved for CalB. For hydrophobic immobilisates, optimizing composition of BASE was implemented to extend their interfacial area with about twofold activity increased. Controlling water content in BASE further increased their catalytic performance for applications. Pickering emulsions were successfully designed to encapsulate two distinct enzymes with remarkable activity increase,

which paves the way for their broad applications in enzyme immobilization. In the future, it will be very interesting to further advance and optimize the present work orientated for real industrial applications.

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9. Publication List

Publication List of My PhD Work

(1) Bai, S.*; **Wu, C.***; Gawlitza, K.; von Klitzing, R.; Ansorge-Schumacher, M. B.; Wang, D., Using hydrogel microparticles to transfer hydrophilic nanoparticles and enzymes to organic media via stepwise solvent exchange. *Langmuir* 2010, 26, (15), 12980-12987. (* equal contribution)

(2) **Wu, C.**; Kraume, M.; Ansorge-Schumacher, M. B., Optimized biocatalytic active static emulsions for organic synthesis in non-aqueous media. *ChemCatChem* 2011, 3, (8), 1314-1319.

(3) **Wu, C.***; Bai, S.*; Ansorge-Schumacher, M. B.; Wang, D., Nanoparticle Cages for Enzyme Catalysis in Organic Media. *Advanced Materials*, in press. (* equal contribution)

(4) **Wu, C.**; Hanske, J.; Ansorge-Schumacher, M. B., Optimizing lipase-containing biocatalytic active static emulsions by engineering water content and solvent phase. *Journal of Molecular Catalysis B: Enzymatic*, under preparation.

(5) Ansorge-Schumacher, M. B.; **Wu, C.**; Schlienz, D.; Janus biocatalysts for organic synthesis. *Patent*, pending.

(6) Gawlitza, K.; **Wu, C.**; Georgieva, R.; Wang, D.; Ansorge-Schumacher, M. B.; von Klitzing, R., Enhanced activity of CalB in organic solvents by immobilization within micronsized p-NIPAM hydrogel particles. *Biomacromolecules*, submitted.

Publication List of My Other Work

(1) Li, P.; Li, J.; **Wu, C.**; Wu, Q.; Li, J., Synergistic antibacterial effects of β-lactam antibiotic combined with silver nanoparticles. *Nanotechnology* 2005, 16, (9), 1912-1917.

(2) Wu, Q.; **Wu, C.**; Liu, H.; Ding, Y., Preparation of uniformly dispersed $PbCrO_4$ nano-luminesence-ellipsoidal spheres via microemulsion approach. *Journal of Tongji University (Nature Science)* 2005, 33, (3), 342-345.

(3) Wu, Q.; Ma, J.; **Wu, C.**; Sun, D. Amoxicillin antibacterial agent containing nanometer silver and its preparation and use. *CN Patent* 1850113, 2006.

(4) Schwiedernoch, R.; Wang, Y.; Xie, G.; Zhao, X.; Wang, G.; **Wu, C.**; Diao, Q.; Zhou, S.; Xie, J. Process and system of preparing ultra pure ionic liquids. *WO Patent* 2008043309, 2008.

(5) Li, P.; **Wu, C.**; Wu, Q.; Li, J.; Li, H., Biosynthesis of different morphologies of $CaCO_3$ nanomaterials assisted by thermophilic strains HEN-Qn1. *Journal of Nanoparticle Research* 2009, 11, (4), 903-908.

10. Curriculum Vitae

Name	**Changzhu Wu**
Date of Birth	August 6, 1981
Nationality	Chinese
Marital Status	Married, one child

Education

07/2008 – 09/2011
- **PhD** – Department of Enzyme Technology, Institute of Chemistry, Technische Universität Berlin (Berlin, Germany)
- Thesis: *Developing Immobilized Biocatalysts with Advanced Interface for Applications in Organic Synthesis*
- Supervisor: Professor Dr. Marion B. Ansorge-Schumacher
- Grade: Very Good (*Magna Cum Laude*)

09/2002 – 08/2005
- **MSc** – Department of Chemistry, Tongji university (Shanghai, China)
- Supervisor: Professor Dr. Qingsheng Wu
- Grade: Excellent Graduate

09/1998 – 07/2002
- **BEng** – Department of Food Science & Technology, Anhui Agricultural University (Hefei, China)
- Grade: Distinguished Graduate

Industrial Experience

05/2005 – 04/2008
- **Associate Scientist –** Accelergy Corporation (Shanghai, China)
- Develop Industrial Heterogeneous Catalysts
- Create Platform for High Throughput Experimentation
- Synthesize Ultra-pure Ionic Liquids

Other Experience

06/2010 – Present
- **Supervisor** of Three Students for Internship at TU Berlin

07/2008 – Present
- **Supervisor** of Lab Course for Enzyme Technology at TU Berlin

08/2010 – 05/2011
- **Supervisor** of a Diploma Student at TU Berlin