MULTI-ENZYME BIOCATALYSIS USING NANO-STRUCTURED MATERIALS
FOR BIOPROCESSING APPLICATIONS

A Dissertation
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ABSTRACT

With the increasing awareness of environmental safety and the need for renewable fuels, enzyme-catalyzed reactions provide convenient substitutes for future industries relative to most catalysis used today. To our date, multienzyme systems involving cofactors have not been fully explored. In this work, novel cofactor-dependent multienzyme biocatalysts were developed for bioprocessing applications. Areas investigated ranging from proof-of-concept to specific applications were: 1) The separation of enzymes from bacteria: to mimic microbes, multienzyme extracts from trichloroethylene degrading bacteria were separated and in vitro degradation was achieved. 2) Immobilization of a multi-enzyme system in nanoporous glass: successful enzyme-cofactor interaction was possible on the same surface if concave structures were used while smaller pores and longer spacers provided remarkable enhancement. 3) Attachment to nanoparticles: efficient cofactor regeneration and reuse for the novel sequential enzyme-catalyzed synthesis of methanol and L-lactate from CO\textsubscript{2}. Theoretical and experimental approaches to optimize the synthesis involved studying the flexibility of the polymeric spacer-arm over a range of solubility conditions. In summary, a green multi-enzyme system able to catalyze complex redox reactions was demonstrated while using a cofactor regeneration mechanism rendering it cost-effective. Such system suggests a wealth of potential in catalysis, remediation, and sensing applications.
DEDICATION

To my love Loubna,

You are the rock that keeps me grounded.
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CHAPTER I
INTRODUCTION

Over the past two decades, enormous advances have been achieved in the fields of protein and genetic engineering, inspiring a rapid growth in the field of industrial biocatalysis for “green chemistry” applications [1-5]. Biocatalysis has long been seen as an area with great promise for chemical synthesis, but industrial applications have been modest due to certain setbacks [2].

One of the setbacks for the industrialization of enzymes would be the competition with the microbial processes. Typically, a single enzyme catalyzes a reaction from a substrate to a product. Thus, it is limiting to find many one-step reactions to produce higher value products using one enzyme. To mimic the microbial pathways, multienzyme systems have to be considered. Multienzyme systems have successfully been used for various applications when single enzyme catalysis was not effective [6, 7].

Another setback is cofactor regeneration. Whole cell catalyses are often used for synthetic reactions which require that cofactors must be regenerated. Because, although cofactor regeneration in vitro is possible, it is generally easier and less expensive to regenerate them in metabolically active cells. If an economically feasible regeneration is devised however, it would revolutionize biocatalysis and supersede this belief, unlocking a wealth of potential for these biocatalysts for applications that cover catalysis, remediation, and sensing.
1.1 Background

To our date, most industrially used enzymes are cofactor-independent enzymes (e.g. hydrolases), which involve relatively straight-forward catalyses. Alternatively, cofactor-dependent enzymes (e.g. oxidoreductases) catalyze more complex reactions for numerous synthetically useful commodity and specialty chemicals. Cofactors are small molecules (e.g. nicotinamide adenine dinucleotide – NAD(H), MW ~ 665 Dal) which are essential for enzymatic reactions. Cofactors like pyridoxal phosphate and biotin are covalently bound to the enzymes and are therefore self-regenerating. Others, such as pyridine nucleotides (e.g. NAD(H)), are co-substrates that are consumed in stoichiometric amounts [8, 9]. Most cofactors are too expensive to be used stoichiometrically, hence, must be regenerated. For industrial application purposes in situ regeneration would be most advantageous. Regenerating the cofactor in situ can consequently reduce the cost of synthesis by increasing the yield [4, 9], reducing unwanted byproducts, and preventing the accumulation of cofactor products which inhibit the preferred route of reaction [5].

Solving the abovementioned setbacks therefore, allows the construction of a novel biocatalyst that is able to mimic microbial pathways. To increase their stability and reusability, enzymes can be immobilized on certain carrier materials. Pioneers in the field successfully immobilized enzymes over four decades ago [10-11]. Immobilizing the enzymes, confines them so that they can be reused continuously. For industrial applications, immobilized enzymes are vastly used, due to the easiness of separation from the reaction medium. Attaching or entrapping enzymes on the carrier materials increases the integrity of the enzyme, thus helps resisting denaturation [11].
Due to the diverse nature of the research in study, various areas of chemical and biochemical engineering are covered. The work comprises fundamentals in the areas of enzyme separation, enzyme and cofactor immobilization, nano-materials preparation and their use to enhance the catalytic activities of cofactor-dependent multienzyme systems. Two types of nano-structured support materials are used, namely, polymeric nanoparticles and nanoporous silica glass.

Nano-structured materials are generally considered materials with at least one its dimensions less than 100 nm. This small scale dimension suggests that those materials will perform differently than when they are in bulk, at the same time differently than molecular level. Nano-structured materials also have large specific area which makes them ideal candidates for enzyme attachment since they provide higher surface density of the biomolecules.

1.2 Objectives

In this Ph.D. dissertation research, five major topics are covered to obtain a supporting understanding of the biocatalytic behavior of multienzyme systems. The approach towards attaining this understanding was achieved by:

(1) studying the feasibility of multi-enzyme cell-free biocatalysis. The model system was the biodegradation of trichloroethylene.

(2) investigating the effect of confining more than one enzyme and cofactor in the cavities of nanoporous materials for increased activity.

(3) developing a process for the immobilization of enzymes and cofactor on nanoparticles for the sequential synthesis of methanol from carbon dioxide.
(4) exploring enzymatic multistep synthesis of L-lactic acid from carbon dioxide and ethanol using both free and nanoparticle-attached systems.

(5) examining the effect of the flexibility of spacer and the spacer length on the transesterification activity of \( \alpha \)-chymotrypsin in organic media.

1.3 Approaches

Different approaches were followed to explore the potential of using multi-enzyme systems for bioprocessing applications. These approaches ranged from the separation of these enzymes from bacteria and extended to immobilizing such multienzyme systems in nanomaterials.

1.3.1 Enzymatic Degradation of TCE Using Enzyme Extracts Isolated From a Bacterial Consortium

Degradation of trichloroethylene (TCE) using crude enzyme extracts from a bacterial consortium is examined for contaminated water treatment. The effects of factors including pH, chemical induction and cofactor to optimize the activity are studied. Initial and overall degradation rates observed by varying those factors were used to identify key factors for the degradation reactions.

The feasibility of enzymatic degradation, enzyme concentrations recovered, and comparison with bacterial degradation are used to draw conclusions of enzymes involved in the degradation and the limitations behind in vitro degradation due to possible enzyme inhibition. The specific objectives of the project are first to achieve successful separation of the intracellular enzymes by rupturing the cells using egg white lysozyme, then to run intracellular and extracellular enzyme degradation assays to comment on the nature of the
TCE degrading enzymes. Then check the effect of pH and cofactor concentration on the degradation rate, to observe if any redox enzyme has key a role in the degradation pathway. Last, to draw conclusions about the advantages/limitations of the enzyme extracts compared to literature viable cells degradation.

1.3.2 Enabling Multienzyme Biocatalysis Using Nanoporous Materials

Multistep reactions catalyzed by a covalently immobilized enzyme-cofactor-enzyme system are studied. Lactate dehydrogenase (LDH), glucose dehydrogenase (GDH), and cofactor NADH are incorporated into porous silica glass supports. The nanoporous structure of the glass supports is suggested to enhance the molecular interactions among the immobilized enzymes and cofactor, thus improves the catalytic efficiency of the system.

Figure 1.1 Configuration of nanostructured enzyme-cofactor-enzyme catalyst in nanoporous silica glass. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [12].

The specific objectives of the study were to investigate the shuttling of the covalently bound NADH between LDH and GDH and the regeneration cycles of
NADH/NAD+. The critical parameters were the effect of different glass pore diameter and polymeric spacers’ length. Those studies were used to ascertain the hypothesis that concave structures enable multistep biocatalysis, even those involving cofactors. Investigating pore wetting, diffusion limitation and geometric arrangement were used to draw conclusion on the advantages of the given cofactor/enzyme system.

1.3.3 Particle-Attached NADH for Enzymatic Reduction of CO₂ to Methanol

Enzymes including formate, formaldehyde, alcohol and glutamate dehydrogenases were coimmobilized onto polystyrene particles. Cofactor NADH is immobilized separately on similar particles (Figure 1.2). CO₂ is provided by bubbling in an aqueous solution containing both enzyme- and cofactor-carrying particles, while glutamate dehydrogenase bearing particles regenerate the cofactors (Figure 1.3).

![Chemical route of enzymatic synthesis of methanol from carbon dioxide with NADH regeneration.](image)

Figure 1.2 Chemical route of enzymatic synthesis of methanol from carbon dioxide with NADH regeneration. (Abbreviations of enzymes: FDH -- formate dehydrogenase; FaldDH -- formaldehyde dehydrogenase; ADH -- alcohol dehydrogenase; GDH -- glutamate dehydrogenase.) Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [13].
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In order to investigate the efficiency of the system, nanoparticles were prepared using emulsion polymerization. Enzymes and cofactors were separately attached on the surface of polystyrene nanoparticles of 500 nm diameter. Yield comparison between the free and immobilized systems was studies, while factors such as regeneration and reusability of the system were explored as well.

1.3.4  Multistep Enzymatic Synthesis of L-Lactic Acid from CO\textsubscript{2} and Ethanol

Three step synthesis of the L-lactic acid was achieved using alcohol dehydrogenase, pyruvate decarboxylase, and lactate dehydrogenase (Figure 1.4). The reaction was a one-pot synthesis starting with ethanol, CO\textsubscript{2} and NAD\textsuperscript{+}. CO\textsubscript{2} in this reaction was supplied by using 250 mM carbonate-bicarbonate buffer with pH 9.5.
The chemical route of the production of L-lactate from carbon dioxide and ethanol.

The key advantage of this process was that it maintains its own cofactor regeneration cycle (Figure 1.4). Meaning, the reaction can regenerate NAD(H) without introducing a “foreign” reaction, thus less contamination due to enzyme, substrate, or product from this reaction. In addition the optimum conditions for the regeneration system would be very close to that of the main reaction system since most enzymes have optimum kinetics at physiological conditions. A kinetic model was devised for the system and was compared to the experimentally obtained data. Similarly, to the section 1.3.3, both enzymes and cofactor were attached on the surface of nanoparticles to explore the effect of nanoparticle size on the reaction. Three different particle materials were used; namely, polystyrene, gold and silica, while each of those materials required a special approach for activating prior to enzyme attachment. Lastly, to further optimize the system, polymeric spacers were introduced to study their effect by providing extra mobility to the system.
1.3.5 Effect of Spacer Flexibility on the Catalysis of Particles-Attached Enzymes

To minimize the effect of reduced mobility and accessibility due to enzyme immobilization, a spacer can be used to link the enzyme to the support. The linkage allows certain flexibility to the enzyme, hence improve its activity (Figure 1.5).

![Diagram](image)

**Figure 1.5** Schematic presentation of the effect of spacer on the mobility of the enzyme on the surface of the solid carrier.

The flexibility of the spacer is dependent on the solvent, so various solvents in the solubility parameter ($\delta$) range of $14.9 - 29.9 \text{ MPa}^{1/2}$ were selected. Since the reaction was designed to be used in anhydrous solvents previously used enzyme system would fail to successfully catalyze the reaction due to substrate solubility problems. Therefore, the model enzyme selected for this study was $\alpha$-chymotrypsin ($\alpha$-CT) that can perform transesterification reactions in organic solvents with relative ease. In this study $\alpha$-CT was bound to nanoparticles of 250 nm in diameter mediated by polyethylene glycol (PEG). Two molecular weights of PEG were selected for this study 550 and 10,000 Dal. The two
different sizes were purposed to extend the investigation to cover the effect of the spacer length.
CHAPTER II

ENZYMATIC DEGRADATION OF TRICHLOROETHYLENE USING ENZYME EXTRACTS ISOLATED FROM A BACTERIAL CONSORTIUM

Trichloroethylene (TCE) is one of the most widely distributed industrial contaminants of water resources. Compared to other technologies such as catalytic oxidation and incineration, biological treatment of TCE is attractive owing to its low energy consumption and environmentally benign nature [14]. However, the success rate of bioremediation at full-scale waste minimization applications has been limited [15].

2.1 Overview

Several factors can be attributed: First, the biotic systems that have been used predominantly for the treatment of municipal wastewater are dramatically different from those that would be effective for chlorinated solvents. As such, it is not surprising that several researchers have shown that chlorinated solvents pass through conventional wastewater treatment plants unaltered [16, 17]. Second, most of the studied processes applied a single bacterium; however, most chlorinated solvents cannot be completely mineralized by a single microbe, and one bacterium can rarely effectively treat multiple contaminants. Third, the use of whole-cell degradation at an industrial scale can be complicated by limitations associated with continuous nutrient amendments, temperature control, pH, cell washout, dilution factors, and toxicity posed by the solvents themselves.
Recent advances in enzymatic biocatalysis have demonstrated the feasibility of using enzymes to conduct biotransformations in harsh environments including high organic solvent concentrations [2, 18, 19]. In many cases, isolated enzymes can provide faster reactions than the microbial processes. In addition, environmental concerns about using genetically engineered cells also make enzymatic processes more attractive. Accordingly, there has been a growing research effort in exploring enzymatic biodegradation as a promising alternative to the microbial approach for large-scale and cost-effective treatment of organic pollutants.

Traditional enzymatic biodegradation studies, however, have focused on the use of individual enzymes. This usually leads to simple one-step conversions, such as dechlorination, generating intermediates that may be quite toxic [18, 20, 21]. Studies have documented that microorganisms usually achieve complete mineralization through interactions of a network of enzymes and cofactors. For instance, depending on the microbial strain used, TCE degradation can occur via four enzymatic steps involving catechol-2,3-dioxygenase, toluene dioxygenase, and cis-dihydriodiol dehydrogenase. Although such mechanisms have been proposed for the living microorganisms, the complete degradation of organic pollutants by using isolated enzyme systems has not been demonstrated. The present study in the chapter evaluates the efficiency of using enzyme systems isolated from biological sources to carry out biodegradation of the chlorinated hydrocarbon TCE. Factors such as the effect of pH and effect of cofactor NADH/NAD+ were explored to probe the enzymatic degradation hypothesis and to assess the contribution of key oxygenases reported in the previous reports [18].
2.2 Materials and Method

All materials used in this study were purchase with the denoted purity grade and no further purifications were performed. All buffers were freshly prepared and used immediately or stored at 4 °C for no longer than one week.

2.2.1 Materials

Bovine serum albumin (BSA), lysozyme (EC 3.2.1.17) from chicken egg white, reduced nicotinamide-adenine dinucleotide (β-NADH), and HCl were purchased from Sigma (St. Louis, MO). Sodium phosphate, hexane, and TCE were purchased in reagent-grade from Fisher Scientific (Fair Lawn, NJ). Protein assay reagent was obtained from Bio-Rad (Hercules, CA). Other chemicals used for making buffers and nutrient solutions were purchased in biotechnical grade from Fisher Scientific.

2.2.2 Growth of Bacterial Consortium

The consortium comprised bacteria initially isolated from soils contaminated with chlorinated solvents and petroleum products were grown in Dr. Teresa Cutright’s Environmental Engineering Laboratory. The bacterial consortium growth procedure follows the protocols detailed in Meza et al [22].

2.2.3 Isolation of Enzyme

Extracellular enzymes were isolated from the microbial growth medium by removing the living cells via filtration. The bacterial solution (800 ml each batch) was first filtered with 11-µm filter paper from Whatman (Florham Park, NJ) to remove visible
suspensions, followed by filtration with polystyrene low protein-binding membrane filter (0.22 µm) from Corning (Acton, MA). The enzyme concentration of the filtrate solution was measured using the Bradford protein assay. The enzyme solutions were then concentrated by evaporating water to a final volume of 200 ml using a rotoevaporator (Yamoto RE500) at 35°C. Intracellular enzymes were obtained by adding lysozyme to the medium before enzyme isolation, followed by the same filtration procedure. Typically, 10 mg of lysozyme was used for each 800-ml bacterial solution, and the solution was stirred at room temperature for 3 h to break the cell walls.

2.2.4 Protein Assay

The total protein concentration was analyzed using the Bradford-Coomassie protein assay [23]. A 2-ml 1:10 (reagent:water) solution was prepared and transferred to a quartz cuvet. Then 50 µl of enzyme solution was added to the cuvet followed by gentle mixing. The absorbance was measured at 595 nm via a spectrophotometer (Shimadzu UV-1601 UV-visible). A calibration curve (using BSA as the standard) was then used to quantify the amount of enzymes in the solution.

2.2.5 TCE Analysis

A gas chromatograph (Shimadzu GC-17A) equipped with a flame ionization detector and a Restek RTX-5 column was used. The analytical method was similar to that reported previously [24]. The initial column temperature was kept at 40°C for 18 min, then ramped at 40°C/min to 120°C. The injector and detector temperatures were both 250°C. Nitrogen was the carrier gas at an inlet pressure of 18 kPa (~0.4 ml/min).
2.2.6 Enzymatic Degradation of TCE

Typically, 200 ml of the concentrated enzyme solution was transferred to a 250-ml Erlenmeyer flask. Degradation was initiated by adding TCE (85.5–171 µl) to the flask. The solution was then distributed into 9-ml aliquots (using 10-ml vials capped with a Teflon lid) and placed in an orbital environ-shaker operated at 25 ± 3°C and 125 rpm. Three aliquots were removed for gas chromatography (GC) analysis every 24 h by extracting the remaining TCE in the reaction solution with hexane (with a phase ratio of hexane:water of 60:40). Control reactions were conducted and analyzed in the same way except that pure buffer solution (50 mM phosphate buffer, pH 6.8) was used instead of enzyme solution. Reactions with additional NADH were also conducted following the same procedure with enzyme solutions with added NADH (5 mg for each 200 ml of enzyme solution). The reaction solutions maintained optically clear during the course of the reactions, indicating no bacterial growth in the reaction solutions.

2.3 Results and Discussions

The isolation of multienzyme systems from living microorganisms to achieve in vitro degradation of organic pollutants was demonstrated. The activities of intracellular and extracellular extracts, in addition to the effect of the growth inducer, cofactor concentration and pH were evaluated.

2.3.1 Activities of Extracellular and Intracellular Enzymes

Toluene monooxygenases (TMOs), which are believed to be the key enzymes in TCE degradation, may exist as either extracellular or intracellular enzymes [25]. To
evaluate the distribution of the enzymes within or outside the living cells in the bacterium consortium, TCE degradation with extracellular extracts and extracts consisting of mixed extra- and intracellular components was conducted. As a result, the initial reaction rate was doubled for extracts containing the intracellular components (Figure 2.1). For experiments with a 700-ppm initial TCE concentration, approx 8% degradation was achieved within 1 d by extracellular extract, whereas the extract of mixed extra- and intracellular components achieved approximately 17% degradation (Figure 2.1). Within 7 d, the mixed extract reached 56% TCE degradation versus 41% observed for the extracellular extract. Control experiments without using enzyme extract showed abiotic losses of TCE in the range of 11–22% within 7 d.

![Figure 2.1 Degradation of TCE using extra- and intracellular enzyme extracts. (◊ Abiotic loss; □ Extracellular extract; ○ Mixed extra- and intracellular extract. Initial TCE concentration was 750 ppm). Reprinted with permission Springer Publishing Company [26].](image-url)
These observations indicated that both the extracellular and the mixed extracts contained enzymes that were effective for TCE degradation. The difference in degradation rates may simply reflect the effect of enzyme concentration. The total protein concentration increased by approximately two-fold in the extracts when the cells were ruptured using lysozyme (excluding the amount of lysozyme) before the isolation of enzymes (protein concentration changed from 0.011 to 0.026 mg/ml). This is in agreement with the increase in the initial reaction rate. For further confirmation, an enzyme extract of 0.015 mg/ml of protein was concentrated to 0.057 mg/ml by evaporating water. The concentrated enzyme solution degraded TCE about 4 fold faster.
than its parent solution (Figure 2.2). Evaluation of the degradation achieved within the first day reflected an initial degradation rate of $3.03 \pm 0.03$ mg of TCE/(mg of protein·d).

### 2.3.2 Effect of Chemical Induction on Growing Bacterial Consortium

One important method to improve the production of effective enzyme components is chemical induction. For whole-cell bioremediation, the chemical induction may introduce toxicity toward the cells, and that usually leads to decreased efficiency for TCE degradation [27]. Both toluene and TCE were used to induce the production of degrading enzymes e.g. TMOs, which were assumed to be the key catalysts for TCE degradation. The results showed that although both chemicals improved the protein concentration in the crude extracts, toluene led to a lower protein concentration (0.031 mg/ml) compared with that achieved with TCE (0.068 mg/ml). TCE degradation rates of 3.24 and 3.40 mg of TCE/(mg of protein·d) were observed for the toluene-induced and the TCE-induced extracts, respectively (Figure 2.3), which are only slightly higher than those achieved without inductions. This observation indicated that although overall protein production was improved by the inductions, the concentration of effective enzyme components (catechol-2,3-dioxygenase, toluene dioxygenase, *cis*-dihydrodiol dehydrogenase, etc.) was not effectively elevated specifically (relative to other protein components) by the inductions. While the fact that enzyme extracts from toluene induced cells performed poorer than their TCE-induced counterpart, suggest that TMO was not necessarily the main degradation enzyme, or at least not for the initial steps. It is however debatable whether the conitions *in vivo* were replicated *in vitro* to draw parallel conclusions.
2.3.3 Effect of pH

The effect of pH on the degradation rate of TCE by the enzyme extracts was experimentally evaluated. The value of pH was varied from 5.5 to 8.5 using sulfuric acid or sodium hydroxide, while the concentrations of enzyme and substrate were kept constant. Table 2.1 tabulated the initial degradation rate (based on the degradation of the first 24 h) at different pH values. As expected, the isolated enzymes showed optimal pH range around 7.0 while more acidic pH seemed to reduce initial degradation rates. Although this pH optimum is the same as what was reported elsewhere for the TMO
enzymes [28], which supports the proposition of enzymatic degradation but without providing further clues to which enzyme is most influential.

Table 2.1 Enzymatic degradation of TCE at different pH values. (Initial TCE concentration was 500 ppm, protein concentration 0.025 mg/ml, and NADH concentration 0.02 mg/ml). Reprinted with permission Springer Publishing Company [26].

<table>
<thead>
<tr>
<th>pH</th>
<th>Degradation % (1 Day)</th>
<th>Degradation % (7 Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>11</td>
<td>39</td>
</tr>
<tr>
<td>6.5</td>
<td>19</td>
<td>45</td>
</tr>
<tr>
<td>7.5</td>
<td>21</td>
<td>40</td>
</tr>
<tr>
<td>8.5</td>
<td>17</td>
<td>30</td>
</tr>
</tbody>
</table>

2.3.4 Effect of the Cofactor

It has been proposed that the first step in the biodegradation pathway of TCE was the enzymatic oxidation of TCE [29]. As mentioned earlier, oxygenases are believed to be the key catalysts enabling the TCE oxidation. Due to their dependence on NADH, the concentration of the cofactor (NADH) may provide an alternative approach to optimizing the enzymatic TCE degradation. TCE degradation with enzyme extract was conducted with the addition of NADH (0.02 mg/ml). The addition of NADH increased the initial reaction rate significantly (Figure 2.4) as compared to the enzymatic degradation without the addition of NADH. Evaluation of the reaction rates revealed the initial rate of TCE degradation was increased to $5.30 \pm 0.05$ mg-TCE/mg-protein.day. The enhancement in degradation rate implied that NADH was a rate-limiting factor in the biodegradation pathway. However, the final degradation over the 7-days course was only slightly
improved (64% with NADH vs 54.4% without NADH). The slower degradation rate in the later stage again suggested the possibility of product inhibition which requires further in-depth investigation.

![Graph showing TCE concentration over time with different conditions](image)

**Figure 2.4** Effect of NADH on the enzymatic degradation of TCE. (Protein concentration was 0.068 mg/ml. ◊ Abiotic losses; □ Without addition of NADH, ○ with 0.02 mg/ml NADH. Initial TCE concentration: 750 ppm.) Reprinted with permission Springer Publishing Company [26].

### 2.4 Conclusion

TCE degradation using crude enzyme extracts isolated from a consortium of aerobic bacteria was demonstrated. Factors such as pH and cofactor have shown noticeable impacts on the enzymatic degradation rates. It is expected that combining with recent advances in cofactor regeneration and enzyme stabilization/activation technologies, it is possible to develop efficient enzymatic degradation technologies for
different organic pollutants using enzyme extracts. Compared to the bacterial approach, enzymatic degradation is promising in that it requires simple operations, can apply concentrated enzymes, can function at harsh reaction conditions, and is free of environmental concerns such as those associated with genetically engineered microorganisms.
CHAPTER III

ENABLING MULTIENZYME BIOCATALYSIS USING NANOPOROUS MATERIALS

The exploration of multistep biotransformations, particularly those involving cofactor-dependent enzymes, constitutes an important part of today’s endeavors in “green” chemistry and chiral materials. A major setback in the advent of these biotransformation is the often requirement of a redox step which would ultimately require a proton acceptor such as cofactors.

3.1 Overview

Generally cofactors can be regenerated via chemical, electro-chemical or enzymatic methods [1, 30, 31]. Compared to other approaches, enzymatic regeneration offers mild reactions and high efficiency that are preferred for most bioprocessing applications. To facilitate the reuse of the often considerably expensive cofactors and enzymes, efforts have been made to immobilize regenerative multi-enzyme systems via both physical entrapment and covalent binding [32-35]. Such immobilized systems have been so far found mostly useful in biosensing [32-35], while it remains as a long-standing challenge to develop efficient multienzyme catalysts for large-scale bioprocessing applications. Cofactor-dependent bioprocessing technologies developed so far mostly use free cofactors [32, 36, 37], even though in some cases the cofactors were modified with
polymers such as poly(ethylene glycol) for enlarged molecular size [38]. In this chapter a triad catalytic system that consists of two enzymes and one cofactor was covalently incorporated into nanoporous glass supports, which have been proven especially effective in stabilizing enzymes at extreme temperature and polar organic solvent denaturing conditions [39]. As shown in Figure 1.1, the nanocavities of the glass will integrate the enzymes and cofactor into molecular vicinity, especially when the pore has fit dimensions. This confinement is expected to enable effective translocation of the cofactor between the active sites of the two coimmobilized enzymes.

3.2 Materials and Method

All materials used in this study were purchase with the denoted purity grade and no further purifications were performed. All buffers were freshly prepared and used immediately or stored at 4 °C for no longer than one week.

3.2.1 Materials

Silica glasses of surface area of 50–100 m²/g were purchased from Silicycle (Quebec, QC, Canada). N-Acryloxsuccinimide (NAS) was obtained from Acros Organics (Morris Plains, NJ). Styrene and sodium hydroxide were obtained from EM Science (Gibbstown, NJ). Epichlorohydrin (ECH), 3-aminopropyltrimethoxysilane (APTMS), and polyvinylpyrrolidone (PVP, Mw 29 kDa) were purchased from Aldrich (Milwaukee, WI). 2,2’-Azobis [2-methyl-N-(2-hydroxyethyl) propionamide] (VA-086) was provided as a gift from Wako Chemicals, Inc (Richmond, VA). 2-Sulfoethyl methacrylate (2-SEM) was purchased from Monomer-Polymer & Dajac Labs, Inc.
Ethanol (HPLC grade), sodium phosphate, glutaraldehyde, poly(ethylene glycol) (PEG), Bovine Serum Albumin (BSA), lactate dehydrogenase (LDH), glucose dehydrogenase (GDH), NADH, NAD$^+$, sodium pyruvate, glucose, glucose oxidase, horseradish peroxidase, o-dianisidine dihydrochloride and divinyl benzene were purchased from Sigma Chemical Co. (St. Louis, MO).

3.2.2 Activation of Glass

Glass particles were activated with APTMS, followed by washing with ethanol then water. Typically 1 g of glass was modified with APTMS (0.05 g/ml) in 8 ml of 80:20 (v/v) ethanol-water solution for 2 h at 40$^\circ$C. The activated glass was separated from the solution by centrifugation, followed by washing with 15 ml of the ethanol-water mixture for at least 3 times. The activated glass was then modified further with spacers of different sizes before the attachment of enzymes and cofactor.

The simplest spacer used was GA, which was attached to the activated glass at room temperature with 0.16 g GA and 1 g of glass in 8 ml of pH 7.0 phosphate buffer. The reaction typically lasted for 4 h. Other spacers were attached through a similar procedure: PEG was attached to the activated glass by first modifying PEG with ECH at both ends of the polymer chains. Typically 1 g of glass was modified with 1 g of ECH-modified PEG dissolved in 8 ml of pH 7.0 phosphate (50 mM) buffer solution for 24 h at room temperature under stirring. For even longer spacers, BSA molecules were attached at the open end of PEG (Mw 10,000) attached to silica glass. BSA was attached in pH 7.0 phosphate (50 mM) buffer solution at a concentration of 30 mg/ml BSA for 12 h under stirring. After extensive washing of the glass till no protein was detected (using the
Bradford method) in the supernatant, the BSA-attached glass was then re-activated using the ECH-modified PEG under the same conditions as the first step of PEG attachment, followed by washing with buffer and deionized water.

![Chemical route of the co-immobilization of enzymes and cofactor.](image)

**Figure 3.1** Chemical route of the co-immobilization of enzymes and cofactor. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [12].

### 3.2.3 Attachment of Enzymes and Cofactor to Glass

The attachment was achieved by contacting the activated glass with aqueous buffer solutions containing enzymes or cofactor (Figure 3.1). Activity tests showed that a
two-step approach ensured enough cofactor loading and gave the best enzyme activity, and that approach was applied in this study: Activated glass was contacted with NADH-containing solution first, and then with a solution containing both LDH and GDH. In detail, one gram of activated glass was mixed with 4 ml of pH 7.0 phosphate buffer containing 30 mg NADH for 48 h at room temperature, followed by washing the glass with fresh buffer. The concentration of NADH was monitored during the attachment and washing processes by UV absorbance at 340 nm, and the amount of immobilized NADH on the glass was calculated based on mass balance. Subsequently, the glass was then contacted with 4 ml of pH 7.0 phosphate buffer containing 0.35 mg of LDH and 0.27 mg of GDH (same molar concentration) for 24 h at 4°C. After extensive washing with fresh buffer, the amount of enzymes attached was calculated based on mass balance as monitored using Bradford protein assay of the immobilization and washing solutions. Depending on the spacers used, the final loadings of cofactor and enzymes were ~ 2 mg NADH and 0.2 mg enzyme per gram of glass.

3.2.4 Activities of Immobilized Enzymes

Reactions of pyruvate reduction and glucose oxidation were conducted at room temperature with shaking at 250 rpm (Figure 3.2). Typically about 250 mg catalyst was applied in 5 ml reaction solution. Initial concentrations of pyruvate and glucose were 1 mM. Aliquots of reaction solutions were taken periodically (0.5 ml under stirring) for analysis after filtration using 0.2 µm PTFE syringe filters (Gelman).
Figure 3.2  Enzyme–catalyzed reactions with in situ cofactor regeneration. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [12].

Pyruvate concentration was determined using a HPLC system equipped with a Waters C-18 column (4.6 x 250 mm). The mobile phase consisted of 50/50 (v/v) of acetonitrile and water with pH 2.1 regulated using sulfuric acid and was applied at a flow rate of 1 ml/min. The pyruvate concentration was detected using the UV-Vis detector at 215 nm. Glucose concentration was measured chemically using the PGO enzymes system (Peroxidase and Glucose oxidase): a 25 µl sample solution was added along with 0.5 ml water to 5 ml of the PGO-colorant solution, which was pre-prepared and contained 5000 U/l glucose oxidase, 1000 U/l horseradish peroxidase and 40 mg/l o-dianisidine dihydrochloride. The solution was mixed well and incubated at 35°C in dark for 30 minutes, followed by absorbance measurements at 450 nm.

3.2.5 Activities of nanoparticle-immobilized enzymes

Poly(styrene) particles of diameter of 500 nm were prepared according to a procedure reported previously [40] and detailed in the following chapter. Cofactor and
enzymes were attached to the particles and were then tested for enzyme activities following the same procedures as described for porous glass.

3.3 Results and Discussion

Commercially available porous spherical glass particles with an average particle diameter of 60 Åm and different pore sizes (30 and 100 nm diameter) were used. The glass was first activated, followed by the covalent attachment of enzymes and cofactor. Control tests with non-active glass showed that physically adsorbed enzymes and cofactor can be stripped out of the glass through this washing procedure and that no activities were detected with the washed glass. In another control test, the immobilized catalyst was incubated in buffer solution (same as that used for the following reaction tests) for 5 days; no active cofactor or enzyme was detected in the supernatant solution, while the washed glass retained its activity.

3.3.1 Successful Regeneration of Cofactors

The cofactor modifications was achieved through the amino group on the adenosine moiety as reported previously in literature [38, 41]. Control tests with non-active glass showed that physically adsorbed enzymes and cofactor can be stripped out of the glass through the washing procedure employed and no activities were detected with the washed glass. The cofactor was immobilized in form of the reduced state, NADH, which was required to enable the reduction of pyruvate into lactate (Figure 3.2). NADH can then be regenerated from NAD$^+$ via the GDH-catalyzed oxidation of glucose. In a test as depicted in Figure 3.3, glucose concentration remained unchanged initially before
the addition of pyruvate; however, the subsequent addition of pyruvate successfully triggered the consumption of glucose. Since the consumption of glucose requires NAD$^+$, the shuttling of cofactor between the two enzymes appeared to be evident. Control tests with activated glass support did not show detectable adsorption of either glucose or pyruvate within three days.

![Graph](image)

**Figure 3.3** Evidence of NADH shuttling between enzymes: Consumption of glucose was triggered by the addition of pyruvate. (□) Pyruvate; (○) Glucose. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [12].

3.3.2 Effect of Spacer Length on Reaction Rate

Conceivably, the thermal vibration of the active components of the catalyst system is critical to the translocation of the cofactor between the two enzymes. The size of the spacer (the chemical linkage to the glass) can be expected to affect the shuttling
frequency and thus impact the overall reaction kinetics. To test this hypothesis, four spacers of different sizes were examined: glutaraldehyde (GA), PEG\textsubscript{550}, PEG\textsubscript{10,000}, and the PEG-BSA-PEG (PEG-attached BSA).

![Figure 3.4 Effect of spacer length on reaction rates. □ Glucose consumption with PEG550 spacer; ◆ Glucose consumption with PEG10,000 spacer; ■ Glucose consumption with PEG-BSA-PEG spacer; ♦ Pyruvate consumption with PEG500 spacer; △ Pyruvate consumption with PEG10,000 spacer; × Pyruvate consumption with the PEG-BSA-PEG spacer. All reactions were carried out with 30 nm pores glass. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [12].](image)

As expected, both the pyruvate and glucose initial reaction rates generally increased as larger spacers were used (Figure 3.4). Particularly, the pyruvate reaction rate for PEG-BSA-PEG spacer was ~18-fold faster than that achieved with GA for glass of 30-nm pores (Table 3.1). Although other factors such as the physicochemical
properties of the spacer may also impact the reaction rates, the evident difference between the results for PEG_{550} and PEG_{10,000} spacers seemed to support the hypothesis that the spacer length is critical in regulating the enzyme and cofactor activities.

3.3.3 Effect of Pore Size on Reaction Rate

Changes in glass pore size also engendered a noticeable impact on the reaction kinetics. Figure 3.5 demonstrates the time courses of reactions catalyzed by the LDH-NADH-GDH system incorporated in glass particles of 30- and 100-nm pores. The initial reaction rates for the consumptions of both pyruvate and glucose for glass of 30-nm pores were apparently faster than that observed for the 100-nm samples (Table 3.1). Experiments with different spacers led to the same observation – higher enzyme activities were achieved with the glass of 30-nm pores. For example, the glucose initial reaction rate was about doubled as the pore size decreased from 100 to 30 nm with PEG-BSA-PEG as the spacer.

The physical dimensions of the system seem to be accountable to this observation (LDH has a dimension of 6 × 8.6 × 13.6 nm with Mw of 36,466 Da, GDH 6.3 × 6.3 × 12.8 nm and 28,117 Da, and BSA 5.5×5.5×12.0 nm and 69,293 Da). It is believed that 30-nm pores provide void spaces that offer a better integration and thus provide more efficient interactions among the enzymes and cofactor; while 100-nm pores may leave open spaces at the center of the pores between enzymes held on opposite sides. In the latter case, the regeneration of the cofactor may be mostly achieved through the interactions of neighboring enzymes on the same side of the wall.
3.3.4 NADH Turnover Number

In addition to the specific reaction rates, the apparent turnover numbers of NADH (specific reaction rate normalized with respect to the amount of cofactor) also reflected the same effects of the sizes of spacers and pores (Table 3.1): while longer spacers displayed faster turnover of NADH, 30-nm pores worked better than the 100-nm pores. The actual shuttling frequency of NADH should be much faster than that reflected by the tabulated turnover numbers though, since excessive amount of NADH was applied (with
an cofactor/enzyme mole ratio of ~400), and only the cofactor molecules placed in the neighborhood of both enzymes have chances to be involved in the reactions.

Table 3.1  Effects of pore size and spacer length on reaction rates. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [12].

<table>
<thead>
<tr>
<th>Spacer</th>
<th>NADH loading</th>
<th>Enzyme loading</th>
<th>Pyruvate reaction rate (turnover #)</th>
<th>Glucose reaction rate (turnover #)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(30) b</td>
<td>(100) b</td>
<td>(30) d</td>
<td>(100) d</td>
</tr>
<tr>
<td>GA</td>
<td>0.83 b</td>
<td>0.79 b</td>
<td>0.11 b</td>
<td>0.11 b</td>
</tr>
<tr>
<td></td>
<td>(1.11)c</td>
<td>(1.06)c</td>
<td>(3.4) d</td>
<td>(3.4) d</td>
</tr>
<tr>
<td></td>
<td>1.88</td>
<td>1.47</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>PEG550</td>
<td>(2.52)</td>
<td>(1.97)</td>
<td>(5.3)</td>
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</tr>
<tr>
<td></td>
<td>1.69</td>
<td>1.83</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td>PEG10k</td>
<td>(2.27)</td>
<td>(2.45)</td>
<td>(4.3)</td>
<td>(5.0)</td>
</tr>
<tr>
<td>PEG-</td>
<td>1.63</td>
<td>2.12</td>
<td>0.18</td>
<td>0.19</td>
</tr>
<tr>
<td>BSA-PEG</td>
<td>(2.18)</td>
<td>(2.84)</td>
<td>(5.6)</td>
<td>(5.9)</td>
</tr>
</tbody>
</table>

a Pore diameter (nm) of glass supports; b Mass loading: mg per gram support; c µmol NADH per gram support; d $10^{-3}$ µmol of enzyme per gram support; e Initial reaction rate: mmol (Pyruvate or Glucose) l$^{-1}$ h$^{-1}$ mg-enzyme$^{-1}$; f NADH turnover number (defined as reaction rate normalized with respect to the amount of cofactor): mmol (Pyruvate or Glucose) l$^{-1}$ h$^{-1}$ mmol-NADH$^{-1}$.

The reaction rate of glucose oxidation was much slower than that of pyruvate reduction. This seemed to be largely owing to the intrinsic nature of the reactions. Tests with free enzymes and cofactor with the same molar ratio in aqueous solutions showed the same observation. Another possible factor is the availability of the immobilized cofactor for the reaction cycles. It can be assumed that only the cofactor molecules located in the neighborhoods of both LDH and GDH have the chance to shuttle between the two enzymes. The cofactor located only close to LDH could not mediate the reaction of glucose. While the glucose reaction showed approximately first-order reaction
kinetics, the reaction of pyruvate slowed down apparently in the later stage of the reaction. More detailed kinetic studies are necessary to examine if this is a result of the complicated concentration-dependence of the reactions, or a result of other factors such as the inactivation or inhibition of enzymes and cofactor.

3.3.5 Diffusion and Transport Properties

Confinement on a nanoscopic scale such as that in nanopores can have a profound effect on the properties of a liquid [42]. For example, phase-transition temperatures of confined liquids can differ dramatically from their bulk values [43] and dynamic processes of liquids may be reduced by up to 10 folds in confined media [44]. This behavior is justified since the surface to volume ratio of a nano-confined liquid is much larger, causing the surface properties of the liquid to be critical. Moreover, the interactions with the walls of the confining medium can play a large role in the behavior of a confined liquid [45].

The surface of the porous glass can contain between two and six hydroxyl groups per square nanometer, depending on the thermal treatment of the silicate during synthesis [46]. These hydroxyl groups are responsible for the strong interactions between the pore surfaces and water, since the oxygen atom in the molecule can accept a hydrogen bond from the surface. This hydrogen bonding is likely to be an influential factor in determining the dynamic properties of confined water. Loughnan et al. [45] observed that this hydrogen bonding inhibits the dynamics at the surface of the pores. This behavior reduced wetting of nanopores 2.4 – 4.4 nm in diameter and induced the reorientation of solvent molecules at the pore surfaces. The same study reported more mobilized
dynamics when the surface hydroxyl protons were substituted with deuterons. Oxygen-deuteron bonds have lower zero-energy than oxygen-hydrogen bonds, consequently weakening these hydrogen bonds. Therefore in this work, in the case of PEG-modified glass, the glass surface was transformed into a neutral and more hydrophobic one. The effect of this modification on wetting was not measured, however was expected to inhibit hydrogen bonding hence promote active transport of water at the pore surface. It is notable in here that the silicate glass used in this dissertation have pores of 30 and 100 nm in diameter, excessively large in comparison to water molecules. In addition, during the initial activation process higher temperatures (40\(^\circ\)C) and ethanol-water mixture were used to decrease the viscosity of water to promote better wetting to all void space in the glass. It is therefore expected that the previously mentioned behavior would be limited to the marginal area of the pore’s surface.

Transport phenomena in porous media involve many singularities. Adsorption on the walls or condensation into a high density phase in the center, are some of those observed trends [47-48]. The transport of molecules into the pore, along the surface and within the pore both play a role in efficient catalysis. To develop a model to calculate the diffusional rate, the model must ideally include intrinsic diffusion coefficients corrected for effects of surface processes, adsorption to walls, and pore size. In the presented work the molecules were assumed not to interact neither with the walls nor with each other. For these conditions the boundary layer is minimal and the thickness is related to geometric constraints determined solely by the pore size.

The multienzyme system in study is different from previously studies systems with the novelty of having both the enzymes and their cofactor immobilized in the porous
support (figure 3.6). Generally, enzyme systems have been studied for diffusion limitation when the enzyme’s catalytic nature was not altered by covalent linkage of cofactor NADH. This would generate an important factor of why such systems can not be compared with free enzyme kinetics to determine the mass transfer effects on the biocatalyst. Therefore diffusion-free kinetic parameters were selected to perform the calculations comparing with the immobilized enzyme-cofactor system.

![Figure 3.6](image)

Figure 3.6  Schematic representation of the porous glass particle (Left) and the pore where enzymes are attached and the reaction takes place.

To tackle this problem, certain assumptions were taken and listed below, accompanied by the practical justification:

1. The reaction obeys the Michaelis rate expression, which was established later in chapter 5 for lactate dehydrogenase).

2. Immobilized cofactor concentration was constant throughout the study due to consistent immobilized enzyme to cofactor ratio and therefore was excluded from the rate expression.
3. The glucose dehydrogenase reaction was considered as the model reaction since it was the rate-limiting reaction in the process.

4. The case of smaller pore (30 nm) was chosen, given that in case of no diffusion limitations for smaller pore, the larger pore (100 nm) should also have no such limitations.

The system was modeled for the diffusion of a substrate to be catalyzed by an enzymatic reaction taking place inside a spherical particle with cylindrical pores of radius $r_0$. In this instance the phenomena of diffusion coupled with a reaction of substrate $S$ inside the pores of the particle can be described by:

$$D \left[ \frac{d^2[S]}{dr^2} + \frac{2}{r} \frac{d[S]}{dr} \right] = E_r V([S])$$  \hspace{1cm} (3.1)

The boundary conditions for this system are obtained from symmetry as:

$$\frac{d[S]}{dr} \bigg|_{r=0} = 0$$  \hspace{1cm} (3.2)

And from the mass balance at the external boundary

$$k([S] - [S])_{r=r_0} = D \frac{d[S]}{dr} \bigg|_{r=r_0}$$  \hspace{1cm} (3.3)
Equations (3.1) through (3.3) have $D$ as the diffusion coefficient of the substrate $S$ in the porous particle (cm$^2$·s$^{-1}$), $E_T$ is the total enzyme concentration (mol·cm$^{-3}$), $V$ is the reaction rate per amount of enzyme (mol·mol-enz.·s$^{-1}$) where $V$ was considered to be only a function of the diffusing substrate, $k$ is the external mass transfer coefficient for the substrate $S$ (cm·s$^{-1}$), $[S]^0$ is the concentration of the substrate in the bulk of the liquid solution where the porous particle is suspended (mol·cm$^{-3}$).

Equation 1 can be made dimensionless using the following substitutions:

$$C = \frac{[S]}{[S]^0}$$

$$x = r/r_0$$

$$\alpha = r_0^2 E_T / D$$

$$\beta = D/(kr_0)$$

$C$, $x$, and $\beta$ are now dimensionless quantities, while $\alpha$ has the dimension of s·cm$^{-3}$. And thus the transformed equation and boundary conditions become as follows:

$$\frac{d^2 C}{dx^2} + \frac{2}{x} \frac{dC}{dx} = \alpha \frac{V([S])}{[S]^0}$$

$$\left(\frac{dC}{dx}\right)_{x=0} = 0$$
\[(1-C)_{x=1} = \beta \frac{dC}{dx} \bigg|_{x=1} \quad (3.6)\]

The overall pseudo-steady state substrate consumption rate, \( R \), expressed per moles per second per cm\(^3\) particle volume is given by

\[
R = \frac{A}{V} \cdot D \frac{d[S]}{dr} \bigg|_{r=r_0} = \frac{3E_T}{\alpha} \frac{dC}{dx} \bigg|_{x=1} 
\quad (3.7)
\]

Where \( A \) is the external surface area of the particles and \( V \) is the volume of the spherical particles. The effectiveness factor \( \eta \) can be obtained by dividing \( R \) by the diffusion-free reaction rate \( E_T V([S]^o) \).

\[
\eta = \frac{(3/\alpha)[S]^o (dC/dx)_{x=1}}{V([S]^o)} 
\quad (3.8)
\]

The Thiele modulus for porous spherical reaction systems that follow the Michaelis rate expression was given by Rovito and Kitrell [49] and by Blais and Lortie [50] as:

\[
\phi = \frac{r}{3} \cdot \frac{V_{\text{max}}}{K_m \cdot D_c} 
\quad (3.9)
\]

And that the relationship between \( \eta \) and \( \phi \) for low substrate concentrations is given by:
Figure 3.7  Schematic representation of the pore. The pore was considered as a long cylinder from the center of the sphere to the surface of the particle. The length of each cylinder is $r_0$ ($5 \, \mu m$) and the diameter is $r'$ (30 nm).

The effective diffusivity of a substrate in porous particles is related to the substrate diffusivity in bulk solution, the particle’s porosity and the geometrical tortuosity by:

$$D_e = \epsilon D_o / \tau$$  \hspace{1cm} (3.11)
The tortuosity, \( \tau \), is a function of the pore size, shape, connectivity, and degree of constrictions. In this work, the tortuosity was considered to be equal to 1 assuming uniform cylindrical pores. When the effective molecular size of the substrate is not negligible with respect to the pore dimensions, the actual effective diffusivity, \( D_e \), will have a value smaller than that calculated using equation 3.11. Smaller effective diffusivity being smaller than \( D_o \) is often the result of steric exclusion effects and augmented hydrodynamic drag on the molecule. The effective diffusivity can be calculated using the following equation [51]:

\[
D_e = \varepsilon D_o K_p(\lambda) K_r(\lambda)
\]  

(3.12)

The equilibrium partition coefficient, \( K_p \), and the hydrodynamic drag coefficient, \( K_r \), are both functions of the ratio the molecule size to pore size, \( \lambda \). Equation 3.13 assumes the pores are uniform nonintersecting cylinders. Spherical molecules in a cylindrical pore with no wall-molecule interactions have \( K_p \) values given by Ferry (1936) [52] as:

\[
K_p = (1 - \lambda)^2
\]  

(3.13)

The effect of viscous drag on the solute transport rate is determined by calculating the local Stokes friction coefficient as a function of position and taking the spatial average. For small \( \lambda \), Brenner et al. [53] gave:
Using equations 13 and 14, $K_P$ and $K_r$ values are calculated for pyruvate and summarized in table 3.1.

Table 3.2  List of the calculated $K_P$ and $K_r$ values for glucose (molecular diameter of 0.9 nm) using equation 3.13 and 3.14.

<table>
<thead>
<tr>
<th>Constants</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_o$</td>
<td>$6.9 \times 10^{-6}$ cm$^2$.s$^{-1}$ [7]</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>0.65</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>0.030</td>
</tr>
<tr>
<td>$K_P$</td>
<td>0.941</td>
</tr>
<tr>
<td>$K_r$</td>
<td>0.888</td>
</tr>
<tr>
<td>$D_e$</td>
<td>$3.8 \times 10^{-6}$ cm$^2$.s$^{-1}$</td>
</tr>
</tbody>
</table>

Therefore, using equation 3.12 the effective diffusivity of glucose, $D_e$, through 30 nm pore is $3.8 \times 10^{-6}$ cm$^2$.s$^{-1}$.

In the case of the free enzyme, the kinetic parameters at reaction conditions of pH 7.0, 23°C temperature, and 50 mM sodium phosphate concentration, were previously evaluated using the Lineweaver-Burk (LB) graphical method. $k_{cat}$ and $K_m$ values were 124 s$^{-1}$ and 0.98 x $10^{-4}$ M, respectively. The particle-immobilized enzyme system’s kinetic constants were evaluated using the same method from data obtained at two different substrate concentrations of 1 and 10 mM. Using initial reaction rate data from these runs an LB plot was produced and immobilized enzyme system’s $V_{max}$ and $K_m$ were calculated at $3.58 \times 10^{-8}$ M.s$^{-1}$ and $2.10 \times 10^{-3}$ M, respectively (figure 3.8).
Figure 3.8  Lineweaver-Burk plot for the calculation of kinetic parameters $K_{\text{cat}}$ and $K_m$. Kinetic parameters were obtained from rate data at different substrate concentrations for the 0.5 µm particles immobilized enzyme system (GDH).

The x-intercept is $-476 \text{ M}^{-1}$ and the slope is $58581 \text{ s}$.

$$K_m = \frac{-1}{-476 \text{ M}^{-1}} = 2.10 \times 10^{-3} \text{ M}$$

and

$$V_{\text{max}} = \frac{2.10 \times 10^{-3} \text{ M}}{58581 \text{ s}} = 3.58 \times 10^{-8} \text{ M} \cdot \text{s}^{-1}$$

Using equation 3.9:

$$\phi = \frac{5 \times 10^{-6} \text{ m}}{3} \sqrt[3]{\frac{3.58 \times 10^{-8} \text{ M} \cdot \text{s}^{-1}}{2.10 \times 10^{-3} \text{ M} \cdot 3.8 \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1}}} = 3.5 \times 10^{-4}$$

the Thiele modulus can be calculated for the nanoporous glass with 30 nm pore diameter.

The obtained Thiele modulus value was approximately $3.5 \times 10^{-4}$ corresponding to an
effectiveness factor of > 0.99. This value suggests according the figure 3.8 of effectiveness factor versus Thiele modulus that the system was reaction rate limited since the diffusion rate was six orders of magnitude faster than the reaction rate. This also suggests that if the reaction rate would be enhanced to increase $10^6$-fold, the system may become mass transfer rate limited.

![Plot of the effectiveness factor versus the Thiele modulus of the reaction system. The red, black, and green portions of the curve denote the reaction-rate limited, intermediate, and mass transfer rate limited behaviors, respectively.](image)

Figure 3.9  Plot of the effectiveness factor versus the Thiele modulus of the reaction system. The red, black, and green portions of the curve denote the reaction-rate limited, intermediate, and mass transfer rate limited behaviors, respectively.

Considering the rate parameters are equivalent to those of a free enzyme reaction with same $E_T$ of $2.73 \times 10^{-7}$ M. In this case, $V_{max}$ and $K_m$ are $1.39 \times 10^{-4}$ M.$s^{-1}$ and $0.98 \times 10^{-4}$ M, respectively. Thiele modulus, $\phi$ value was found to be 0.10 which is still below a critical $\phi$ value of 0.3, corresponding to a $\eta$ value of 0.99. Therefore, the effectiveness factor was still in the red margin confirming a reaction rate limited system.
Diffusions in nanopores however, might be very different from the traditional view points, such as Fickian diffusion, since there is a lack of bulk phase fluid property. Therefore, the Weisz-Prater criteria was used to check for any pore diffusional limitations on the reaction rates.

The Weisz-Prater criterion is usually used to estimate the influence of pore diffusion on reaction rates in heterogeneous catalytic reactions. Therefore, for this system, the Weisz-Prater criterion was calculated to assess if the criterion is satisfied [54].

\[
N_{W-P} = \frac{R \cdot r^2}{[S]^0 \cdot D_e} \leq 3\beta
\]  

(3.15)

Where \( R \) is the reaction rate per volume of catalyst and \( r \) is the radius of the particle.

Table 3.3  List of values of used variables for the Weisz-Prater Criterion.

<table>
<thead>
<tr>
<th>( R ) (( mol.s^{-1}.m^{-3} ))</th>
<th>( R_p ) (( m ))</th>
<th>([S]^0) (( mol.m^{-3} ))</th>
<th>( D_e ) (( m^2.s^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.24</td>
<td>5 x 10^{-6}</td>
<td>1.0</td>
<td>3.8 x 10^{-10}</td>
</tr>
</tbody>
</table>

The \( N_{W-P} \) value for the presented system is 0.082, which when compared to the value of \( \beta \) which is given by the following equation [54]:

\[
\beta = \frac{4(1-\eta)}{n}
\]  

(3.16)
for significant effectiveness of $\eta \geq 0.95$ and considering a virtual first order kinetics
($n=1$) as shown previously with $K_m \gg [S]$, the Weisz-Prater critical value is $3\beta = 0.6$.

Considering the obtained values, $N_{w.p} (0.082) < 3\beta (0.6)$ which reconfirms that
the pore diffusion limitations were negligible, therefore internal diffusion limitations can
be neglected and observed reaction rates can be used for comparison between systems.

All the speculations presented are predictions using currently available theories;
verification of such theories will be subject to future systematic investigations on this
topic.

3.3.6 Effect of Leached Cofactor on reaction rate

The effect of possible leakage of cofactor or enzyme to the reaction solution is
tested by first immobilizing the enzyme system using the outlined method. The
immobilized enzyme-cofactor system after being washed following the previously
described method prior to the application in the activity assay was mixed in buffer and
left mixing at room temperature for 1 week at reaction conditions. After 1 week of
mixing, the filtered solution was assayed for free cofactor activity by adding enzymes
glucose dehydrogenase and lactate dehydrogenase and substrates lactic acid and glucose.

Figure 3.7 shows that no significant activity (approximately 4% in 5 days) was observed
from any leached cofactor that might have came off the porous particles. Therefore, the
previously observed activities are considered to be due to oxidation/reduction taking
place with the immobilized cofactors predominantly.
Figure 3.10  Cofactor leaching test. Immobilized glass was left mixing for 5 days in buffer and LDH assay with pyruvate as substrate were done at pyruvate initial concentration of 1 mM to check if there were any free cofactor.

3.3.7 Concavity Effect: 500 nm Particle as Carrier

To examine the effect of the concave configuration (Figure 3.1), a comparison study with nonporous particles was conducted. Individual enzymes attached to submicron particles have demonstrated high enzyme activities that were close to those of free enzymes [49, 50]; however, no work has been reported in using these particles for multienzyme systems. In this work, the triad catalytic system was co-immobilized onto the outer surface of 500 nm (diameter) polystyrene particles (with PEG_{10,000} as the spacer) prepared according to a procedure reported previously [40] with final loadings as 3.65 mg NADH and 0.38 mg enzyme per gram of particles (molar ratio of cofactor to enzyme: 416, similar to those of nanoporous glass). Reactions were carried out at the
same pyruvate and glucose initial concentrations (1 mM), the same as that applied in experiments with porous glass. Initial reaction rates of 0.021 and 0.038 mmol$^{-1} \cdot l^{-1} \cdot h^{-1} \cdot mg$-enzyme$^{-1}$ were observed for pyruvate and glucose, respectively, indicating much slower reactions as compared to those observed for nanoporous glasses. For example, the pyruvate reaction rate was less than 2% of that achieved for glass of 30-nm pores with the same spacer. Since the non-porous particles do not suffer internal mass transfer limitations, usually much higher reaction rates can be expected than porous materials. [40, 55, 56]. This observation particularly demonstrated that the nanopores afforded efficient integration of the enzymes and cofactor, and thus fostered faster reactions.

Considering the geometry in figure 3.11, the nanoparticle/nanopores -attached enzymes and cofactors were considered as spherical particles of different sizes while the PEG spacer-arm was considered as a coiled string connecting the enzyme/cofactor to the particle’s surface. Based on the shown arrangement in figure 3.11, the enzyme (and cofactor) can reach within their vicinity in a space analogous to that of a cone. The overlap volume between two reaching zones was regarded as the probability of a collision that will lead to a reaction. Hence this overlap can be considered proportional to the reaction rate of the system. The critical interaction in this case was between an enzyme and cofactor, since substrates were assumed to be readily available at the active site of the enzyme at all times due to a much simpler transport dynamic.

Using a simulated folding of a given surface from convex spherical (500 nm in diameter) distribution to a concave cylindrical (30 nm in diameter) structure using AutoCAD 2002 by Autodesk (San Rafael, CA), the overlap increased by roughly 4 folds (The cone height was 15 nm for the enzyme and 5 nm for the cofactor with both having
45° angle rotation from the vertical axis). This increase in reaction probability however was for one given reaction. In the present study to achieve continuous reaction, a full cofactor regeneration cycle has to be completed so that the same cofactor will have to be accessible to the other half-reaction enzyme. Therefore, the probability of achieving both reactions simultaneously is going to be \( p \cdot p = p^2 \) instead of \( p \), and therefore will have \( \sim 16 \) folds higher probability in catalyzing the reaction system.

Figure 3.11  Schematic presentation of expected arrangement of immobilized enzyme and cofactors on the surface of nanoparticles (A) Side view, (B) top view, and confined in nanopores (C) Side view and (D) Top view.

This observation although did not predict the extent of enhancement of activity observed (50 folds), provides a simplified explanation to the behavior. The effect of
cofactors and enzymes on different nanoparticles interacting with each other was not explored geometrically, due to the size of the enzymes covering the cofactors (Figure 3.12). Using this arrangement, the enzymes were prevented of accessing the cofactors when monolayer coverage or higher is achieved. To solve this problem, cofactors have to be immobilized separately (discussed in following chapters).

![Schematic presentation of the obstructed interaction between enzyme on nanoparticles and cofactors on other nanoparticles.](image)

Figure 3.12 Schematic presentation of the obstructed interaction between enzyme on nanoparticles and cofactors on other nanoparticles.

3.4 Conclusion

In summary, it was demonstrated that nanopores enabled multienzyme reactions by integrating the components of a catalytic system within a molecular distance. Efficiency of such a co-immobilized system can be improved by optimizing the flexibility of spacers and the size of pores, both of which manipulate the interactions among the immobilized components. Applications of such catalysts in industrial
chemical processing may provide efficient *in-situ* cofactor regeneration, easy catalyst/cofactor reuse and simple product purification. It is anticipated that more complicated systems that perform biological pathways mimicking living cells can be ultimately constructed through the same approach.
CHAPTER IV
PARTICLE-ATTACHED NADH FOR THE REDUCTION OF CO₂ FOR METHANOL PRODUCTION

Cofactor-dependent enzymatic reactions are of interests to a great variety of organic syntheses, especially for chiral chemicals [5, 58-63]. Cofactors mediate such reactions by functioning as electron carriers. In particular, NAD(H) and NADP(H) are widely needed cofactors for the biosyntheses of chiral alcohols, amino acids, hydroxy acids, aldehyde, ketones, epoxides, etc. [61-63]. On the other hand, the cofactors cost ~$1000/mol. That price makes the large-scale applications of cofactor-dependent biotransformations largely incompetent without cofactor regeneration and reuse measures [65]. Cofactor regeneration, however, is often a daunting task. This has led to a long-lasting tradition that microbial process is preferred when the transformation requires cofactors [60]. Microbial cultures operate multienzymic pathways efficiently in microbial terms, but in most cases complicate the bioprocessing with the maintenance of cell viability and growth, which are not necessarily conducive to efficient biotransformations. In addition, the water-insolubility and toxicity of organic compounds may also significantly constrain the applicability of microbial processes. Some of these difficulties may be alleviated through the latest development in genetic engineering; but that approach raises many other concerns in the eventual endpoint of releasing the
engineered microbial strains into the natural environment. Accordingly, the construction and use of multienzyme systems that allow efficient \textit{in-situ} regeneration of cofactors is an appealing yet considerably challenging subject in the area of biocatalysis.

4.1 Overview

There are different ways to regenerate cofactors [66-68]. One of that is electrochemical regeneration. This method has been generally applied for electrode-based biosensors and biofuel cells [69, 70]. Its application in bioprocessing seemed to be much less attractive [71]. The most extensively examined cofactor regeneration method for bioprocessing is the use of substrate-driven enzyme-catalyzed reactions. In continuous bioprocessing operations, the task of cofactor regeneration is always tethered with the driving to retain both the enzymes and cofactor in bioreactors. Various immobilization methods, both physical entrapment and covalent binding, have therefore been examined for this purpose. Earlier representative examples included the complexation of NAD(H) with Sepharose [72] and entrapment in fibroin membranes [73]. Functioning as physical barriers to retain the cofactors, the capsule membranes or matrices usually introduce great mass transfer resistance to the biotransformation reactions. Use of ultrafiltration-cell reactors with catalysts and substrates contained in the same bulk solution can improve reaction kinetics by allowing homogenous reactions [74]. The productivities of such reactors are, however, rather limited owing to the constrained flow rates of the reaction media through the nanometer pores of the membranes. Entrapments using capsules or membranes of larger pores are possible when
cofactors are grafted with water-soluble polymers [75, 76] or attached to enzymes [77]; still, the processes remain inefficient for large-scale applications.

Covalent binding to insoluble materials can provide much more efficient cofactor retention or recovery. This approach is hitherto most widely practiced for metal electrodes [78, 79], while studies have demonstrated the feasibility of using covalently immobilized cofactor to mediate reactions catalyzed by free enzymes in bioprocessing or analysis [65, 80]. In the present work, a new approach is reported to cofactor immobilization and enzymatic recycling. The cofactor is covalently immobilized to polymeric particles, while the enzymes are immobilized on the same type of particles but separately from the cofactor. The collision between these two types of particles is expected to facilitate the interaction between the cofactor and enzymes, and thus effect the desired reactions. The catalytic system can be recovered for reuse from the reaction media at the end of reactions by means of centrifugation or filtration.

This new approach was tested for the enzymatic production of methanol from carbon dioxide. The feasibility of using isolated enzymes to convert CO₂ to malic, aspartic, isocitric, and formic acids has been demonstrated over two decades ago [81, 82]. As a result of the rapidly growing demand in the environment’s well-being, the formate dehydrogenase-catalyzed CO₂ reduction has been examined more extensively in recent years as a promising approach to greenhouse gas fixation for the production of fuels and chemicals [83-86]. In particular, it has been shown feasible to produce methanol from CO₂ through the use of multienzyme systems [83-85]. Similar to many other enzymatic redox reactions, the enzymatic reduction of CO₂ requires cofactors such as NADH, pyrroloquinolinequinone, and methyl viologen to function as electron donors [82-85].
While the enzymatic production of methanol from CO$_2$ is greatly appealing, one mole of methanol requires the consumption of three moles of cofactor [85]. The regeneration and reuse of the cofactor are then especially critical to such bioconversion processes.

4.2 Materials and Method

All materials used in this study were purchase with the denoted purity grade and no further purifications were performed. All buffers were freshly prepared and used immediately or stored at 4 °C to minimize the CO2 degasing. The experimental setup was built in-house with glass and dry ice was used for the methanol trap.

4.2.1 Materials

Methanol of HPLC grade, formate dehydrogenase (FDH), formaldehyde dehydrogenase (FaldDH), alcohol dehydrogenase (ADH), glutamate dehydrogenase (GDH), NADH/NAD$^+$, $\alpha$-ketoglutarate, formic acid, formaldehyde, divinyl benzene, and polyethylene glycol were purchased from Sigma Chemical Co. (St. Louis, MO). N-acryloxy succinimide (NAS) was obtained from Acros Organics (Morris Plains, NJ). Styrene was obtained from EM Science (Gibbstown, NJ). L-glutamate and polyvinylpyrrolidone (PVP, $M_w$ 29 kDa) were purchased from Aldrich (Milwaukee, WI). 2,2’-Azobis [2-methyl-N-(2-hydroxyethyl) propionamide] (VA-086) was kindly provided as a gift from Wako Chemicals, Inc (Richmond, VA). 2-Sulfoethyl methacrylate (2-SEM) was purchased from Monomer-Polymer & Dajac Labs, Inc. (Feasterville, PA). Bio-Rad Bradford protein assay reagents and protein standard were obtained from Bio-Rad Laboratories (Hercules, CA).
4.2.2 Preparation of Polystyrene Particles

Polystyrene particles with a diameter of ~500 nm were prepared by emulsion polymerization following a procedure as reported previously [40]. In brief, an emulsifier solution was first prepared by dissolving 5 g of 2-SEM in 50 g DI water, followed by diluting it with water to 100 g while the pH was adjusted to 3.5 by adding 10 wt% NaOH solution. For the emulsion polymerization reaction, an aqueous solution consisting of 0.7 ml of the 2-SEM solution, 7 ml ethanol, 12 ml DI water, 90 mg polyvinyl pyrrolidone and 100 mg VA-086 was constituted in a 20-ml vial, to which a 1.2-ml of styrene solution containing 196 mg NAS and 16.0 µl divinyl benzene was then added. The head space of the vial was purged with nitrogen before it was sealed. The mixture was then emulsified on a vortexer, and then heated to and maintained at 70°C in a water-bath under stirring. The polymerization reaction was stopped after 10 h. The particles were washed with ethanol and DI water in a stirred ultrafiltration cell with a polyethersulfone membrane of a cut-off Mw of 300 kDa. Clean particles were stored in DI water at 4 °C if they were not used immediately. The number average diameter of the particles was measured as 490 ± 10 nm by accounting at least 100 particles from SEM images.

4.2.3 Enzyme and Cofactor Immobilization

Enzyme immobilization was conducted by adding 3 g of particles to 18 ml of 0.1 M pH 7.0 phosphate buffer solution containing formate, formaldehyde and alcohol dehydrogenases (36 mg of each) and 108 mg of glutamate dehydrogenase (Figure 4.1). The solution was stirred for 24 h at room temperature before centrifugation to stop the reaction. The enzyme-bearing particles were washed extensively with fresh phosphate
buffer solution till no protein was detectable in the supernatant solution. The particles were further purified by ultrafiltration with a 500-kDa cut-off membrane and stored in pH 7.0 phosphate buffer at 4°C. The washing solutions were collected and assayed for protein concentration using the Bradford method. Enzyme loading (ranged from 1.9 to 2.9 mg-enzyme/g-nanoparticles) on the particles was calculated by dividing the missed enzyme mass with the mass of the particles. Since there is no easy method available to distinguish the enzymes attached to the particles, the same mass ratio of enzymes added to the immobilization solution was assumed for the particle-attached enzymes in our calculations, i.e., 50% of the immobilized protein was assumed to be GDH while the remaining protein mass was that of the methanol-producing enzymes (including GDH, FaldDH, and ADH). The cofactor NADH was immobilized following a similar procedure with a recipe of 22 mg NADH for 500 mg of particles in 3 ml of the same buffer solution. NADH concentration in the solution was monitored by the absorbance at 340 nm. The loading of the cofactor on the particles ranged from 2.3 to 3.7 mg-NADH/g-nanoparticles as calculated using the same mass balance calculation.

![Chemical route for the attachment of enzymes onto polystyrene particles.](image)

Figure 4.1   Chemical route for the attachment of enzymes onto polystyrene particles. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [13].
4.2.4 Enzymatic Synthesis of Methanol

Reactions were carried out in a 20-ml glass vial with CO₂ bubbling (Figure 4.2). CO₂ gas from a cylinder was first saturated with H₂O before it entered the reactor. The flow rate of the gas was controlled the same for all the experiments by controlling the pressure valve. The flow rate was measured to 80 ml/min by floating-ball flowmeter. The exiting gas stream was fed into a methanol trap which was held in dry ice.

![Experimental setup for the bioconversion of CO₂ to methanol. (1 – compressed carbon dioxide cylinder; 2 – controlling valve; 3 – humidifier to saturate carbon dioxide with water; 4 – reactor, 20 ml; 5 – dry ice methanol trap; 6 – gas vent.) Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [13].](image)

The reaction was conducted by adding 4 ml of 1 mM glutamate solution in 0.1 M pH 7.0 phosphate buffer to the reaction vial. The solution was bubbled with CO₂ for 30 min before the addition of polystyrene particles carrying NADH and enzymes to initiate
the reaction. About 800 mg of immobilized enzyme (to result in an enzyme concentration of 0.5 mg-protein/ml) and 40~600 mg of immobilized cofactor (to result in cofactor concentration ranging from 50 to 500 µM) were added to initiate the reaction. For comparison reactions with free enzymes, since GDH was not used, the protein content (including GDH, FaldDH, and ADH with the same mass ratio) was controlled as 0.25 mg-protein/ml to keep the concentration of the methanol-producing enzymes the same as that of the immobilized enzymes in the reaction solutions. The reaction typically lasted for 30 min before it was stopped for methanol concentration analysis. A Shimadzu GC-17A gas chromatography equipped with a FID and a Restek RTX-5 column (15 m x 0.35 mm x 1.0 µm) was used for the analysis of methanol concentration. GC analysis showed no residual methanol in the gas stream after passing through the dry ice trap (methanol in the gas was detectable before the trap). The total amount of methanol produced was determined by GC analyses of both the reaction solution retained in the reactor and the ice formed in the dry ice trap after melting. The volume of the solution in the reactor was typically reduced by 0.2~0.3 ml for a 30-min reaction, while 0.8~1.2 ml liquid was collected in the dry ice trap. The reaction solution was filtered with Gelman® 0.2 µm PTFE syringe filter before GC analysis. Typically aliquots of 200 µl of clear sample solutions were taken for GC analysis. The initial column temperature was kept at 45°C for 4 min, then ramped at 40°C/min to 120°C. The injector and detector temperatures were 150°C and 250°C, respectively. Nitrogen was used as the carrier gas at an inlet pressure of 18 kPa (~0.4 ml/min). The injection volume was 10 µl. The concentration in the liquid from the trap was typically 3-7 µM, while the concentration in the reactor was typically 2-5 µM, indicating ~40% of methanol produced was stripped
out of the reactor by CO$_2$. To account the variation of solution volume during the reaction, a nominal final product concentration was calculated by dividing the total amount of methanol by 4 ml, the initial volume of reaction solution. This nominal final concentration is what was used in our comparison at different conditions.

4.3 Results and Discussion

The reactions with free cofactor and immobilized enzymes produced much more methanol than other combinations between immobilized/free enzymes and cofactors, while the reactions with immobilized enzymes and cofactor provided the lowest productivity throughout the test cofactor concentration range.

4.3.1 Activity of the Immobilized System

A productivity of 31 \( \mu \text{mol-methanol/h/g-enzyme} \) (based on the amount of methanol-producing enzymes) was achieved with immobilized cofactor and enzymes at an NADH concentration of 200 \( \mu \text{M} \). That was lower than but comparable to the 45 \( \mu \text{mol-methanol/h/g-enzyme} \) observed for free enzymes and cofactor at the same reaction conditions. Overall, it appeared that immobilized enzymes were more active than free enzymes when free cofactor was applied. This observation is in controversy to what is usually expected for immobilized enzymes. Similarly, for reactions with free enzymes, the immobilization of cofactor slightly yet consistently improved the methanol production (Figure 4.3). Immobilized enzymes usually exhibit reduced activities, mostly as a result of the substrate mass transfer resistance encountered in heterogeneous reactions and conformational changes of the enzymes. For sequential reactions as those
concerned in this work, enzyme co-immobilization integrated different enzymes into a molecular vicinity on the surface of the particles after immobilization. This may substantially reduce the distance for the intermediate chemicals to travel between the active sites of the enzymes, and thus increase the overall rate of methanol production [87]. For the comparison between the reactions of free and immobilized cofactor with free enzymes, we believe that the cofactor-carrying particles may absorb enzyme molecules from the solution, and thus improve the overall reaction via the postulated enzyme-integration mechanism.

![Graph](image_url)

**Figure 4.3** Effect of cofactor concentration on methanol produced with different reaction systems. (□) Immobilized enzymes and free cofactor; (Δ) Free enzymes and immobilized cofactors; (○) Free enzymes and free cofactor, (◊) Immobilized enzymes and immobilized cofactor. Reaction time was 30 min at room temperature.) Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [13].
4.3.2 Reusability of the Immobilized System

To examine the feasibility of recycling and reusing the immobilized enzymes and cofactor, comparison reactions were conducted: one with immobilized enzymes and cofactor in the presence of glutamate to allow cofactor regeneration, one with the same immobilized enzymes and cofactor but without the addition of glutamate, while the other was with immobilized enzyme but free cofactor without the addition of glutamate. The reactions were performed with 50 µM NADH and 0.5 mg-protein/ml. The reactions were allowed to last 30-min before were stopped to recover the catalysts for next round of activity tests at the same reaction conditions. Immobilized enzymes and cofactor are recovered by centrifugation, and wash with fresh DI water for three times. For reactions with immobilized cofactor, the cofactor and catalyst particles were reused without addition of fresh cofactor. For the reactions with immobilized enzyme but free cofactor, the enzymes were recovered by centrifugation and washing; for each reuse of these enzymes, the same amount of free cofactor was supplemented. The results of these tests are shown in Figure 4.4.

For the reactions with immobilized cofactor, the methanol production capability of the catalysts reduced quickly without NADH regeneration (Figure 4.4). The first round of reaction resulted in a methanol concentration of 2.1 µM, which corresponds to a yield of 13%. No significant amount of methanol was produced after 5 cycles of reusing, indicating the point of full consumption of NADH. The 11 reusing cycles of this reaction system accumulated a total of 0.029 µmol of methanol, corresponding to a 43% cumulative yield. Interestingly, that is approaching the 50% yield observed for reactions
with free cofactor. One cause for the yield being less than 100% may come from the balance of the reaction equilibrium and kinetics, which limit the amount of the intermediates that can be transformed to methanol for a given period of time. The best productivity was achieved with immobilized cofactor and free cofactor with cofactor regeneration. The catalysts showed no apparent loss of activity after 11 cycles of reusing with the cumulative yield increased almost linearly with increase in reusing cycles. The cumulative yield after 10 cycles reached 127%. Much higher yield can be expected as the reusing continues.

![Cumulative Percentage-Yield of Methanol](image)

Figure 4.4 Cumulative methanol production yield as a function of the number of reusing cycles of the enzymes and cofactor. ((Δ) Immobilized enzymes and free cofactor with cofactor supplement for each reuse; (◊) Immobilized enzymes and cofactor with in situ cofactor regeneration; (□) Immobilized enzyme and cofactor system without cofactor regeneration or supplement.) Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc [13].
4.4 Conclusion

It was demonstrated that cofactor attached to solid particles can be used to mediate reactions of immobilized enzymes with \textit{in situ} cofactor regeneration. The cofactor can be easily recovered and reused in the same way as immobilized enzymes. The use of immobilized cofactor can substantially improve the efficiency of cofactor utilization, and thus improve the potential of cofactor-dependent bioprocessing technologies.
CHAPTER V
MULTISTEP ENZYMATIC SYNTHESIS OF L-LACTIC ACID FROM CO₂ AND ETHANOL

Bioprocessing is becoming increasingly desirable for chemical production and drug synthesis [88-91]. Often microbial processes involve complicated culturing conditions, and in many cases the separation and purification of the products are a daunting task. Enzymatic processes, on the other hand, can be much faster, cleaner and simpler. The current arts of enzymatic bioprocessing are primarily for single-enzyme systems. Biotransformations that require concerted interactions among multiple enzymes and/or cofactors mostly have to rely on microbial processes. How to manipulate and apply multienzyme systems in vitro to carry out complex reactions represents one of the greatest challenges in bioprocessing.

5.1 Overview

Cofactor-dependent redox reaction systems are of tremendous interests to many organic syntheses, especially for enantio-pure compounds [92-95]. For example, interacting with different enzymes, nicotine amide adenosine NAD(H) can mediate the syntheses of chiral alcohols, amino acids, hydroxy acids, aldehyde, ketones, epoxides, etc. [96, 97]. Large-scale operations of such biotransformations, however, are still limited by the high cost of cofactors. Efficient cofactor regeneration is therefore essential.
to the development of enzymatic processes to replace microbial processes [98]. So far, bioprocessing processes that require cofactors have been traditionally approached with microbes.

There are different ways to regenerate cofactors [99-101]. One of these is electrochemical regeneration. This method has been generally applied for electrode-based biosensors and biofuel cells [102, 103]. Its application in bioprocessing seemed to be much less attractive [104]. The most extensively examined cofactor regeneration method for bioprocessing is the use of substrate-driven enzyme-catalyzed reactions. This requires the cofactor shuttles between two different enzymes. In continuous bioprocessing operations where immobilized enzymes are preferred, it becomes quite challenging to balance the immobilization of catalyst and the shuttling of cofactor. In a previous chapter, it was found that cofactors immobilized on small particles can interact via particle collisions with enzymes immobilized in the same way. When particles carrying the cofactor and enzymes are suspended in the same aqueous solution, biotransformation reactions were observed and cofactor regeneration was achieved. In the present work, we explore the use of a particle-carried multienzyme system for the synthesis of lactate from carbon dioxide and ethanol. The interest in this reaction system comes from two considerations. First, this unique reaction system has an internal cofactor regeneration loop, thus provides an ideal model to examine the enzyme-cofactor interactions for the particle-immobilized system. Second, it is closely related to the issue of carbon dioxide fixation, which has been subject to extensive research as a result of the worldwide drive to reduce the emission of the greenhouse gases.
5.2 Materials

Alcohol dehydrogenase (ADH), pyruvate decarboxylase (PyDC), lactate dehydrogenase (LDH), ethanol, pyruvic acid, thiamine pyrophosphate, polyethylene glycol, 3,3-dithiopropionic acid bis-N-hydroxysuccinimide ester (DTSP), glutaraldehyde, ethylene diamine, and cofactor NADH/NAD\(^+\) were purchased from Sigma Chemical Co. (St. Louis, MO). N-acryloxsuccinimide (NAS) was obtained from Acros Organics (Morris Plains, NJ). Styrene was obtained from EM Science (Gibbstown, NJ). Sodium carbonate and sodium bicarbonate were purchased from Fisher Scientific (Fair Lawn, NJ). Acetaldehyde, polyvinylpyrrolidone (PVP, M\(_w\) 29 kDa), epichlorohydrin (ECH), dimethyl sulfoxide (DMSO) and 3-aminopropyltrimethoxysilane (APTMS) were purchased from Aldrich (Milwaukee, WI). 2,2’-Azobis [2-methyl-N-(2-hydroxyethyl) propionamide] (VA-086) was kindly provided as a gift from Wako Chemicals, Inc (Richmond, VA). 2-Sulfoethyl methacrylate (2-SEM) was purchased from Monomer-Polymer & Dajac Labs, Inc. (Feasterville, PA). Lactate standard was purchased from ICN (Costa Mesa, CA). Bio-Rad Bradford protein assay reagents and protein standards were obtained from Bio-Rad Laboratories (Hercules, CA). Gold nanoparticles (250 nm) were purchased from BB-International (UK). Silica nanoparticles (250 nm) were purchased from Spherotech Inc. (Libertyville, IL).

5.3 Free Enzyme System

Reactions were conducted in 4-ml UV-grade polymethylmethacrylate cuvettes. The reaction rates of ADH- and LDH-catalyzed reactions were monitored by following the concentration change of NADH using a Shimadzu UV-1601 UV-Vis
spectrophotometer at 340 nm. The concentration of enzyme was controlled at 0.25 mg/ml; while the concentration of substrates, either ethanol or pyruvate, varied from 10 to 500 µM in 250 mM carbonate-bicarbonate pH 9.5 buffer solution. Reactions for PyDC were conducted in 2-ml vials at the same pH.

5.3.1 Evaluation of Kinetic Parameters

Reaction rates in the 2-ml vials were obtained by measuring changes in ethanol concentration using gas chromatography (Shimadzu GC-17) equipped with an FID detector. The column temperature was maintained at 45°C, and the injection port temperature 220°C, detector temperature of 250°C. The injection volume was 1 µl. The kinetic parameters were then graphically evaluated and summarized in Table 5.1.

Table 5.1 Summary of the values of kinetic parameter of the enzyme ADH, PyDC, and LDH. The parameters were evaluated experimentally at same enzyme concentration as the reaction (0.25 mg/ml) in 250 mM carbonate/bicarbonate buffer pH 9.5.

<table>
<thead>
<tr>
<th></th>
<th>Φ₀</th>
<th>Φₐ</th>
<th>Φ₉</th>
<th>Φ₅₆</th>
<th>Kₘ (mM)</th>
<th>kₕ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td>0.0904</td>
<td>11.02</td>
<td>197.63</td>
<td>69320</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>ADH⁻¹</td>
<td>0.0083</td>
<td>38.605</td>
<td>54.433</td>
<td>4642</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PyDC</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.35</td>
<td>7.10</td>
</tr>
<tr>
<td>PyDC⁻¹</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1.58</td>
<td>66.3</td>
</tr>
<tr>
<td>LDH</td>
<td>0.0013</td>
<td>29.189</td>
<td>6.6</td>
<td>8356</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>LDH⁻¹</td>
<td>0.8665</td>
<td>251.9</td>
<td>1774.8</td>
<td>718200</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
5.3.2 Activity Test

The activity tests to obtain the concentration data were done using a carbonate-bicarbonate buffer. The 250 mM buffer would perform as the carbon dioxide source as well as keep the reaction’s pH at 9.5. Typically to a reaction volume of 10 ml of buffer, enzymes ADH, PyDC, and ADH were added to a concentration of 0.25 mg/ml for each enzyme. To the solution, NAD$^+$ and thiamine pyrophosphate (PyDC coenzyme) were added to concentrations of 100 and 10 $\mu$M, respectively. Then, to initiate the reaction, ethanol was added from a stock solution of 100 mM of ethanol in buffer.

Aliquots of 200 $\mu$l were taken periodically and the concentration of each component was determined separately: the concentrations of lactate and pyruvate were determined using Shimadzu HPLC system with a mobile phase containing 0.05% v/v sulfuric acid in an acetonitrile-water (1:10, v/v) mixture. A Thermo-Aquasil C18 column was used with a flow rate of 1 ml/min and 50 $\mu$l injection volume at 222 nm. Similarly, 200 $\mu$l were taken for analysis of ethanol and acetaldehyde using gas chromatography. Concentration of NADH was measured by using its absorbance at 340 nm by taking 2 ml into a quartz cuvette. The 2 ml were then returned after the reading.

5.3.3 Kinetic Model

Figure 5.1 shows the chemical route of the suggested pathway for the production of lactate from carbon dioxide and ethanol. The synthesis pathway involves one oxidation and one reduction reactions of the cofactor NAD(H), and each regeneration cycle of the cofactor produces 1 molecule of L-lactate. The carbonate and bicarbonate in the buffer solution (pH of 9.5, 250 mM) provide carbon dioxide for the enzymatic reactions, similar
to reactions reported for the carboxylation of acetaldehyde [104]. Thiamine pyrophosphate was used as the co-decarboxylase.

Modeling of enzyme kinetics have been previously studied [97-99]. Mathematical models provide a fast and frequently accurate estimation of the behavior of biocatalytic systems. Multistep enzyme models have gained increasing interest especially to optimize the substrates ratios and identify the rate limiting step. Models that involve cofactor regeneration in particular provide valuable information to explore both \textit{in vivo} [100] and \textit{in vitro} recycling [101, 102]. For the kinetic model, both dehydrogenases, ADH and LDH were considered to follow the steady state bi bi mechanism [99, 103]. The PyDC catalyzed carboxylation of acetaldehyde – although being bi uni reaction – was considered to be dependent solely on the concentration of acetaldehyde, since CO$_2$ was in excess in the solution (~8 mM). A key factor in the reaction was the freshness of the buffer, while buffers were stored after purging the head-space with carbon dioxide gas. In different experiments, buffers more than few days old did not perform as well as fresh buffers since soluble CO$_2$ would escape the solution till equilibrium is reached with the head space.

The kinetic parameters in the model were experimentally obtained at the reactions conditions of temperature, pH, and ionic strength. The parameters were evaluated experimentally due to the lack of literature data reporting values at the given conditions. Table 5.1 summarizes the values of the kinetic parameters of enzymes. The concentration used to obtain those parameters, were in the range of 10 to 500 µM, covering the range of concentrations used for the multienzyme L-lactate synthesis.
The change of substrate concentrations with time is modeled with the differential equations (1) through (6). The \( v_i \) symbol denotes for the reaction velocity of the corresponding reaction. The minus sign is for a reaction proceeding in the reverse direction (Figure 5.1).

\[
\frac{d[EtOH]}{dt} = -v_1 + v_{-1} \quad (5.1)
\]

\[
\frac{d[Acet]}{dt} = v_1 - v_{-1} - v_2 + v_{-2} \quad (5.2)
\]

\[
\frac{d[Pyr]}{dt} = v_2 - v_{-2} - v_3 + v_{-3} \quad (5.3)
\]

\[
\frac{d[Lac]}{dt} = v_3 - v_{-3} \quad (5.4)
\]
\[
\frac{d[NAD^+]}{dt} = -v_1 + v_{-1} + v_2 - v_{-3} \tag{5.5}
\]

The most general form of the rate equation for bireactant mechanisms using the Cleland’s nomenclature for an \( A + B \rightarrow P + Q \) reaction is \([97]\):

\[
v = \frac{V_{\text{max}} [A][B]}{K_{iA}K_{iB} + K_a[B] + K_b[A] + [A][B]} \tag{5.6}
\]

when expressed in reciprocal form in terms of \( \Phi \)’s \([98]\):

\[
\frac{1}{v} = \Phi_0 + \frac{\Phi_A}{[A]} + \frac{\Phi_B}{[B]} + \frac{\Phi_{AB}}{[A][B]} \tag{5.7}
\]

where,

\[
K_a = \Phi_A / \Phi_0 \tag{5.8}
\]

\[
K_b = \Phi_B / \Phi_0 \tag{5.9}
\]

\[
K_{ia} = \Phi_{AB} / \Phi_B \tag{5.10}
\]

\[
V = E_0 / \Phi_0 \tag{5.11}
\]

Thus, using equation (7) the rate expressions of each reaction would correspond to:

\[
v_1 = \left( \frac{1}{\Phi_{\text{ADH,0}} + \frac{\Phi_{\text{ADH, ADH-A}}}{[\text{EtOH}]} + \frac{\Phi_{\text{ADH, B}}}{[\text{NAD}^+]} + \frac{\Phi_{\text{ADH, AB}}}{[\text{EtOH}][\text{NAD}^+]}} \right) \tag{5.12}
\]

\[
v_{-1} = \left( \frac{1}{\Phi_{\text{ADH,0}} + \frac{\Phi_{\text{ADH, ADH-A}}}{[\text{Acet}]} + \frac{\Phi_{\text{ADH, B}}}{[\text{NADH}]} + \frac{\Phi_{\text{ADH, AB}}}{[\text{Acet}][\text{NADH}]} \right) \tag{5.13}
\]
\[ v_2 = \left( \frac{k_{\text{cat},2} \cdot E_2 \cdot [\text{Acet}]}{K_{m,2} + [\text{Acet}]} \right) \quad (5.14) \]

\[ v_{-2} = \left( \frac{k_{\text{cat},-2} \cdot E_2 \cdot [\text{Pyr}]}{K_{m,-2} + [\text{Pyr}]} \right) \quad (5.15) \]

\[ v_3 = \left( \frac{1}{\Phi_{\text{LDH},0} + \Phi_{\text{LDH},A} \cdot [\text{Pyr}] + \Phi_{\text{LDH},B} \cdot [\text{NADH}] + \Phi_{\text{LDH},AB} \cdot [\text{Pyr}] \cdot [\text{NADH}]} \right) \quad (5.16) \]

\[ v_{-3} = \left( \frac{1}{\Phi_{\text{LDH},-1,0} + \Phi_{\text{LDH}^{-1},A} \cdot [\text{Lac}] + \Phi_{\text{LDH}^{-1},B} \cdot [\text{NAD}^+] + \Phi_{\text{LDH}^{-1},AB} \cdot [\text{Lac}] \cdot [\text{NAD}^+]} \right) \quad (5.17) \]

The Φ’s, \( K_m \) and \( k_{\text{cat}} \) were experimentally determined using the graphical methods described by Purish 1983 [99] and their values are summarized in Table 5.1.

5.3.4 Results and Discussions

The chemical route for the synthesis of L-lactate from carbon dioxide and acetaldehyde has been previously explored [100-101]. In the present work, ethanol was used as the co-substrate. For this purpose, alcohol dehydrogenase (ADH) was introduced to oxidize ethanol to acetaldehyde and to provide an \textit{in situ} regeneration of the cofactor NAD(H). The \( \text{HCO}_3^-/\text{CO}_3^- \) provided the necessary conditions of pH, ionic strength and \( \text{CO}_2 \) in the solution for the carboxylation reaction to proceed. At higher pH and ionic strength, it was reported that PyDC favors the carboxylation of acetaldehyde rather than the decarboxylation of pyruvate [101].
The L-lactate synthesis reactions consisted of batches of 10 ml containing the triad enzyme system (ADH, PyDC and LDH), cofactor NAD\(^+\), thiamine, and alcohol. Samples were taken at time intervals and the concentration profile of each of the substrate (ethanol and NAD\(^+\)), products (lactate) and byproducts (acetaldehyde, pyruvate, and NADH) were plotted. The differential equations shown in the kinetic model were fed into a linear differential equation solver, Polymath v5.1 (Ben-Gurion University of the Negev, Israel) and concentration profiles were compared with experimental data (Figures 5.2-5.4). The mathematical model contained the concentration equations (1) to (5) and the rate expressions (12) to (17). The model was provided with \(\Phi\)'s, \(K_m\), and \(K_{cat}\) values listed in table 5.1 and initial concentrations of the chemicals. While the output of the software had the concentration information of all the six species involved in the reaction as function of time. Concentrations in the model were predicted at 10-seconds intervals.

To study the validity of the model and the effect of initial cofactor concentration on the production of lactate, three sets of reactions of NAD\(^+\) concentrations of 50 (Figure 5.2), 100 (Figure 5.3), and 500 (Figure 5.4) \(\mu\)M were conducted. The concentration of different species changed differently in the reaction system. The reason was the difference in rate mechanism, rate constants’ values, and whether the species was involved in the regeneration loop of the reaction. For instance, the initial rate of consumption of both ethanol and NAD\(^+\) were closely comparable in all reactions. However, the post 1-h data show that the concentration of NAD\(^+\) levels off and in other scenarios increasing. This phenomenon occurred because of production of NAD\(^+\) from NADH regeneration reached steady rate.
Figure 5.2 Concentration profiles of reactant and products. Ethanol (◊), acetaldehyde (□), pyruvate (△), NAD+ (×), lactate (○), and for the model simulated concentrations of ethanol (ـــ), acetaldehyde (ـــ), pyruvate (ـــ), NAD+ (ـــ), lactate (ـــ).

Figure 5.3 Concentration profiles of reactants and products. Ethanol (◊), acetaldehyde (□), pyruvate (△), NAD+ (×), lactate (○), and for the model simulated concentrations of ethanol (ـــ), acetaldehyde (ـــ), pyruvate (ـــ), NAD+ (ـــ), lactate (ـــ).
As seen in Table 5.4, the 12-h concentrations—not necessarily steady state—show the effect of the initial cofactor concentration on the consumption of ethanol and the production of lactate. Although initial NAD\(^+\) concentration has considerable effect on individual cofactor-dependent enzymes LDH and ADH, it did not provide the expected effect of boosting the lactate production, instead promoted accumulation of byproducts. Similarly, more NAD\(^+\) shifted the thermodynamic equilibrium of the LDH reaction more towards lactate oxidation to pyruvate. The carboxylation of acetaldehyde therefore, would be the rate limiting reaction for this process. It is thus suggested to use less cofactor and more ethanol to improve the rate of the overall lactate production.

Figure 5.4 Concentration profiles of reactants and products. ethanol (◊), acetaldehyde (□), pyruvate (△), NAD\(^+\) (×), lactate (○), and for the model simulated concentrations of ethanol (−−−), acetaldehyde (−), pyruvate (−−−), NAD\(^+\) (−), lactate (——).

The model prediction of the concentration of the reaction’s intermediates was a good fit with experimental data. Few regions of sharp change in concentrations e.g. at $t =$
15 min did not fit well experimentally. This misfit was caused when providing initial amounts of ethanol and NAD\(^+\) that would solely stimulate \(v_3\), while other reactions proceed slower at low substrate concentration. Another driving force for the total reaction is equilibrium, both in the model and experimentally, the ADH reaction proceeded till equilibrium, thereafter it progressed at a constant rate until ethanol or NAD\(^+\) were exhausted. Mathematically, the equilibrium for each reaction would be reached once the forward and the backward reaction rates are equal. Due to its role as the middle reaction, PyDC-catalyzed \(v_2\) and \(v_2^{-1}\) would not reach equilibrium until both ADH and LDH reactions are balanced, since both acetaldehyde and pyruvate are product and substrate of the corresponding reactions. However, since ADH has comparable kinetic parameters for the forward and backward rates, the reaction would drastically drop in rate once acetaldehyde concentration starts rising due to accumulation. Lactate accumulation on the other hand would still remain of minor effects since the kinetic parameters favor \(v_3\) over \(v_3^{-1}\) to a ratio of 1 to 20 based on single-enzyme experimental values.

<table>
<thead>
<tr>
<th>NAD(^+):Ethanol (μM: μM)</th>
<th>Ethanol Consumed (μM)</th>
<th>NAD(^+) Consumed (μM)</th>
<th>Lactate Produced (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:100</td>
<td>58</td>
<td>12</td>
<td>38</td>
</tr>
<tr>
<td>100:100</td>
<td>70</td>
<td>20</td>
<td>39</td>
</tr>
<tr>
<td>500:100</td>
<td>73</td>
<td>42</td>
<td>41</td>
</tr>
</tbody>
</table>

In Figure 5.2, monitoring the NAD\(^+\) concentration shows that its concentration did not drop more than 20 μM. However lactate reached a final concentration of 38 μM.
For each 1 mol of lactate produced 1 mol of NAD$^+$ must be consumed if there was no regeneration. Thus, recycling must have taken place during the course of the reaction. To prove this hypothesis, a reaction with continuous ethanol feed was carried out until lactate reached a concentration higher than the initial NAD$^+$. 

![Graph showing concentration profiles of reactants and products. Ethanol (◇), acetaldehyde (○), pyruvate (Δ), NAD$^+$ (×), lactate (○), and for the model simulated concentrations of ethanol (−−−), acetaldehyde (−−), pyruvate (−−), NAD$^+$ (⋯), lactate (―).](image)

Figure 5.5 Concentration profiles of reactants and products. Ethanol (◇), acetaldehyde (○), pyruvate (Δ), NAD$^+$ (×), lactate (○), and for the model simulated concentrations of ethanol (−−−), acetaldehyde (−−), pyruvate (−−), NAD$^+$ (⋯), lactate (―).

Figure 5.5 shows that continuously feeding ethanol (at variable rate in the range of 0.01 – 0.5 µl /min of an ethanol solution of 10 mM in buffer) kept the ethanol concentration at 100 ± 15 µM. NAD$^+$ concentration used was 10 µM since using less cofactor, although slowed down the reaction, forced the reaction to visibly regenerate the cofactor. The final concentration of lactate was 87 µM, which corresponds to ~ 9-fold the amount of cofactor initially added to the reaction vessel. Therefore, one can consider that
at least 9 regeneration cycles have taken place over the course of 4 days. As a result, the turnover number of the cofactor would be 9.6 mmol-Lac.L$^{-1}$.d$^{-1}$.mmol-NAD$^+$.$^{-1}$.

![Figure 5.6](image)

**Figure 5.6** Model simulated produced lactate amount for a reaction with various initial concentrations. (A) 100 μM NAD$^+$ and 100 μM ethanol where profile of lactate with continuous removal of lactate (---) and without removal (---) and (B) 100 μM NAD$^+$ and 100 μM ethanol were profile of lactate with continuous feed of acetaldehyde at a rate of 1.6 μg/h (- - -), 2.4 μg/h (⋯), without addition/removal (---), at a removal rate of 1.6 μg/h (---), and 2.4 μg/h (⋯). Reaction were over the course of 12 hours and with individual enzyme concentration of 0.25 mg/ml.

In Figure 5.6A the model estimation of the effect of the continuous removal of lactate on the productivity of the system is provided. The system with continuous removal of lactate was predicted to accumulate 60 μg-lactate, whereas without removal...
the amount would be 47 \( \mu \)g-lactate in a 12 hours reaction. Similarly, the effect of the removal of acetaldehyde on the productivity was estimated (Figure 5.6B); however it only showed that adding more acetaldehyde would boost the lactate production. The observation can be explained since more acetaldehyde increases the rate of the PyDC reaction, which is considered to be rate-limiting for the synthesis system. Note that the model does not take into account the product inhibition or any type of inhibition.

5.4 Immobilized Enzyme/Cofactor System

Particles with sizes ranging from 0.1 – 1.0 \( \mu \)m were prepared using different recipes via emulsion polymerization [40]. In brief, an emulsifier solution was first prepared by dissolving 5 g of 2-SEM in 50 g DI water, followed by diluting it with water to 100 g while the pH was adjusted to 3.5 by adding 10 wt% NaOH solution.

5.4.1 Preparation of Polystyrene Nanoparticles

For the emulsion polymerization reaction, an aqueous solution of the 2-SEM solution (0.5-1.5 ml), ethanol (2.5-9.5 ml), DI water (9.5-16 ml), polyvinyl pyrrolidone (0-110 mg) to certain proportions were constituted in a 20-ml vial with 100 mg of VA-086, to which styrene solution (0.6-1.2 ml) containing NAS (98-196 mg) and divinyl benzene (8.2-16.0 mg) was then added. The head space of the vial was purged with nitrogen before it was sealed. The mixture was then emulsified on a vortexer, and then heated to and maintained at 70\(^{\circ}\)C in a water-bath under constant stirring of 600 rpm. The polymerization reaction was stopped after 10 h and particles were washed using the standard procedure described in chapter IV.
5.4.2 Enzyme and Cofactor Immobilization on Polystyrene Nanoparticles

The batches of the multienzyme system were immobilized on nanoparticles of 0.1, 0.25, 0.5, and 1.0 µm. In details, in 3 ml of 50 mM phosphate buffer pH 7.0, 5 mg of each enzyme (ADH, PyDC, and LDH) were added to a solution containing 500 mg of the particles. The mixture was allowed to mix for 12 hours. After the immobilization the nanoparticles were washed by ultrafiltration with a 500-kDa cut-off membrane with buffer. The amount of the enzymes washed was measured with Bradford protein assay and the loading was calculated by mass balance. The values of the loadings and their corresponding percent of monolayer coverage on the particles are summarized in Table 5.2. Using the same procedure, 60 mg of NAD$^+$ were added to 250 mg of nanoparticles suspended in 4 ml of the same buffer. The mixture was allowed to mix for 48 hours and then it was washed by ultrafiltration. The loaded cofactor was accounted for by mass balance using the values obtained from the permeate solution analyzed using HPLC. The NAD$^+$ loadings values are summarized in Table 5.3.

Table 5.3 Summary of the enzyme loading values and the percent of the monolayer coverage on polystyrene nanoparticles.

<table>
<thead>
<tr>
<th>Particles size (nm)</th>
<th>100</th>
<th>250</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading (wt%)</td>
<td>10.9%</td>
<td>5.1%</td>
<td>2.8%</td>
<td>1.3%</td>
</tr>
<tr>
<td>Monolayer (%)</td>
<td>89%</td>
<td>93%</td>
<td>100%</td>
<td>98%</td>
</tr>
</tbody>
</table>

82
Table 5.4  Summary of the NAD$^+$ loading values on polystyrene nanoparticles.

<table>
<thead>
<tr>
<th>Particle size (nm)</th>
<th>100</th>
<th>250</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading (wt%)</td>
<td>4.9%</td>
<td>3.3%</td>
<td>1.0%</td>
<td>0.6%</td>
</tr>
</tbody>
</table>

5.4.3  Enzyme/Cofactor Immobilization Using PEG as Spacer

To the solution of suspended nanoparticles, ethylene diamine was added to a concentration of 0.1 M. The mixture was left to mix for 1 hour, then particles were washed with D.I. water by ultrafiltration (500 kDal cutoff). Meanwhile, 1 g of PEG (MW=10 kDal) was dissolved in 1 ml ECH and 1 ml of water. The mixture was allowed to mix for 12 hours. The PEG-ECH solution was then dried by nitrogen blowing to remove all residual ECH and water. As a standard condition, 100 mg of the activated particles were mixed with 100 mg of epoxy-terminated PEG. The mixture was allowed to mix for overnight. Then the particles were washed with water to remove excess PEG. Enzymes and cofactor were then contacted with the particles for attachment. Typical loading values were comparable to no-spacer assays.

5.4.4  Enzyme Immobilization on Gold Nanoparticles

First, 134 mg of gold nanoparticles (250 nm diameter) were activated by chemisorption using 5 mM DTSP in DMSO for 2 hours as suggested by [102]. Then the gold particles were centrifuged at 14,000 g for 15 minutes to remove the DMSO phase and the particles were washed with methanol then with DI water. The gold nanoparticles
then were left mixing for 8 hours at 4°C in 1 ml of 3 mg/ml of each of the enzymes. The particles were washed after immobilization by centrifugation and protein content was assayed. The loading was ~50 mg-protein/g-particles, corresponding to close to 100% of a monolayer assuming the particle size is a homogenous 250 nm.

![Chemical route for the attachment of enzymes to 250 nm gold nanoparticles](image)

**Figure 5.7** Chemical route for the attachment of enzymes to 250 nm gold nanoparticles.

### 5.4.5 Enzyme Immobilization on Silica Nanoparticles

One hundred milligram of silica nanoparticles of 250 nm diameter were first washed with water to remove the stabilizers in the solution. Then nanoparticles were contacted at 40°C with APTMS (0.05 mg/ml) for 2 hours in 5 ml volume. The particles were then washed using an ultrafiltration cell with 500 kDal cutoff membrane. After the surface activation with amine groups, 1.5 mg of each enzyme were contacted with the
100 mg of particles for 24 hours in a 5% (v/v) solution of glutaraldehyde. The unattached enzymes were washed out using ultrafiltration with 38 mg-protein/g-particles (73% of monolayer) obtained.

Figure 5.8 Chemical route for the attachment of enzymes to 250 nm silica nanoparticles.

5.4.6 Activity Test of Nanoparticles-Attached Enzyme/Cofactor

Same preparation, sampling and analysis procedures of that of the free system were used for the immobilized system. Enzyme/cofactor bearing nanoparticles were added to a reaction volume of 4 ml of buffer to a total enzyme concentration of 0.75 mg/ml and 100 µM concentration of cofactor. Thiamine pyrophosphate was used in free solution at 10 µM throughout all reactions. Similarly, ethanol was added to initiate the L-lactate synthesis reaction at concentration of 100 µM.
5.4.7 Results and Discussion

The conditions at which optimum free system activity and detection occurred were selected to be the set conditions for the immobilized system. The size of the nanoparticles is expected to dramatically affect the activity of the system [41]. For this purpose the activity of multiple variations of polystyrene particle sizes (100-1000 nm) were tested and summarized in Table 5.5, where zero size corresponds to the free state of the enzyme or cofactor. The observed activity of the system with both enzyme and cofactor in immobilized state represents an interesting behavior of interaction between the enzyme and its cofactor even when attached to a solid support. Also interestingly, the immobilization did not reduce the activity by few orders of magnitude as expected, instead kept the activity of the immobilized system within comparable range to that of the free (e.g. two fold decrease in the case of enzyme immobilized on 100 nm particles reacting with free cofactor). For the different sizes, the obtained results showed that the activity of the system decreased with increasing particles size. It was noticeable that the activity of the system would radically drop when particles of 500 nm or larger are used. The cofactor especially was very sensitive to its carrier size, which implies that the mobility of the cofactor in the system was key design parameter for a highly active biocatalyst. A parallel comparison to the initial reaction rate activity is given for the lactate final concentration (Table 5.6). The 12-h lactate concentration did not have high sensitivity to the particles size as was the initial rate. In the 250 nm enzyme and 250 nm cofactor example, the initial rate was 9-fold lower than the free system, whereas it was
only 4-fold difference in lactate concentration which implies that most lactate production was at the steady state part of the reaction rather than the initial stage. Therefore, the difference in initial rate would not project its effect on the final product concentration as significantly, due to byproducts accumulation.

Table 5.5 Summary of the initial reaction rate of the immobilized system for combinations of particles size. The reaction was at 100 µM ethanol, 100 µM NAD+, 10 µM thiamine, and 0.75 mg-enzyme/ml.

<table>
<thead>
<tr>
<th>µmol-EtOH.L⁻¹.h⁻¹</th>
<th>Particles-cofactor (nm)</th>
<th>0</th>
<th>100</th>
<th>250</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles-Enzyme (nm)</td>
<td>0</td>
<td>104</td>
<td>31</td>
<td>28</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>52</td>
<td>24</td>
<td>15</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>48</td>
<td>18</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>32</td>
<td>15</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>26</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5.6 Summary of the 12-h lactate concentration synthesized by the immobilized system for combinations particles size. The reaction was at 100 µM ethanol, 100 µM NAD+, 10 µM thiamine, and 0.75 mg-enzyme/ml.

<table>
<thead>
<tr>
<th>µmol-Lac.L⁻¹</th>
<th>Particles-cofactor (nm)</th>
<th>0</th>
<th>100</th>
<th>250</th>
<th>500</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles-Enzyme (nm)</td>
<td>0</td>
<td>42</td>
<td>24</td>
<td>20</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>33</td>
<td>18</td>
<td>13</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>24</td>
<td>17</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>21</td>
<td>11</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>19</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

From the 12-h lactate data the yield of the reaction can be calculated using the initial reactants used. Consequently, the highest observed yield for the immobilized system was 18% based on initial ethanol. Similarly, the cofactor turnover would be 0.18
mol-Lac/mol-NAD⁺. A turnover number smaller than 1, usually implies that the system did not complete a cofactor regeneration cycle. To take advantage of the reusability of the immobilized system, an experiment was designed with continuous ethanol feed and concentration of 1 mM, lower NAD⁺ concentration (10 µM), and longer reaction time (9 days). The reaction shown in Figure 5.9 produced a total of 4 µmol of lactate, while initially had 0.1 µmol of NAD⁺. The reaction afforded 40 cofactor regeneration cycles e.g. the cofactor turnover number was 40 mol-Lac/mol-NAD⁺. The number would be significantly higher if byproducts acetaldehyde and pyruvate were taken into account.

![Figure 5.9](image-url) Concentration profile of reactants and products. Ethanol (◊), acetaldehyde (○), pyruvate (△), lactate (□). Initial concentrations: 1 mM ethanol, 10 µM NAD⁺ (immobilized on 100 nm nanoparticles), 10 µM thiamine, 0.75 mg/ml enzyme (immobilized on 100 nm nanoparticles).

Since the material of the enzyme carrier has been proven as significant design parameter [103], gold and silica spherical nonporous nanoparticles with 250 nm in
diameter were selected for a comparison test with the polymeric nanoparticles. Factors such as the surface morphology, charge, and density would be appealing if significant enhancement in performance is observed. The comparison of synthesis reactions using each of those nanoparticles with free cofactor are shown in Figure 5.10.

Figure 5.10 Concentration of lactate for 250 nm nanoparticles for different materials. Polystyrene (○), Silica (△), Gold (□). Initial concentrations: 100 µM ethanol, 100 µM NAD+, 10 µM thiamine, 0.75 mg/ml enzyme (immobilized).

The polystyrene particles showed the highest activity compared to its metal and glass counterparts. The 12-h lactate concentrations were 24, 16, and 10 (Figure 5.10), while the initial rates were 48, 27 and 13 µmol-EtOH.L\(^{-1}.h^{-1}\) for the polystyrene, silica, and gold, respectively. Few possible phenomena might have contributed to this observation. First, the density of polystyrene, glass and gold are 1.05, 2.5, and 19.32 g/ml [98]. This difference in density creates different mixing patterns and partitioning in the
solution. Polystyrene having the density closest to that of water will be the one to have to most stable suspension. This suspension is essential higher frequency of collision between particles, which has been shown to have a strong correlation with activity [40].

Second, the modification procedure of the particle surface prior to enzyme immobilization. For polystyrene, the surface was activated with free succinamide containing monomers during the synthesis of the particles. The modification is a very moderate procedure that is carried out at neutral pH, room temperature or 4°C, and do not require or release any reagents [40]. The silicate modification however, involves first modifying the glass surface with amine groups, then crosslink the α-amine groups on the enzyme and glass using glutaraldehyde. Glutaraldehyde, although used gently is still denaturing to enzymes [99]. DTSP, similar to glutaraldehyde, is a bifunctional molecule which can crosslink/fix enzymes [100]. Therefore, considering the density, the moderate immobilization conditions, and the fact that the particles are of same size and with close to monolayer coverage of protein, polystyrene was the easiest to synthesize and most efficient carrier.

To further optimize the system, and since the mobility of the enzyme was proven in earlier chapters to be critical, a polymeric spacer was employed to improve the catalytic activity of the system. PEG was selected as the model spacer with 2 different molecular weights 550 and 10,000 Dal. Activities for the spacer enzyme were more pronounced with 13% increase over the no-spacer system when the enzyme system was attached using the spacer arm (Table 5.2). Similarly, the all-immobilized reaction had 13% enhancement using the longer spacer. The results suggested a minor impact on the mobility of the enzyme/cofactor compared to results from chapter III. A practical
explanation for this behavior is that unlike the confinement in nanopores, nanoparticle-enzymes are highly mobile and although a spacer would still provide extra mobility, its effect is less pronounced. The other possibility is the choice of polymer for the spacer arm. Since typically the more soluble the polymer is, the higher the flexibility the chain is, hence a better spacer arm. For that reason, the next chapter will be answering the question on whether more soluble polymer provides a better spacer for enzyme attachment.

Table 5.7 Summary of initial reaction rate and 12-h lactate concentration for comparison reaction between the PEG550 and PEG10k spacers. Initial reaction conditions were: ethanol (100 µM), NAD+ (100 µM), thiamine (10 µM), and total enzyme (0.75 mg/ml).

<table>
<thead>
<tr>
<th>Particles-Enzyme (nm)</th>
<th>μmol-EtOH.L⁻¹.h⁻¹</th>
<th>Particles-cofactor (nm)</th>
<th>μmol-Lac.L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µmol-Lac.L⁻¹)</td>
<td>Free</td>
<td>PEG550</td>
</tr>
<tr>
<td>Free</td>
<td>104</td>
<td>24</td>
<td>34</td>
</tr>
<tr>
<td>PEG550</td>
<td>(42)</td>
<td>(12)</td>
<td>(19)</td>
</tr>
<tr>
<td>PEG10k</td>
<td>54</td>
<td>18</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>(28)</td>
<td>(11)</td>
<td>(N/A)</td>
</tr>
<tr>
<td></td>
<td>68</td>
<td>N/A</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>(36)</td>
<td>(N/A)</td>
<td>(23)</td>
</tr>
</tbody>
</table>

5.5 Conclusion

The multienzyme synthesis of L-lactate from carbon dioxide and ethanol was demonstrated in this chapter. The in situ cofactor regeneration cycle in the synthesis route proved effective in reducing the amount of cofactor required by the process by continuous oxidation/reduction back to its original state. The devised kinetic model predicted the initial rates and the final concentration data with good accuracy. For this system, a conversion of 41% was achieved. While a 4-days reaction gave 9.6 mmol-
Lac.L$^{-1}.d^{-1}.mmol-NAD^{+1}$ with continuous ethanol feed. The immobilized multienzyme system achieved remarkable activity comparable to that of the free system (4-fold lower using the 100 nm particles).
CHAPTER VI

EFFECT OF SPACER FLEXIBILITY ON THE CATALYTIC BEHAVIOR OF

PARTICLE-ATTACHED ENZYMES

Immobilized biocatalysts are increasingly being applied in industrial processes (e.g. waste treatments using tyrosinase [101], acrylamide synthesis using nitrile hydratase [102, 103], and milk processing using β-galactosidase [102, 104]). This advent initiated interest in researches to answer fundamental questions of the behavior of enzymes. The immobilization of enzyme has been the most commonly applied method to increase the stability and reusability of enzyme [105]. Physical adsorption, entrapment, encapsulation and covalent bonding are the currently used methods [106]. Covalent attachment, presents when achieved successfully a relatively non-leaking enzyme from its carrier compared to its physical adsorption and ionic bonding counterparts. The strength and the permanence of the bond is more resilient to changes in ionic strength and or pH and would only be cleaved by a chemical agent of heat. This advantage proves useful for stability and reusability purposes. A tradeoff is evident however, since the loss in activity of the enzyme due to multiple factors introduced by attachment to insoluble carrier. Chemical and physical changes in the protein structure, diffusion limitations of substrate and products, and mobility of the enzyme are few of those factors. To reduce these effects, a spacer can be used to link the enzyme to the support. The spacer on the other hand should neither interfere in the chemical reaction nor in the structure of catalytic active site [107].
6.1  Overview

The hydrophobicity/hydrophilicity of the spacer is likely to be a critical factor since it provides higher flexibility for the attached enzyme (Figure 1.5). For this purpose the transesterification reaction of aminopropyl propyl ester and methanol, catalyzed using \( \alpha \)-chymotrypsin was used as model reaction and tested in various organic solvents [108]. Enzyme catalysis in nonaqueous media has been proven, nonetheless, the catalytic efficiencies of enzymes in those media are orders of magnitude lower than those in aqueous solutions [109]. Immobilizing enzymes increases the nonaqueous catalysis by facilitating the enzyme distribution in the solution, since most enzymes are insoluble in organic solvents. When insoluble, enzymes tend to gather into larger aggregates that may introduce substrate and/or product diffusion limitation [100]. The anhydrous organic solvent is expected to provide the polymeric spacer with higher solubility when its solubility parameter (\( \delta \)) is closer to that of the spacer.

In this chapter, the results obtained with nanoparticles attached \( \alpha \)-chymotrypsin by using polyethylene glycol of 550 and 10,000 Dal molecular weights are presented. Comparison with the activity of the native enzyme and no-spacer particles is also presented to demonstrate the spacer lengths’ significance.

6.2  Materials and Method

All materials used in this study were purchase with the denoted purity grade and no further purifications were performed. Solvents were stored with molecular sieves in dry environment to eliminate water traces in the solvents, especially the polar ones.
Samples were taken from solvent bottles using a syringe and needle to minimize exposure of solvent to humidity from air.

6.2.1 Materials

Polyethylene glycol, α-chymotrypsin from bovine pancreas (α-CT), and n-acetyl-L-phenylalanine ethyl ester (APEE) were purchased from Sigma Chemical Co. (St. Louis, MO). N-acryloxsuccinimide (NAS) was obtained from Acros Organics (Morris Plains, NJ). Styrene, hexane, cyclohexane, iso-octane, benzene, o-diclorobenzene, ethylene diamine, ethylene glycol, glycerol, and n-propanol (HPLC grade) were obtained from EM (Gibbstown, NJ). 2,2’-Azobis[2-methyl-N-(2-hydroxyethyl) propionamide] (VA-086) was kindly provided as a gift from Wako Chemicals USA, Inc. (Richmond, VA). Polyvinylpyrrolidone (PVP, Mₜ 29 kDa) was purchased from Aldrich (Milwaukee, WI). 2-Sulfoethyl methacrylate (2-SEM) was purchased from Monomer-Polymer & Dajac Labs, Inc. (Feasterville, PA). Bio-Rad Bradford protein assay reagents and protein standards were obtained from Bio-Rad Laboratories (Hercules, CA).

6.2.2 Organic Solvents

To explore the effect of the flexibility of the polymeric spacer, a wide variety of solvents were selected over a Hansen solubility parameter δ range of 14.9 to 29.9 (MPa)^{1/2} listed in table 6.1. Hansen solubility parameters were originally developed by Charles Hansen to predict the solubility of one material in another to form a uniform solution. The idea that like dissolves like is consistent with this concept while materials with close δ values are more likely to be miscible. Based on this concept the hypothesis is
to identify from the list of selected solvents the solvent at which PEG is most flexible. This solvent is assumed initially to be the solvent closest in $\delta$ to that of PEG.

Table 6.1  Selected solvent and their solubility parameter values.

<table>
<thead>
<tr>
<th></th>
<th>$\delta$ (MPa)$^{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>14.9</td>
</tr>
<tr>
<td>c-Hexane</td>
<td>16.8</td>
</tr>
<tr>
<td>Benzene</td>
<td>18.8</td>
</tr>
<tr>
<td>Chloroform</td>
<td>19.0</td>
</tr>
<tr>
<td>Dichlorobenzene</td>
<td>20.5</td>
</tr>
<tr>
<td>Pyridine</td>
<td>21.9</td>
</tr>
<tr>
<td>Nitroethane</td>
<td>22.7</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>24.3</td>
</tr>
<tr>
<td>Ethylene diamine</td>
<td>25.2</td>
</tr>
<tr>
<td>Nitromethane</td>
<td>26.0</td>
</tr>
<tr>
<td>Ethylene Glycol</td>
<td>29.9</td>
</tr>
</tbody>
</table>

6.2.3 Preparation of the Polystyrene Particles

Polystyrene particles with a diameter of 250 nm were prepared by emulsion polymerization following a procedure reported previously [110]. In brief, an emulsifier solution was first prepared by dissolving 5 g of 2-SEM in 50 g DI water, followed by diluting it with water to 100 g while the pH was adjusted to 3.5 by adding 10 wt% NaOH solution. For the emulsion polymerization reaction, an aqueous solution consisting of 1.0 ml of the 2-SEM solution, 5 ml ethanol, 14 ml DI water, 75 mg polyvinyl pyrrolidone and 100 mg VA-086 was constituted in a 20-ml vial, to which a 1.0-ml of styrene solution containing 164 mg NAS and 14.0 µl divinyl benzene was then added. The head
space of the vial was purged with nitrogen before it was sealed. The mixture was then emulsified on a vortexer, and then heated to and maintained at 70°C in a water-bath under stirring. The polymerization reaction was stopped after 10 h. The particles were washed with ethanol and DI water in a stirred ultrafiltration cell with a polyethersulfone membrane of a cut-off Mw of 300 kDa. Clean particles were stored in DI water at 4 °C if they were not used immediately. The number average diameter of the particles was measured as 258 ± 10 nm by accounting at least 100 particles from SEM images.

6.2.4 Preparation of the α-CT-Attached Particles

Enzyme immobilization was conducted by adding 250 mg of particles to 1 ml of 0.1 M pH 7.0 phosphate buffer solution containing 50 mg/ml α-CT. The solution was stirred for 12 h at 4°C before centrifugation. The particles were washed by ultrafiltration with a 500-kDa cut-off membrane and stored in pH 7.0 phosphate buffer at 4°C. The washing solutions were collected and assayed for protein concentration using the Bradford method. Enzyme loading was in the range of 33–39 mg-enzyme/g-nanoparticles.

6.2.5 Preparation of Spacer Mediated α-CT Attached Particles

Using a similar procedure, 250 mg of particles were first contacted for 1 h with ethylene diamine (0.1 M) in 1 ml to modify the surface with amine groups. Then the particles were washed from excess ethylene diamine by ultrafiltration. At the same time, PEG was modified at its both ends with ECH by dissolving 1 g of PEG in 3 ml of ECH and allowed to react for 12 hours. The modified PEG was recovered by evaporating ECH.
To attach PEG to the particle surface, 250 mg of PEG were added to 1ml containing 250 mg of particles. The mixture is left mixing for 24 hours, then particles were washed with water. Enzyme was then attached to the particles using the same procedure as above and loading ranged from 27-31 mg-enzyme/g-particles.

6.2.6 Activity Test

Typically, 374 µl of n-propanol are added to 10 ml of the selected solvent (conditioned for 3 days with 3 Å molecular sieves) to make a concentration of 0.5 M. Then 5.3 mg of APEE are dissolved in the solution to a concentration of 2.5 mM. From the reaction solution 200 µl were taken and analyzed using a Shimazu GC-17A gas chromatography equipped with a FID and a Restek RTX-5 column (15 m x 0.35 mm x 1.0 µm). For the analysis, 1 µl was injected with 100°C column temperature ramped to 190°C at a rate of 20°C/min then kept constant for 3.5 min. The injection port and detector temperature were set to and left at 250°C. APPE GC chromatograms were calibrated with APPE solutions in hexane. The reaction was initiated by adding 5 mg of α-CT (or particles containing 5 mg of enzyme) while the reaction was left mixing at 200 rpm on a shaker. The reactor was repeatedly sampled at time intervals to calculate the activity of the enzyme system.

6.3 Results and Discussion

The observed changes in the activity of the free α-CT enzyme when used with different organic solvents are summarized in Table 6.2. The free enzyme activity is expected to be dependent on the polarity of the organic solvent. The transesterification
activity of the native enzyme was highest using cyclo-hexane, performing 20 times better than polar solvent acetonitrile. It is believed that the water-immiscible solvents would preserve the water layer around the enzyme necessary for the catalysis. While water-miscible solvents can permeate through this layer thus reducing the catalytic activity [19].

Contrary to what is usually observed in enzyme biocatalysis, the apparent activities of nanoparticle-CT in organic solvents were found to be higher than those of the native. As shown in Table 6.2, nanoparticles-CT exhibited activities that are up to 1168 times higher than those of the native CT suspended in the same organic solvents. This enhancement was due to multiple factors contributing in the reaction such as dispersion of enzyme and the structural changes induced by the chemical bonding to the solid carrier.

Table 6.2 Activity data for the native and no-spacer particles-attached enzymes.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>δ (MPa)$^{1/2}$</th>
<th>Native-Enzyme Activity (µM-APPE·h$^{-1}$)</th>
<th>Particles-Enzyme Activity (µM-APPE·h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>14.9</td>
<td>1.30 ± 0.36</td>
<td>574 ± 211</td>
</tr>
<tr>
<td>Cyclo-Hexane</td>
<td>16.8</td>
<td>1.41 ± 0.42</td>
<td>512 ± 184</td>
</tr>
<tr>
<td>Benzene</td>
<td>18.8</td>
<td>1.07 ± 0.33</td>
<td>234 ± 49</td>
</tr>
<tr>
<td>Chloroform</td>
<td>19.0</td>
<td>0.00 ± 0.00</td>
<td>47 ± 16</td>
</tr>
<tr>
<td>o-Dichlorobenzene</td>
<td>20.5</td>
<td>0.89 ± 0.67</td>
<td>54 ± 30</td>
</tr>
<tr>
<td>Pyridine</td>
<td>21.9</td>
<td>0.41 ± 0.13</td>
<td>97 ± 63</td>
</tr>
<tr>
<td>Nitroethane</td>
<td>22.7</td>
<td>0.37 ± 0.08</td>
<td>432 ± 76</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>24.3</td>
<td>0.07 ± 0.04</td>
<td>53 ± 21</td>
</tr>
<tr>
<td>Ethylene diamine</td>
<td>25.2</td>
<td>0.43 ± 0.10</td>
<td>223 ± 91</td>
</tr>
<tr>
<td>Nitromethane</td>
<td>26.0</td>
<td>0.34 ± 0.08</td>
<td>382 ± 37</td>
</tr>
<tr>
<td>Ethylene Glycol</td>
<td>29.9</td>
<td>0.43 ± 0.13</td>
<td>128 ± 31</td>
</tr>
</tbody>
</table>
Figure 6.1 Percent (%) activity differences compared to no-spacer. (◊) No-spacer particles, (□) PEG\textsubscript{550} spacer particles, and (△) PEG\textsubscript{10k} spacer particles.

Figure 6.1 suggests that the activity of the no-spacer enzyme has advantage over the spacer enzymes at lower δ (14.9-19.0 MPa\textsuperscript{1/2}). This behavior was unexpected since even if the spacer did not provide any extra mobility, it is not expected to reduce the activity. To analyze this behavior, one have to take a closer look at the solvents in this range benzene, c-hexane, and n-hexane. All of those solvents are highly hydrophobic and thus commonly used in liquid-liquid extraction and delipidation process. Typically, every protein has some hydrophobic and some hydrophilic moieties, and while such protein is in hydrophobic solvent, the solvent may cause denaturation of the protein and hence loss of activity. Immobilization with no spacer has the advantage of possible multipoint attachment which may stabilize the protein. The absence of spacer would also bring the protein on the surface to cluster together to resist any hydrophobic denaturation. At these conditions the no-spacer enzyme may perform better than the spacer enzyme.
Figure 6.2 Transesterification activity of α-CT at (A) 4 oC, (B) 23 oC, and (C) 40 oC in different solvents. (◊) No-spacer particles, (□) PEG\textsubscript{550} spacer particles, and (△) PEG\textsubscript{10k} spacer particles.
When different temperatures were tested, high temperatures provided chance for higher mobility of the PEG (23-25 MPa\(^{1/2}\)) and thus reduce the effect observed at lower δ values. The maximum observed activity was at δ = 24.3 MPa\(^{1/2}\) for PEG\(_{10k}\) spacer and provided 220% increase in activity compared to the no-spacer. This behavior allows a potential design parameter for the selection of polymeric spacers or solvent to obtain highest mobility.

6.4 Conclusion

The use of spacer is expected to provide the enzyme with more accessibility, which makes it more active. In this chapter the issue of “which spacer should be selected?” was raised. It was observed that highest improvement in activity (220%) was observed at δ neighboring that of the spacer. This observation sustains the claims that the flexibility issue is of key importance for selecting a spacer. While selecting a polymer with δ close to that of water would result in a more active biocatalyst.
CHAPTER VII

CONCLUDING REMARKS

In the presented research, five interconnected studies were presented bridging between enzyme separation, immobilization, and the use of nano-structured materials in constructing multienzyme biocatalysts. Those studies outlined in chapters 2-6 were presented in the objectives of chapter I.

In the first study, enzymatic degradation of TCE was studied to explore the possibility of using cell-free enzymes for environmental biodegradation. The results uncovered that enzymes were able to degrade TCE, however at a rate lower than viable bacteria. While degradation was proven, possible inhibition hindered the *in vitro* degradation.

The results in the second study demonstrated that highly nanoporous materials enabled the multienzyme reaction of lactate and glucose dehydrogenases. Proof of cofactor shuttling has been evident and the hypothesis of concave structure effect was established. The obtained results provided a viable potential for the immobilization of the multi-enzyme system from bacteria. The suggested continuation for this study is to explore a higher temperature range for the catalysis. The nanoporous surfaces are known to provide supreme stability for the enzymes; it would be interesting to explore the extent of the increased stability at higher temperatures, thus the advantage of this system.
Methanol producing enzymes from carbon dioxide have been immobilized to polymeric nanoparticles, while the cofactor was immobilized on similar but separate particles. Immobilized systems showed productivity comparable to the free system and successfully achieved a cumulative yield exceeding of 100% after 10 reuse cycles. The evidence of an active cofactor attached to solid particles to mediate reactions of immobilized enzymes suggest a wealth of potential for these expensive redox mediators. In addition, these cofactor can be easily recovered and reused in the same way as immobilized enzymes.

Due to the multidisciplinary nature touching the fields of enzyme, nano-, and environmental technologies, multienzyme L-lactate synthesis inspired from the nanoparticles-attached system was studied. The interest in this reaction system comes from two considerations, the internal cofactor regeneration loop and carbon dioxide fixation. The system was explored in details in its free state, compared with a kinetic model, particle sizes, materials of particles, and spacer length. Likely continuation for this study is to further optimize the reaction for higher activity and reusability.

To establish solid criteria for the selection of spacer other then its length, the effect of spacer flexibility (solubility) was investigated. The extent of improvement of more flexible spacer was accounted for by performing the reaction in different organic solvents. Transesterification reaction catalyzed by α-CT was used as model reaction. In conclusion, highest activity increase was observed at solvent solubility parameter neighboring that of the spacer. To have more comprehensive understanding of spacers, the study can be continued to verify the line between stability effect and flexibility effect.
To achieve that, a parallel study is suggested to be performed in aqueous solutions using different polymeric spacers of different $\delta$ values.
REFERENCES


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LIST OF PUBLICATIONS


