ORGANIC PHASE ENTRAPMENT OF GLUCOSE OXIDASE IN POLYMERIC NANOPARTICLES

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ORGANIC PHASE ENTRAPMENT OF GLUCOSE OXIDASE IN POLYMERIC NANOPARTICLES

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ABSTRACT

Immobilizing enzymes using polymeric particles is an excellent means to increase the reusability of a biocatalyst. Recovering active enzyme free in solution is difficult. However, the immobilized enzyme-particle system can be easily removed using methods such as filtration or centrifugation. One interesting immobilization technique is to entrap enzymes within the polymer matrix. To achieve this, polystyrene particles of varied size (100 to 750 nm) and degree of cross-linking (monomer: cross-linker molar ratios ranging from 1:1 to systems with no cross-linker) were treated with glucose oxidase in toluene solution. In toluene, the polymer expands, allowing enzyme to diffuse into the matrix. Then, the particles are treated with hexane, a worse solvent, which forces the polymer to shrink, entrapping the enzyme. The enzyme-polymer particle complexes were tested using a standard enzyme activity assay to quantify the various systems of varied size and cross-linking. The effect of cross-linking appears to be that there is a minimum level at about 0.1 moles/mole required to support reaction, which had an observed reaction rate of about 65 µmol min⁻¹ mg solid⁻¹. Kinetic studies at varied polymer particle size indicate that smaller particles result in higher reaction rates. Kinetic studies at varied polymer particle size indicate that smaller particles result in higher reaction rates; for the case of 250 nm particles, the observed rate was close to 30 µmol min⁻¹ mg solid⁻¹.
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# TABLE OF CONTENTS

| LIST OF TABLES | viii |
| LIST OF FIGURES | ix |
| LIST OF SCHEMES | x |

## CHAPTER

### I. INTRODUCTION

1.1 Enzymes as Catalysts ................................................................. 1
1.2 Objectives .................................................................................. 4
1.3 Approaches .................................................................................. 5
   1.3.1 Enzyme Modification .............................................................. 5
   1.3.2 Particle Synthesis ................................................................. 5
   1.3.3 Enzyme Immobilization ......................................................... 6
   1.3.4 Enzyme Mass Loading ............................................................ 7
1.4 Scope ............................................................................................ 8

### II. LITERATURE REVIEW

2.1 Biocatalysis ................................................................................. 9
2.2 Glucose Oxidase .......................................................................... 10
2.3 Enzyme Modification ................................................................. 11
2.4 Enzyme Immobilization ............................................................... 13
2.4.1 Surface Attachment ......................................................... 13
2.4.2 Internal Entrapment ...................................................... 14
2.5 Nanoparticle Preparation and Characterization ...................... 14

III. KINETICS AND ENZYME LOADING RESULTS ......................... 16
3.1 Introduction ........................................................................ 16
3.2 Materials and Methods ..................................................... 19
  3.2.1 Preparation of Nanoparticles ..................................... 19
  3.2.2 Enzyme Solubilization ............................................... 20
  3.2.3 Enzyme Entrapment .................................................. 21
  3.2.4 Protein Loading Measurement .................................... 22
  3.2.5 Kinetic Activity Measurement .................................... 23
  3.2.6 Particle Characterization .......................................... 24
  3.2.7 The Effect of Cross-Linking on Particle Size ............... 25
3.3 Results ............................................................................. 27
  3.3.1 Methodology for Particle Size Control ....................... 28
  3.3.2 System Comparisons of Immobilized, Modified, Free Enzyme .... 30
  3.3.3 Enzyme Loading Measurements ................................ 35
  3.3.4 The Effect of Particle Size ........................................ 36
  3.3.5 The Effect of Cross-Linking ...................................... 38

IV. THEORETICAL CONSIDERATIONS .......................................... 41
  4.1 Introduction .................................................................. 41
  4.2 Enzyme Loading Kinetic Theory ..................................... 42
  4.3 Internal Mass Transfer Discussions ................................. 43
4.4 External Mass Transfer Discussions

V. FURTHER DISCUSSIONS

5.1 Oxygen Limited Reaction

5.2 Discussions Relating the Experimental and Theoretical Sections

VI. CONCLUSIONS

BIBLIOGRAPHY
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>Output for Light Scattering of 218 nm Particles</td>
<td>25</td>
</tr>
<tr>
<td>3-3</td>
<td>Experimental Design, Adjusting the Aqueous Phase to Control Particle Size</td>
<td>29</td>
</tr>
<tr>
<td>3-3</td>
<td>Experimental Design, Adjusting the Organic Phase to Control Particle Size</td>
<td>30</td>
</tr>
<tr>
<td>4-1</td>
<td>Calculating the Thiele Modulus for Different Particle Sizes As a Function of Deff and the Porosity Factors</td>
<td>46</td>
</tr>
<tr>
<td>4-2</td>
<td>The Damköhler Number and the External Effectiveness Factor as Functions of Thin Film Thickness</td>
<td>51</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>SEM Image of 100 nm Particles</td>
<td>24</td>
</tr>
<tr>
<td>3-2</td>
<td>Output for Light Scattering Measurements of Particle Size and Particle Size Distribution</td>
<td>25</td>
</tr>
<tr>
<td>3-3</td>
<td>The Observed Effect of Cross-Linker on Particle Size</td>
<td>27</td>
</tr>
<tr>
<td>3-4</td>
<td>Linearization of Michaelis-Menten Kinetics to Find the Effective Rate Constant for Free Natural Enzyme</td>
<td>32</td>
</tr>
<tr>
<td>3-5</td>
<td>Comparing Best Particle Reaction System to Free Enzyme Reaction</td>
<td>33</td>
</tr>
<tr>
<td>3-6</td>
<td>Comparing Free Natural Enzyme to Modified Enzyme</td>
<td>34</td>
</tr>
<tr>
<td>3-7</td>
<td>The Experimental Results for Mass Enzyme Loading, $[E]_0$</td>
<td>36</td>
</tr>
<tr>
<td>3-8</td>
<td>Reaction Rate as a Function of Particle Size</td>
<td>38</td>
</tr>
<tr>
<td>3-9</td>
<td>Reaction Rate as a Function of Cross-Linking</td>
<td>40</td>
</tr>
<tr>
<td>4-1</td>
<td>The Effect of the Particle Porosity Constants on the Thiele Modulus</td>
<td>45</td>
</tr>
<tr>
<td>4-2</td>
<td>The Effect of the Thiele Modulus on the Internal Substrate Concentrations</td>
<td>48</td>
</tr>
</tbody>
</table>
## LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>11</td>
</tr>
<tr>
<td>4-1</td>
<td>42</td>
</tr>
</tbody>
</table>

2-1  The Glucose Oxidase Reaction

4-1  Overall Oxidation Reaction of Glucose Using the Enzyme GOx
CHAPTER I

INTRODUCTION

1.1 Enzymes as Catalysts

Enzymes are becoming of greater interest to chemical engineers due to their amazing capacity to solve some of sciences most complicated and interesting problems. It has been reported many times to the point where it is common knowledge in the field of bio-engineering that enzymes have the potential to catalyze many important industrial reactions both more efficiently and without the need of extreme temperatures, pressures or pH values. Indeed, at extremes most of all enzymes actually become ineffective (32). Furthermore, there is a large variety of enzymes that act as natural catalysts. Most of these natural catalysts function best in aqueous phase. Some function best in organic phase, and yet others have naturally optimized at phase interfaces (32-33, 39-40).

Enzymes have already found their way into many applications. As some examples, a great deal of research is under way to use cellulases for the degradation of organic wastes such as wood pulp and old newspaper into fermentable sugars to form ethanol, a potential fuel source (27, 28). Another important application of enzyme reactions is glucose sensors, for example for diabetes patients. The enzyme glucose oxidase is very sensitive to glucose concentrations and can thus be calibrated to detect glucose levels in blood (29). Furthermore, research is very active in the development of
biofuel cells. Moreover, in the food industry, enzymes are already used to process dairy, alcohol and many other products (30).

A great deal is already known about the biochemistry of these large proteins, such as binding sites, active sites, 3-D folding conformation and even how and why the proteins are synthesized in vivo (32). However, there is still much to be learned. Even so, one very interesting area of research has to do with the practical processes revolving around biocatalysts and their use. Because it is difficult to recover free enzyme out of aqueous solution without precipitation or other methods which inherently damage the protein, it is a significant challenge to efficiently recover enzyme from reactants and products in a chemical process. Recovering the enzymes allows for catalysts recycle, for further use. In answer to this, in recent years much focus has been centered on the immobilization of enzymes (5, 14-15, 18, 22-27, 41, 43, 46-49).

In the process of immobilizing an enzyme, the protein has essentially become part of a much larger system. Instead of having Stokes radius (relating to the dynamic volume) on the order of $10^{-10}$ m in solution (31), the enzyme can be immobilized with a particle much larger, on the order of $10^{-7}$ m. A common consequence of enzyme immobilization is reduced activity as compared to enzyme free in solution, probably due to factors revolving around a hindered active site, a misfolded protein, or reduced mobility of the peptide in solution (32). Smaller immobilization scaffolds tend to reduce this effect (5, 15), but a point would eventually be reached where the scaffolds are so small they can not be recovered from solution.

There are already many methods of enzyme immobilization as reported previously (5, 11, 14-17, 22-26, 29). Some of these methods include surface attachment
(physical or covalent) to nano-structures, such as carbon nanotubes, polymer particles and polymer fibers. Other methods, including the one which was the basis for the experimentation discussed below in this paper, involves the entrapment of enzyme within a polymer matrix. The differences between these methods can be exploited depending upon the problem to be solved.

In the surface attachment methods, commonly the nano-structure is activated with a functional group which will create covalent bonds with the protein (5, 51-54, 57). In contrast, with enzyme entrapment discussed in the following thesis, the process is completely physical in nature. No chemical reactions take place between the polymer support and the protein (23). A polymer particle will swell in different solvents; good solvents, by definition, will swell the polymer more than poor solvents (1-4, 8, 13). If enzyme is modified in such a way to be soluble in a good solvent for a particular polymer, the enzyme could be introduced into the particle via equilibrium swelling and diffusion. Then, once equilibrium volume swelling has been reached, the polymer particle can then be shrunk by a bad solvent, ideally trapping at least some of the enzyme within the polymer matrix. Finally, the enzyme’s natural substrate can be introduced to the particles, where the new system would act much like a catalytic pellet.

Enzymes have been classified based on their specific reaction types as per The International Union of Biochemistry and Molecular Biology and the International Union of Pure and Applied Chemistry. Oxidoreductases catalyze oxidation-reduction reactions; i.e. reactions with electron transport using cofactors such as FAD, as in reactions with Glucose Oxidase. Transferases catalyze functional group transferring reactions, such as phosphorylation using ATP. Hydrolases catalyze the hydrolytic cleavage of various
bonds, such as those in cellulose to form fermentable sugars. Lyases are those enzymes which add groups to or cleave groups from double bonds, which, for example, is important in processes such as the pyruvate pathway. Isomerases assist in intramolecular rearrangements. As the name suggests, an isomer of the original compound is formed. The final category, ligases catalyze the joining of two molecules, often with the assistance of ATP free energy (32).

1.2 Objectives

The goal of the research work was to explore the novel method of enzyme immobilization. The quantitative measurement to study the new immobilization technique was observed reaction velocity. The results are then discussed in terms of diffusion and activity terms, specifically with regards to the Thiele Modulus (a measure of the internal effectiveness factor) and the external effectiveness factor. Surface immobilization of enzyme onto polymer nanoparticles has been studied before (5, 14, 51-54). The concept of organic phase entrapment of enzyme within a polymer nanoparticle matrix is a new approach. Consequently, the research objectives are summarized below.

1. Synthesize and characterize polystyrene nanoparticles of varied size and degree of cross-linking.
2. Develop GOx- nanoparticle complex by entrapping the enzyme inside the nanoparticles and measure the resulting reaction rates.
3. Estimate the enzyme loading of the prepared biocatalyst.
4. Understand the effects of particle size and degree of cross-linking on the activity of the immobilized glucose oxidase.
1.3 Approaches

In this study, the method for creating the enzyme-nanomaterial composites was entrapment within the polymer matrix. The enzyme carrying nano-structure material was polystyrene cross-linked with divinyl-benzene, and the enzyme glucose oxidase (GOx) was used as a model enzyme to quantitatively study the immobilization technique. More specifically, the effects of different particle size and degree of cross-linking were explored. In addition, the immobilized enzyme was compared to free natural enzyme, as well as free modified enzyme to free natural enzyme. Finally, enzyme yield of the modification protocol was considered. The GOx-entrapped particles were applied to catalyze biotransformation reactions, and the product was monitored using a standard GOx activity assay.

1.3.1 Enzyme Modification

As discussed in more detail below with references (methods section, chapter III), enzymes have been modified following a procedure reported previously (11) in such a way that the protein was paired with a surfactant to became organic soluble. Standard reaction assay tests were run to see the effect of the modification procedure on enzyme activity, which was found to be roughly 10% retained activity. Furthermore, during the modification process, roughly 50% of the protein by weight is lost.

1.3.2 Particle Synthesis

Polystyrene particles cross-linked with divinyl-benzene have been synthesized before and are well understood, as described below (5). The particle size is controlled by varying the reaction protocol, in both the organic and the aqueous phases of the emulsion.
reaction. The emulsion reaction was initiated using free radicals. The particle size distribution is normally very narrow in emulsion polymerization (13). The degree of cross-linking can be controlled experimentally by varying the relative amounts of monomer (styrene) and cross-linking agent (divinyl-benzene).

The reaction is initiated by free radicals. Finally, particle characterization was completed primarily by light scattering, which estimates the effective particle diameter based on the theory that particles of different sizes have different velocities in solution. The theory revolves around the fact that the particles have the same kinetic energy in solution (all at the same temperature), and the assumption that all of the particles have the same density (to convert from mass to volume and thus diameter), a reasonable assumption, so long as all the particles under study are of the same material. Finally, the particle average velocities are measured as a function of the degree of laser beam scattering by sample compared to a reference (65).

1.3.3 Enzyme Immobilization

Enzyme immobilization is an important step in bio-processing, as it allows for the recovery of the catalyst. Because immobilization is usually accompanied with reduced activity, a great deal of focus is on enzyme immobilization using nanoscale structures, so as to minimize the effects of lost mobility (5, 14-15).

The immobilization technique was to entrap glucose oxidase within the matrix of nano-scale polystyrene latex particles, cross-linked with divinyl-benzene. The particle sizes and the degree of cross-linking were varied and the effects were analyzed.
In general, smaller particles yielded higher reaction rates, a result of greater particle mobility and reduced diffusion limitations. The effect of cross-linking appeared to be more discontinuous. At low degrees cross-linking, the observed reaction rates were quite low; at higher degrees the reaction rate seemed to jump to a higher level, but no trend was observed. One explanation is that the cross-linking needed to be high enough to create a sufficiently tight particle to entrap glucose oxidase inside. In other words, looser particles allowed some enzyme to seep out during the washing procedures.

1.3.4 Enzyme Mass Loading

The loading of GOx in the PS particles was calculated by monitoring the amount of enzyme applied before and after entrapment. Equilibrium conditions were confirmed assuming Fickian diffusion of solvent into the spherical polymer pores at unsteady state. The mass loading of enzyme was also measured using analytical techniques, exploiting the fact that GOx is yellow in color and absorbs light at 450 nm.

The amount of GOx loaded into the polystyrene particles was determined via a materials balance. The mass of enzyme in the bulk phase was measured before the introduction of nanoparticles. After adequate time was allowed for equilibrium swelling, the particles were washed with increasing dilutions of a poor solvent, hexane. Each of these washes was analyzed using UV-Vis spectrophotometry and compared to a calibration curve to calculate the mass concentration of GOx. In addition, after each of the nanoparticle enzyme systems was dried and lyophilized, it was washed with aqueous solutions. These aqueous washing solutions were also measured using the calibration curve for glucose oxidase in water.
1.4 Scope

In outline, this thesis is presented as follows. Chapter II reviews some current literature about biocatalysis, enzyme immobilization, and nanobiotechnology. Chapter III focuses on the methods and results of experiments conducted on the enzyme-nanoparticle complex systems. In chapter IV some of the theory behind modeling the entrapped enzyme systems is explored. The pertinent theory in chapter IV includes Michaelis-Menten kinetics, the Thiele Modulus, internal diffusion coefficients (resulting from pore geometry and porosity) and external diffusion limitations. Finally, the conclusions are summarized in chapter V.
2.1 Biocatalysis

Some of the first known examples of biocatalysis for commercial use involved the use of whole cells, or live micro organisms rather than purified enzymes. The advantage of the whole cell approach is the inherent availability of cofactors and coenzymes (18). Examples of applications are ethanol fermentation and vitamin C production. More recently, some applications of enzymes have turned to the use of purified enzymes, which have been isolated from the cell, most commonly from bacteria or the more simple eukaryotes like yeast and fungus. Probably the most important and widely used method of generating a variety of enzymes from these sources is directed evolution (33, 34). These applications are becoming more and more significant. In 2002 the global market for enzymes was $1.89 billion; in 2004 it was $2.00 billion; it is estimated that in 2009, the market will be $2.35 billion (35).

Current applications include a wide variety of different fields, from fuel production and waste management to surfactants to pharmacy and medicine, and almost everything in between. For example, lipases (enzymes which catalyze the degradation of lipid molecules) have been used in applications such as biodiesel, pharmaceuticals and
biosurfactants (36). In one example of many pharmaceutical applications, enzymatic systems (involving amino acid oxidases, catalase, and many dehydrogenases) are used to produce a stereoselective drug family of satins. These drugs are used to control cholesterol, a multi-billion dollar market (37). In waste water treatment and bioremediation, an example of one major problem is the removal of halogenated compounds. One group of such biocatalysts is the hydrogen-halide lyases (38). Glucose oxidase has been studied in biofuel cell applications (16, 17).

As the commercial and academic use of biocatalysts continues to grow, the availability of enzymes and other biocatalysts will only continue to be mainstreamed and commercialized. The common problems involved in biocatalysis will be answered more efficiently, because both the availability and applicability of enzymes will be increased by site-directed mutagenesis and directed evolution (39). Enzymes, if in their natural, free state are often difficult to recover and are relatively unstable in higher temperatures or pressures, and in extremely acidic, alkaline, and saline conditions (32). Methods such as immobilization and enzyme and/or process modifications can be used to minimize these problems (5, 11, 22-26, 39, 40).

2.2.1 Glucose Oxidase

The enzyme used in this study was glucose oxidase. Glucose oxidase is a glycoprotein consisting of two homodimers, each at 80 kDa. The enzyme is considered a flavoprotein, indicating the necessity of flavin adenine dinucleotide (FAD) which is a coenzyme part each of the two subunits (66).
Glucose oxidase falls under the class of oxidoreductase enzymes (chapter I, section 1). The enzyme uses a coenzyme (not covalently bound) as an electron transport molecule for catalysis. Substrate binding depends on glutamine residues to hydrogen bond with the hydroxyl groups of glucose. Once the glucose is bound to the active site of glucose oxidase, FAD is reduced to FADH$_2$ and glucose is oxidized to gluconic acid (one of the –OH groups is oxidized to a ketyl =O group), assisted in part by histidine and glutamine residues in the active site. To complete the cycle, molecular oxygen is reduced to hydrogen peroxide as FADH$_2$ is oxidized back to FAD which can then be reused to oxidize another molecule of glucose and produce another molecule of hydrogen peroxide. The reaction scheme is presented in schematic 2-1 (66-69).

![Scheme 2-1 The glucose oxidase reaction, including the formation of hydrogen peroxide](image)

2.3 Enzyme Modification

Many important industrial syntheses, such as pharmaceutical and fine, specialty chemicals occur in organic phase. Most enzyme systems are naturally aqueous, and consequently their direct application to organic phase syntheses is difficult. Indeed, unmodified aqueous enzymes used in organic solvents can experience a reduced activity by a factor of $10^5$ (40). Consequently, there is great motivation to engineer systems in a
manner such that the loss of activity is less drastic. Methods to enhance the enzyme system so that natural activity is retained include solvent engineering and protein engineering.

Solvent engineering is often simpler and involves the use of co-solvents. Because the solvent system must be organic (reactants), co-solvents such as organic solvents or even a very small amount of water (< 1% v/v) can be added that help the protein to maintain its 3-D shape.

In contrast, protein engineering involves the mutation of proteins in such a way that their structure changes to optimum configuration in a new solvent (40). Alternatively, the protein can exposed to a surfactant, leading to solubility in organic phase (11).

Finally, the last method of enzyme system modification discussed here is an interesting compromise of solvent and protein engineering. The protein is engineered, but in a quasi-reversible way. Removal of organic solvent (i.e. by drying) and addition of aqueous environment reverses the modification step and the enzyme could again be dissolved in water. As discussed in the results in chapter III, the re-dissolved protein lost some of its natural activity.

The cause of the lost activity was not explicitly explored, but there are a couple of possible reasons. In one case, the non-covalent interaction between the protein and the coenzyme may have been disrupted during the manipulation steps. In addition, in the context of organic phase it is indeed possible that the 3-D structure of the protein is compromised and only retains a fraction of the normal activity.
2.4 Enzyme Immobilization

The immobilization of a biocatalyst, be it an enzyme or micro-organism, is a common solution to the problem of difficult biocatalyst recovery and low protein stability in conditions that deviate from the protein’s natural environment. A common problem with immobilization is that biocatalysts often lose some of their natural activity (41). Interestingly, in some cases, the active site exposure to substrate is actually enhanced (42). Immobilization involves a two part system. The first part is the biocatalyst itself and the other is the solid support. Some common supports include sol-gel and mesoporous silica glass, polymeric particles, polymeric fibers, carbon nanotubes and membranes (5, 11, 14, 43-48).

2.4.1 Surface Attachment

Many methods for enzyme immobilization have been reported and discussed. Some of these include attachment to structure surfaces either by physical adsorption (49-50) or covalent bonds (5, 14, 15, 29, 51-55). Physical adsorption exploits specific regions of the protein that associate strongly (i.e. by salt bridges or hydrophobic interactions) with a specific structure. In contrast, covalent bonding of proteins to the surface normally involves activation of the structure with functional groups. For example, Zhang, et. al, covalently attached glucose oxidase to a gold electrode by use of gold nanoparticles activated stepwise with amine groups, which will form covalent bonds (via peptide bonding) with the carboxylic terminus of the protein (29). In another report (5), the catalytic activity (as $k_{cat}/K_M$) showed very little change compared to free enzyme
for cases surface attachment to very small particles (100 and 270 nm). However, the enzyme loading was shown to be quite low, less than 10%.

2.4.2 Internal Entrapment

In contrast to surface attachment, biocatalysts have been immobilized by entrapping them inside a given structure, such as a polymer particle (46-47). In fact, Lei, et. al. (47) entrapped organophosphorous hydrolase inside silica pores (12 to 15 µm silica beads). The pores were functionalized with carboxy and amino groups, which covalently bind to the amino acid residues of the enzyme. The enzyme retained as much as 84% of its original activity, but protein loading inside the pores was low, 0.2% w/w. No discussions were found regarding the entrapment of enzyme via physical interaction within a nanosized polymeric cross-linked particle.

2.5 Nanoparticle Preparation and Characterization

Of great interest for immobilization of enzymes are nanoscale structures. The smaller dimension allows for greater enzyme mobility in solution (5). Geometrically speaking, based on the same total mass of structure, those structures with smaller dimensions will have greater surface: volume ratios. Consequently, such structures have higher potential for enzyme mass loading. It will be interesting to see, as technology allows for smaller and smaller structures, when a point is reached where the structure is too small to effectively immobilize the protein.

Nanoparticles have been synthesized in a variety of ways. One method that is particularly effective is from polymers. There are some important advantages to polymer synthesis. First, emulsion polymerization has been shown to be an extremely efficient
and reliable technique to synthesize polymeric particles of narrow size distribution (5, 6). Second, polymers can be easily modified with co-polymers, wherein multiple functional groups can be very easily incorporated into the particle (5, 12, 14, 19, 24, 26, 33, 42, 54). In addition, because polymers are not completely solid structures, polymers have a very high surface area to volume ratio, as long as the pores can be efficiently exploited in enzyme immobilization (5, 15, 22-24).

Methods for polymer nanoparticle synthesis often include emulsion polymerization with free radical initiation (5, 5, 58-63, 65). The organic phase typically is made up of monomer and co-monomer(s) and cross-linking agent. Water, sometimes an alcohol, initiator, surfactant and stabilizers normally constitute the aqueous phase. The reaction is commonly initiated by heat, but other mechanisms such as radiation can be used. Finally, the reaction can be terminated by any number of mechanisms, including the effective removal of monomer, and the removal of initiator and initiating mechanism (heat, radiation). In addition, there are many other molecular mechanisms for termination which much be controlled, like chain transfer and inefficient initiation (13).

Polymeric nanoparticles are characterized in many different ways. Some of these methods include scanning electron microscopy (SEM), transmission electron microscopy (TEM), and light scattering. SEM of polymers normally requires sputter coating of a thin layer of conductive metal, like gold (63, 65). TEM imaging requires staining agents, such as osmium tetroxide (58-64). Finally, light scattering is commonly used in particle characterization. This technique involves focusing a laser at the sample in a known solution (where the light refraction is already known and calibrated against as a zero) and then measuring the new refracted light at a specific angle (65).
3.1 Introduction

The use of enzymes as an engineering tool to convert reactants to desired products has been studied for many years, by many groups (5, 11, 14-24). To this end, it has been found desirable to immobilize the biocatalyst so that the enzyme can be separated easily from reaction medium, thus allowing for enzyme recycling. Nanostructures, such as nanoparticles and carbon nanotubes, have been of particular interest for enzyme immobilization, due to their inherent advantages over larger structures such as higher accessible surface area and increased mobility in solution (5, 25, 26).

Polymeric structures lend their own added advantages to the equation. Polymers can be synthesized from a large variety of monomers, and techniques have been established to efficiently produce a variety of particle morphologies and compositions, allowing for a great deal of system control for structure optimization (11-14, 23). In addition, co-monomer systems can be used to add functional groups and cross-linking to the nanostructure, depending on the needs of the application (13).

Extensive work has been applied into the understanding of catalytic nanoscale materials such as nanofibers, nanoparticles and carbon nanotubes (5, 11, 14-17, 22, 24-
In fact, some applications involving around these new nanotechnologies include bio-fuel cells, bio-activated particles, and activated membranes (5, 14, 16, 17). In a great deal of the successful applications studied so far, the biocatalysts are covalently attached to the surface of the nanostructure.

One of the possible drawbacks of surface activation of nanostructures is the inherent limitation of protein loading. Typical industrial biocatalysts have enzyme loadings below 1%-wt (15, 27). Moreover, in a previous study where enzyme was covalently attached to a polymeric nanoparticle surface, enzyme loading was considered using mono-layer theory (5, 14, 15, 25). That is, only the amount of enzyme that can fit on the particle mono-layer surface can be immobilized.

A novel immobilization technique is the physical entrapment of enzyme inside a polymer matrix. Comparing the potential immobilization volume, entrapment of enzyme within a sphere has potential for higher enzyme loading than would mono-layer surface coverage of the same sphere (so long as the particle radius is at least three times the mono-layer thickness. For a glucose oxidase hydrodynamic radius on the order of $10^{-10}$ m, so the nano-scale spherical volume is still larger than the mono-layer volume). Internal entrapment of enzyme inherently leads to diffusion limitations. As such, smaller structures are paramount to minimize this effect (14, 25, 26).

In order to entrap the enzyme in the polymer matrix, three steps must occur. First, the solvent must be a good enough solvent (13) to cause the matrix to swell. If no swelling occurs, there will be too little diffusion of the protein into the particle. Second, to successfully manufacture a biocatalytic particle, the enzyme must be able to form a homogeneous solution with the solvent that fits this description, and not denature. If the
protein does not dissolve, any amount of solvent diffusion into the particle would have no benefit and if the protein denatures, the catalyst would be inactive. Third, and finally, once the protein has formed a homogeneous solution with the solvent and has penetrated into the polymer matrix, the matrix must be shrunk to force the entrapment of the enzyme.

The protocol of modifying a protein so that it will dissolve in organic phase has been established and discussed (11). The protein complexes with a surfactant and the complex is able to dissolve into the organic phase. Finally, after the solvent and enzyme are in equilibrium with the polymer, a poorer solvent must be introduced to the system to cause the polymer matrix to constrict and entrap the enzyme (13). The poor solvent must be compatible (i.e. miscible) with the first solvent, or it would not be able to interact with the polymer. Furthermore, the poor solvent must be poor enough to shrink the particle to an extent where enzyme cannot leech back out.

Experiments were conducted to better understand the process of enzyme entrapment. To quantify the loss in activity of surfactant-complexed enzymes compared to natural enzymes, kinetic tests have been run on enzyme that has been modified, dissolved in organic phase and then re-dissolved into aqueous phase, and the activity compared to the free enzyme in aqueous. Second, the swelling and enzyme loading process was considered by using existing polymer swelling theoretical models (2, 4). In addition, to examine the process and effect of particle shrinking, experiments were conducted to test the effect of cross-linking on the reaction kinetics. Finally, to better understand the system as a whole and its potential practical applications, experiments were run with varied particle sizes to see if the system would follow the expectation the
smaller particles yield higher observed reaction velocities. In the case of entrapped enzyme, this expectation is based on shorter diffusion resistances. As will be discussed later in this chapter, the effect of particle size in the nanometer range likely has nothing to do with diffusion (due to severe reaction limitation), but rather enzyme availability and particle mobility on solution. These forces tend to favor smaller particle diameters (5, 6).

3.2 Materials and Methods

Glucose oxidase (GOx) from A. niger, Peroxidase from soybean, sodium acetate trihydrate, divinyl-benzene (DVB), β-D-glucose, and o-Dianaisidine were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol and sodium hydroxide (NaOH) were purchased 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). 2-Sulfoethyl methacrylate (2-SEM) was purchased from Monomer-Polymer & Dajac Labs, Inc. (Feasterville, PA). Polyvinylpyrrolidone (PVP, MW 29 kDa) and didocecyldimethyl ammonium bromide (DAB) were purchased from Aldrich (Milwaukee, WI). Styrene was purchased from Acros Organics (Belgium). HPLC-grade toluene was obtained from Omnisolv (Charlotte, NC).

3.2.1 Preparation of Nanoparticles

Polystyrene particles were synthesized using emulsion polymerization. Particles varying from 100 to 750 nm were prepared by varying the reaction mixture recipe. To control particle size, the amount of surfactant, total amount of monomer and cross-linker, and the concentration of particle stabilizer were all adjusted. More specifically, adding less surfactant will produce larger particles, adding more monomer will increase particle
size and adding stabilizer (PVP) helps to control particle size and morphology (5). These parameters were systematically adjusted in order to control particle size.

Surfactant stock solution was prepared by dissolving 5 g of 2-SEM in 50 g DI water, followed by dilution to 100 g with water and pH adjustment to 3.5 by adding 10% NaOH. The reactor emulsions were prepared by mixing organic phase with aqueous phase. The organic phase contained monomer (styrene, 0.45 to 5 ml) and cross-linking agent (DVB, 1.25 to 560.55 µl). The aqueous phase contained initiator (VA-086, 2.5 mg ml⁻¹), stabilizer (PVP, up to 10 mg ml⁻¹), ethanol (0.125 to 0.5 ml ml⁻¹), surfactant (2-SEM stock solution, 5 to 75 µl ml⁻¹) and 20 ml water. The mixture vial was purged with nitrogen, sealed, and emulsified using a vortexer. Polymerization reaction was initiated at 70 to 75 °C in a water bath with stirring. The reaction was stopped after 10 to 12 hours and the particles washed with three washes of ethanol and DI water. Each consecutive wash was completed by centrifuging the particles and removing the supernatant liquid then re-dispersing the particles in the next wash solution.

The particles were characterized using both Scanning Electron Microscopy and light scattering. Based on light scattering data, the particles proved to be of uniform distribution with typical particle dispersions (Dₘ/Dₙ) between 1.0 and 1.1

3.2.2 Enzyme Solubilization

The organic-soluble enzyme was produced by mixing two phases: enzyme in 20 mM pH 5.5 sodium acetate buffer with organic soluble surfactant (DAB). Specifically, 7 mg of glucose oxidase were dissolved into 10 ml buffer and 9 mg surfactant was dissolved into 10 ml toluene. The corresponding enzyme concentration in the aqueous
phase was 4.4 µM and the surfactant concentration in organic phase was less than 2.0 mM. The critical micelle concentration of DAB is on the order of 14 mM (9). It is important that the surfactant concentration be lower than the CMC. If micelles form during the mixing, the enzyme would be inside the micelle, surrounded by but not dissolved into toluene.

Furthermore, high concentrations of surfactant impair the ability of polystyrene to swell with toluene (3). The two solutions were mixed and stirred at 500 rpm for two minutes and the resulting mixture was centrifuged at 9.3 times gravity for five minutes and the organic phase was recovered (11). The recovered organic phase was completely dried under air stream for three to four hours to ensure the removal of any residual water. The dry weight yield of enzyme after the series of these steps was about 50%. That is, from the initial 7 mg protein dissolved in the buffer, a typical yield was between 3 and 4 mg protein in toluene. The organic-soluble enzyme was then re-dissolved into toluene and filtered using 200 nm syringe filters to remove any left over denatured protein and other large particles and structures and added to particles for entrapment (11).

3.2.3 Enzyme Entrapment

The enzyme glucose oxidase was physically entrapped within the polymeric particle matrix. In order to accomplish this, the aqueous soluble natural enzyme must be in organic solution, as discussed in 3.2.2. Specifically, a solution of organic-soluble enzyme (typically 0.5 ml with a concentration of 1.0 mg ml⁻¹, as measured using a calibration curve from enzyme in water) was added to 5.0 mg of particles and allowed to
equilibrate for three hours. The organic solvent causes the polystyrene particles to swell, allowing enzyme to diffuse into the polymer matrix.

Next, the particles were treated with increasing concentrations of poorer solvent, causing the particles to shrink, thus entrapping the enzyme. Typical dilutions of poorer solvent were the pure toluene, solution, followed by increasing dilutions with hexane. First, the toluene phase was diluted to 75% (v/v) with the addition of pure hexane. The mixture was shaken gently and allowed to settle. Some of the liquid was physically removed and fresh, pure hexane was added to replace the removed liquid volume, resulting in a 60% toluene: hexane solvent mixture. This procedure was repeated by pipetting away solvent mixture and adding pure hexane to 50%, then 33%, down to 25% and 10% toluene: hexane.

Finally at the last step, all of the solvent mixture was removed and replaced with pure hexane. Prior to use in kinetic studies, the particles were dried under air stream for one hour and then lyophilized to ensure removal of residual toluene.

3.2.4 Protein Loading Measurement

The approach to experimentally calculate the amount of GOx loaded into the polystyrene particles was to perform a materials balance. That is, the total mass of GOx in bulk phase before entrapment must equal the combined mass of GOx entrapped in the particles and the mass of GOx recovered from all of the washing steps. The quantity of enzyme in the bulk phase was measured before equilibrating with the nanoparticles. After enough time was allowed to ensure equilibrium swelling, the particles were washed with increasing dilutions with a poor solvent, hexane. Each of these washes was
analyzed using UV-Vis spectrophotometry and compared to a calibration curve to calculate the mass concentration of GOx. In addition, after the enzyme complex systems were dried and lyophilized, they were washed with aqueous solutions. These solutions were also measured as described above for the enzyme mass balance.

3.2.5 Kinetic Activity Measurement

First, 1 mg of the enzyme-containing nanoparticles was weighed out and washed a total of three times, twice in DI water and once (the last wash) in 50 mM pH 5.5 sodium acetate buffer. After the last wash, 2 ml of the buffer was added to the particles, which were used in the kinetics studies.

The oxidative activity of GOx was measured using a multi-enzyme assay as suggested by Sigma Aldrich. Reaction stock solution was prepared as follows: 22.5 ml glucose solution (10% w/v) in water, 54 ml (0.415 mM in 50mM pH 5.5 sodium acetate buffer) dianisidine solution and 54 ml of 50 mM pH 5.5 sodium acetate buffer. For each reaction, 0.1 ml (0.2 mg ml$^{-1}$) HRP solution was added to 2.9 ml of reaction stock solution in a 4 ml cuvette. Then, 0.1 ml of particle-enzyme complex solution was added, the mixture quickly stirred, and the reaction was monitored using UV-Vis kinetic method for 2.5 minutes, according to the standard assay.

The reaction was monitored according to the solution absorbance at a wavelength of 500 nm, detecting the oxidized dianisine product. The reaction system is discussed later, in chapter IV. For reaction control, the 0.1 ml enzyme complex solution was substituted with 0.1 ml solution containing enzyme-free particles of the respective size.
and cross-linking. For quantitative free enzyme activity measurements, 0.1 ml free enzyme solution (in DI water) was added.

3.2.6 Particle Characterization

The light scattering and SEM results agree well with the general observation that emulsion polymerization is an effective method for the production of polymeric particles at the nanometer scale (see figures 3-1, 3-2 and table 3-1) (5, 13). As one of the studied parameters was particle size, polystyrene particles were synthesized of varied size (between 100 and 750 nm). The experimental design used to control particle size is discussed later in section 3.3.1.

Figure 3-1 SEM image of 100 nm particles
Table 3-1 Output for Light Scattering Calculations of 218 nm Polymer Particles.

Included are the solutions to increasingly complex light scattering equations.

<table>
<thead>
<tr>
<th>Gamma [s⁻¹]</th>
<th>Diff. Coel. (cm² s⁻¹)</th>
<th>Elf. Diam. (nm)</th>
<th>Poly</th>
<th>Skew</th>
<th>Kurtosis</th>
<th>RMS Error</th>
<th>Amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear: 7.528e+02</td>
<td>2.154e-08</td>
<td>227.8</td>
<td></td>
<td></td>
<td></td>
<td>2.087e-03</td>
<td>0.543</td>
</tr>
<tr>
<td>Quadratic: 7.859e+02</td>
<td>2.250e-08</td>
<td>218.1</td>
<td>0.101</td>
<td></td>
<td></td>
<td>2.993e-04</td>
<td>0.548</td>
</tr>
<tr>
<td>Cubic: 7.856e+02</td>
<td>2.243e-08</td>
<td>218.2</td>
<td>0.099</td>
<td>-0.07</td>
<td></td>
<td>3.038e-04</td>
<td>0.548</td>
</tr>
<tr>
<td>Quartic: 7.879e+02</td>
<td>2.255e-08</td>
<td>217.6</td>
<td>0.116</td>
<td>1.07</td>
<td>5.94</td>
<td>1.254e-03</td>
<td>0.548</td>
</tr>
</tbody>
</table>

3.2.7 The Effect of Cross-Linking on Particle Size

The effect of DVB on particle size was also analyzed. Because the effect of particle size and the effect of cross-linking are considered in the study (later, in section...
3.3), it is important to understand the interaction of these two variables. In controlling particle size, the relative degree of cross-linking, i.e. the ratio of monomer to cross-linking agent, was held constant. Similarly, in controlling cross-linking, all of the parameters which are determined to control particle size (i.e. surfactant concentration) were held constant.

Furthermore, in order to fully understand the system, it is important to quantify the effect that varied degree of cross-linking might have on particle size. In other words, it is necessary to be able to separate any observations regarding effects on reaction velocity from being a result of particle size or cross-linking, or even both. The following experimental results are used separate these issues. At low ratios of DVB (below 0.1 moles/mole), the particles are smaller than expected (195 vs. 218 nm), and at high ratios of DVB (i.e. at 1 mole/mole) the particles are larger than expected (322 compared to 225 nm). These results are in agreement with other observations (see figure 3-3) (12). However, the range in variation of particle size is still much smaller than that of the range between the sizes studied in the effect of particle size.

Moreover, because the particles with more cross-linking were slightly larger than expected, if the cross-linking effect were due solely to the effect of cross-linking on particle size, then the observed reaction rates would be lower with more cross-linking agent. Therefore, any effect of cross-linking on observed reaction rates is not confounded with the effect that cross-kinking has on particle size.
3.3 Results

The immobilized glucose oxidase was studied using the standard enzyme assay. The parameters of interest were particle size and degree of cross-linking. More specifically, the objective was to determine how these two experimental factors impact observed reaction velocities. Furthermore, because cross-linking has an effect on particle size, any possible interaction between these two parameters was examined. A study was completed to determine if cross-linking itself had an effect on particle size, and if so, to what extent so it could be inferred if the effect of cross-linking was a result of particle size alone.
3.3.1 Methodology for Particle Size Control

The control of particle size is an extremely important part of the results discussed below. Indeed, without particle size control the understanding of the system would be severely limited. Discussions of reaction vs. diffusion control would be impossible and from an operational standpoint there would be would one fewer parameter available for process optimization. While it is commonly accepted that emulsion polymerization is an excellent and efficient method for particle size and particle size distribution control, it is not necessarily a trivial point to synthesize nanoparticles of known and desired size.

To achieve this, the entire emulsion polymerization reaction was considered and all of the factors were taken into account. First, the continuous (aqueous) phase was examined and optimized for control of particle size. Next, the emulsion (organic) phase was studied. Finally, all of the knowledge was put together to best experimentally control the particle size for this specific system. Polystyrene nanoparticles were synthesized with divinyl-benzene cross-linker with an aqueous phase consisting of water, ethanol, the surfactant 2-SEM and the stabilizer PVP. The amount of initiator was kept constant in all of the following discussions.

It has been shown previously (5) that for the polystyrene particle system the ratio of ethanol to water is a factor in controlling particle size. More specifically, increased ethanol yields larger particles. In addition, it has been shown (6) the more surfactant will lead to more, smaller particles. Thus, experiments were run to determine the quantitative impact of these parameters, including the effect of stabilizer on particle size. These results are summarized in table 3-2.
Table 3-2 Experimental Design, Adjusting the Aqueous Phase to Control Particle Size

<table>
<thead>
<tr>
<th>Run</th>
<th>Ethanol:Water (ml: ml)</th>
<th>Stabilizer (mg)</th>
<th>Surfactant (ml)</th>
<th>Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10:09</td>
<td>110</td>
<td>0.3</td>
<td>316</td>
</tr>
<tr>
<td>2</td>
<td>11:08</td>
<td>110</td>
<td>0.3</td>
<td>304</td>
</tr>
<tr>
<td>3</td>
<td>10:09</td>
<td>150</td>
<td>0.3</td>
<td>356</td>
</tr>
<tr>
<td>4</td>
<td>11:08</td>
<td>150</td>
<td>0.3</td>
<td>345</td>
</tr>
<tr>
<td>7</td>
<td>10:09</td>
<td>150</td>
<td>0.3</td>
<td>273</td>
</tr>
<tr>
<td>8</td>
<td>10:09</td>
<td>150</td>
<td>0.4</td>
<td>248</td>
</tr>
<tr>
<td>11</td>
<td>10:09</td>
<td>200</td>
<td>0.1</td>
<td>195</td>
</tr>
<tr>
<td>12</td>
<td>10:09</td>
<td>200</td>
<td>0.15</td>
<td>182</td>
</tr>
<tr>
<td>13</td>
<td>10:09</td>
<td>200</td>
<td>0.075</td>
<td>308</td>
</tr>
<tr>
<td>14</td>
<td>10:09</td>
<td>200</td>
<td>0.05</td>
<td>269</td>
</tr>
</tbody>
</table>

The runs indicated in italics were a $2^{3-1}$ design, holding surfactant concentration constant to determine the effect of the parameters, ethanol: water ratio and stabilizer concentration. However, the range of ethanol: water was limited because above 11: 8 ml: ml the particles tended to form large, clumpy (unusable) aggregates in the reaction vessel. Furthermore and as expected, increased stabilizer concentration (runs 1 and 2 vs. runs 3 and 4) yielded larger particles. Similar observations can be made comparing runs 7 and 8 (bold) regarding the effect of surfactant concentration. It should be noted that runs 1-4; 7-8; and 11-14 were run on separate days, which explains the variation between similar runs 3 and 7. However, despite the batch to batch variation, it became apparent that this line of experimentation would not be able to yield particles larger than 500 nm in diameter.

To solve this, rather than further modifying the aqueous phase, which was already optimized, the organic phase was studied. The only real variable available to alter was
the amount of styrene, because the amount of divinyl-benzene in the reaction is fixed for a constant degree of cross-linking. The limit of monomer was chosen because the volume of reaction vessel became a limiting factor. The reaction volume was held constant, so that scale-up considerations (temperature control, agitation rates) could be avoided. The results of the experimental runs to determine the effect of monomer concentration on particle size are shown in table 3-3.

Table 3-3 Experimental Design, Adjusting the Organic Phase to Control Particle Size

<table>
<thead>
<tr>
<th>Run</th>
<th>stabilizer</th>
<th>surfactant (ml)</th>
<th>monomer (ml)</th>
<th>cross-linker (ml)</th>
<th>Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>original</td>
<td>110</td>
<td>0.5</td>
<td>1.2</td>
<td>16</td>
<td>313</td>
</tr>
<tr>
<td>15</td>
<td>110</td>
<td>0.5</td>
<td>1.8</td>
<td>24</td>
<td>321</td>
</tr>
<tr>
<td>16</td>
<td>110</td>
<td>0.5</td>
<td>2.4</td>
<td>32</td>
<td>582</td>
</tr>
<tr>
<td>20</td>
<td>110</td>
<td>0.5</td>
<td>3.6</td>
<td>48</td>
<td>631</td>
</tr>
<tr>
<td>21</td>
<td>110</td>
<td>0.5</td>
<td>5</td>
<td>66.7</td>
<td>747</td>
</tr>
<tr>
<td>24</td>
<td>110</td>
<td>0.15</td>
<td>5</td>
<td>66.7</td>
<td>742</td>
</tr>
<tr>
<td>25</td>
<td>110</td>
<td>0.2</td>
<td>5</td>
<td>66.7</td>
<td>740</td>
</tr>
<tr>
<td>26</td>
<td>110</td>
<td>0.1</td>
<td>5</td>
<td>66.7</td>
<td>823</td>
</tr>
</tbody>
</table>

In table 3-3, runs 24-26 (underlined) were completed to examine the possibility of synthesizing larger particles by using the knowledge obtained from the runs summarized in table 3-2. However, because there was little gain in size from these additional considerations and trials, run 21 (bold) was chosen for the largest particle to be studied.

3.3.2 System Comparison of Immobilized, Modified, and Free Enzyme

One of the central parameters in biocatalysis is the effective reaction kinetic constant. For this glucose oxidase study, the comparative interplay between $V_{\text{max}}$ for the
natural free GOx system and that of the immobilized GOx system were evaluated. The Michaelis-Menten rate equation 3-1 was analyzed by a reciprocal plot for the free system, and then this was compared to the best reaction velocity for immobilized system, as shown in figures 3-4 and 3-5.

\[
V_{app} = \frac{k_{cat} \cdot [E]_0 \cdot [S]}{K_M + [S]}
\]  

(3-1)

In equation 3-1, \(V_{app}\) is the apparent (measured) reaction rate, \([E]_0\) is enzyme loading and \([S]\) is the (constant) bulk glucose concentration. Examination of equation 3-1 shows the importance of the enzyme loading in the reaction kinetic considerations.

Glucose oxidase enzyme loading can be theoretically considered using polymer swelling equilibrium theory, and is discussed in chapter IV. The model used in this study considers the volume swelling fraction of the organic solvent in the polymer matrix (4). The equilibrium volume is assumed to contain the same concentration of enzyme as the bulk solution. However, from a practical standpoint, in order to estimate a rate constant, \(V_{max}\) was used. \(V_{max}\) is expressed as \(k_{cat} \cdot [E]_0\), and is used in equation 3-2.

\[
\frac{1}{V_{app}} = \frac{k_M}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}}
\]  

(3-2)
Figure 3-4 Linearization of Michaelis-Menten Kinetics to Find the Effective Rate Constant for Free Natural Enzyme. Evaluating the slope and intercept from and using equation 3-2, $V_{\text{max}} = 4.9 \text{ mmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg GOx}^{-1}$ and $k_M = 0.0010 \text{ mg ml}^{-1} = 0.0056 \text{ mM}$.

It was apparent that the effective reaction rate is much lower than that of free enzyme. In comparison, the highest observed rate, $0.1 \text{ mmol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg GOx}^{-1}$, relates to a free substrate concentration in the range of $\mu\text{g ml}^{-1}$, as read in the x-axis of figure 3-5. The first order reaction rate based on this concentration is about $5 \times 10^{-2}$ times the calculated $V_{\text{max}}$ in free enzyme solution, which was calculated above in figure 3-4. While it is difficult to directly compare the reaction constants between the two systems (the glucose concentration was not varied in the immobilized enzyme case), in can be
noted that the most active immobilized system had an apparent reaction velocity which was two orders of magnitude lower than the observed maximum velocity of the same reaction carried out with free enzyme.

![Free Enzyme and Immobilized System Reaction Velocity Comparison](image)

Figure 3-5 Comparing Best Enzyme Particle Reaction System to Free Enzyme Reaction.

The reaction velocity of the best particle system is on the order of $10^{-2}$ times the $V_{\text{max}}$ of the free enzyme.

To delve deeper into the understanding of the large decrease in observed enzyme activity, the reaction velocity of modified free enzyme was explored. Experiments were run to determine whether the reduced activity was due to the enzyme modification step itself, or rather to the subsequent entrapment protocol.
First, the enzyme was exposed to the procedure to modify it to organic solubility, as described in section 3.2.3. Thereafter, instead of the consequent entrapment steps, the organic soluble enzyme was dried of organic phase under air stream and then dissolved back into aqueous buffer. For low concentrations of enzyme with roughly linear reaction kinetics, the natural free enzyme is about ten times (estimated by comparing the linear slopes, figure 3-6 below) more reactive than the modified free enzyme. Therefore, some of the mentioned loss in enzyme activity (as discussed in the two orders of magnitude loss) can be attributed to the procedure to render the enzyme organic-soluble, and the rest must be due to immobilization effects.

![Graph comparing enzyme activity](image)

Figure 3-6 Comparing Free Natural Enzyme to Modified Free Enzyme
3.3.3 Enzyme Loading Measurements

The GOx loading was measured for each system by materials balances over the enzyme. Unfortunately, large uncertainties accompanied the procedure, probably at least in part because of the very low (sub-μg) levels of protein, where highly-precise quantitative detection is difficult using the standard UV-vis calibration. The average loading over three repetitions was roughly \(10^2\) times the theoretical loading, as shown in figure 3-7. However, it is impossible to deduce a trend regarding loading and particle size, which is expected; as long as equilibrium is reached, the particle dimensions have no bearing on volume swelling.

The theoretical mass loading, as given by the equilibrium enzyme concentration, the volume fraction of solution inside the polystyrene particle and the particle density, is 0.29 mg protein per g particle. The enzyme concentration in toluene was held constant at 1.0 mg ml\(^{-1}\), the swelling fraction of toluene in polystyrene is 0.3 ml ml\(^{-1}\), (1) and the density of the polystyrene spheres is 1.05 g ml\(^{-1}\).

The total volume of polystyrene particles is constant in each case because the total mass of particles was held constant for each experiment and the density of the polystyrene should be constant with constant cross-linking. Furthermore, with constant cross-linking, the equilibrium volume swelling of toluene into the polystyrene particles should be constant. As long as equilibrium volume swelling is reached for each particle size (i.e. larger particles would take longer for equilibrium swelling to occur), then the total fraction of immobilized enzyme within the particles will be the same. If the fraction of enzyme is constant and the total volume of polystyrene particles is constant, then the total mass of enzyme must be constant.
Figure 3-7 The Experimental Results for Mass Enzyme Loading, $[E]_0$. Error bars represent the standard deviation of three experimental repetitions.

3.3.4 The Effect of Particle Size

One of the parameters studied was the effect of particle size on observed reaction velocities. The importance of studying this parameter is that it is one which is easily adjustable from an operational standpoint (section 3.2.1). Based on diffusion models, it is expected that smaller particles will yield faster reaction velocities because the diffusion distances are shorter (i.e. one-half or one-fourth). With shorter distances, substrate concentrations will be higher within the entire particle, and thus so will the reaction velocity (which is proportional to substrate concentration for lower concentrations see equation 3-1). However, as determined by experiment, the system is highly reaction
limited and thus the diffusion assumptions do not suffice. For the case where theoretical enzyme loading is not a function of particle size, an important factor is that for the same weight of particles, smaller particles will have more total surface area per unit particle weight than will larger particles. Higher catalytic surface area will result in higher reaction rates. Finally, because smaller particles will move more quickly in solution, smaller particles should yield higher reaction rates, as suggested previously (5).

Thus, from a practical standpoint, the question is not particle size optimization from a reaction standpoint but rather size optimization from standpoint of being able to separate the immobilized enzymes from reaction solution after the reaction is complete. In other words, the balance between small immobilization substrates and filtration or biocatalyst recoverability needs to be optimized.

The oxidation of glucose as catalyzed by glucose oxidase entrapped within polystyrene particle matrix was carried out using four different particle sizes. The results of these studies are shown in figure 3-8 and indicate that smaller particles yield faster reaction rates. For example, the initial reaction rate for particles of 250 nm diameter was about two-fold the rate for 750 nm particles.
Figure 3-8 Reaction rate as a function of particle size. Error bars for reaction velocity dimension represent the standard deviation of three repetitions and for the particle size dimension are from light scattering standard deviations.

3.3.5 The Effect of Cross-Linking

In addition to particle size, the effect of cross-linking on the GOx oxidation rate was studied. The ratio of DVB to styrene ranged from no DVB to a 1:1 molar ratio. Again, this parameter can be easily adjusted from an operational standpoint by simply varying the ratio of moles of monomer to moles of cross-linker. From a theoretical view, the point of the study is to observe the effect on particle tightness on reaction velocity. As discussed in chapter IV, in the Thiele Modulus discussions, one very important factor is the particle porosity. Because this was not directly measured, the effect of this
parameter was studied by varying the degree of cross-linking. More relative amount of cross-linking agent will lead to a more close-knit particle, with more connecting bridges between the polystyrene chains (6). Qualitatively, this is saying that more cross-linking agent yields a less porous particle. It is expected that less cross-linking (a more porous biocatalyst) will result in lower diffusion resistance and allow for faster reactions.

However, as shown experimentally, this system is highly reaction limited. In this case, more porous particles may be less effective at entrapping enzyme. In other words, if the particle is too porous, most of the entrapped enzyme simply leeches back out during the washing steps and the particle is rendered less active. Indeed, from the data, it appears that there is point with enough cross-linking agent to support reaction, and below which the reaction is quite slow (see figure 3-9).

The observations and conclusions regarding reaction velocity and degree of cross-linking are subject to the uncertainty which results from the relationship between cross-linking and particle size (analyzed in figure 3-3). The change in particle size from the points represented the lowest cross-linking (none) and those representing the highest cross-linking (1:1) is roughly 100 nm. It may be noted by comparing figures 3-8 and 3-9 that the “jump” change in observed reaction velocity from low cross-linking to high cross-linking is on the order of 3- to 5-fold, but the change in observed reaction velocity from even 200 to 500 nm particles is only 2 fold. The effect of cross-linking appears to be much greater than what can be accounted for with the particle size effect alone.
Figure 3-9 Reaction rate as a function of cross-linking. Error bars for reaction velocity dimension represent the standard deviation of three repetitions.
CHAPTER IV

THEROETICAL CONSIDERATIONS

4.1 Introduction

To understand the catalytic system, both reaction limitations and diffusion limitations were considered. As far as the reaction terms are concerned, the relationship between the enzyme reaction constants, which evaluate $V_{\text{max}}$, as discussed in section 3.3.2 are paramount. For the case of enzyme entrapment, the effective interplay is observed to be a result of the extent to which enzyme is entrapped within the polymer matrix, a function of the equilibrium volume swelling due to the chosen solvent.

Additionally, the observed reaction kinetics is a function of the retained activity of the enzyme after the consecutive immobilization steps. The diffusion limitations are connected to how fast the substrate can diffuse inside the polymer particle to interact with the enzyme. Furthermore, the particular system of interest has the additional consideration of the diffusion of hydrogen peroxide (the product of the GOx reaction) back out of the particle, where it reacts with the bulk solution to produce the detected product. This is necessary so that the reaction can be monitored, as suggested by the standard assay. These discussions ideas are represented in scheme 4-1.
4.2 Enzyme Loading Kinetic Theory

This section is devoted to the consideration of enzyme loading as a function of
time, and has nothing to do with glucose or hydrogen peroxide diffusion, which will be
discussed in section 4.3 assuming steady-state conditions.

The kinetic model is to shows that, for glucose oxidase loading within the
carbohydrate particle, the assumption that equilibrium swelling is reached is adequate. As
discussed in chapter III, to entrap enzyme, the organic-soluble enzyme was allowed to
equilibrated for three hours with the polymeric particles. The model shows that the time
needed for diffusion is much faster, on the order of ms. Therefore, the time allowed was
over $10^3$ times that needed. The rigorous model is not shown here for brevity. However,
one can note that the time constant for the diffusion of toluene into the particles ($r^2/Deff$)
is on the order of $10^{-5}$ min. To arrive at this, the diffusion coefficient of toluene in
polymer is on the order of $10^{-6}$ cm$^2$ min$^{-1}$ (8) and the particle radius is on the order of
$10^{-5}$ cm (particle size characterization was discussed in chapter III).

![Scheme 4-1 Overall Oxidation Reaction of Glucose Using the Enzyme GOx](image)

Scheme 4-1 Overall Oxidation Reaction of Glucose Using the Enzyme GOx
4.3 Internal Mass Transfer Discussions

A common method of judging a system as far as diffusion and reaction limitations are concerned is the Thiele Modulus (equation 4-4), which is a quantitative method to determine if a system is reaction limited, diffusion limited, or somewhere in between.

For spherical particles (6)

\[ \phi = R \times \left( \frac{krxn}{Deff} \right)^{0.5} \]  

(4-4)

R = particle radius
krxn = first order rate constant
Deff = effective diffusivity (described below)

These last two terms, krxn and Deff require further discussion and evaluation. The reaction term will be evaluated based as a first order rate constant, which is valid for the reaction in substrate concentrations for which the reaction velocity is less than ½ of Vmax. The diffusion term will be evaluated based on the catalyst porosity.

A pseudo first-order rate constant was used in the kinetic discussions regarding the immobilized enzyme system. The first order assumption is valid for the case where the available substrate concentration is much lower than the value for kM. The first order rate constant is here defined in equation 4-5.

\[ krxn = \frac{V_{\text{max}} \times [E]_0}{k_M} \times f \]  

(4-5)
In the above equation, \( f \) is an efficiency factor (here taken to be 1/10, see figure 3-6). The reaction constant is calculated for \( V_{\text{max}} = 4.9 \text{ mmol min}^{-1} \text{ mg Gox}^{-1} \) (figure 3-4), \([E]_0 = 0.29 \text{ mg Gox g PS}^{-1}\) (section 3.3.3) \( k_M = 0.0056 \text{ mM} \) (figure 3-4) and \( f = 0.1 \) (figure 3-6), \( k_{\text{rxn}} = 12.7 \text{ min}^{-1} \).

\[
\begin{align*}
    k_{\text{rxn}} &= \frac{4.9 \text{ mmol} \times 0.29 \text{ mg Gox} \times L}{0.0056 \text{ mol min}^{-1} \times \text{ mg Gox g PS}^{-1} \times 1 \text{ g PS} \times 1 \text{ g PS} \times 1000 \text{ mol PS} \times 2 \text{ ml} \times 1000 \text{ ml} \times 0.1} \\
                   &= 12.69 \text{ min}^{-1}
\end{align*}
\]

In order to fully consider diffusion limitations, one important factor is the geometry of the pores. In this system, the particle porosity was not directly measured. To take this into account (and its effect on the internal diffusivity), common values were used as an estimate (6) and the effect of the porosity factors on the reaction system was analyzed (see equation 4-6 and figure 4-2).

\[
D_{\text{eff}} = \frac{D \times \varepsilon \times \sigma}{\tau}
\]  \hspace{1cm} (4-6)

\( D \) = diffusivity in solution (area/time)

\( \varepsilon \) = pellet porosity = void space fraction of sphere

\( \sigma \) = a constriction factor = a measure pore radius

\( \tau \) = tortuosity = a measure of pore geometry

A common product for these factors is about 0.1 (6), which is in the range where the Thiele Modulus is sensitive to these diffusion factors. As judged on the y-axis, the values for the Thiele Modulus are still very low, indicative of a severely reaction limited system.
The Effect of the Porosity Factors on Thiele Modulus for Given D and krxn constants

Figure 4-1 The Effect of the Particle Porosity Constants on the Thiele Modulus. Deff is varied as a function of porosity parameters.

The calculations considered in figure 4-2 are shown in more detail in Table 4-1. For these calculations, the krxn used was the empirical estimate as discussed above. Deff is the effective diffusivity of glucose within the pores, which is the diffusion coefficient of glucose (7) in water multiplied by the porosity factors in equation 4-5: ε, σ, and τ. For the calculations described in figure 4-2 and in table 4-1, the porosity factors range from 0 to 1. A combined factor greater than 1 would imply that the diffusion of substrate is enhanced by the pores, which has no physical meaning in the present context.
Table 4-1 Thiele Modulus for different particle sizes as a function of Deff and the porosity factors

<table>
<thead>
<tr>
<th>k rxn (min⁻¹)</th>
<th>12.7</th>
<th>112</th>
<th>218</th>
<th>521</th>
<th>747</th>
</tr>
</thead>
<tbody>
<tr>
<td>diameter (nm)</td>
<td>Deff (nm² min⁻¹)</td>
<td>Thiele</td>
<td>Thiele</td>
<td>Thiele</td>
<td>Thiele</td>
</tr>
<tr>
<td>0.01</td>
<td>27000000</td>
<td>0.03840679</td>
<td>0.074756</td>
<td>0.17866</td>
<td>0.25616</td>
</tr>
<tr>
<td>0.11</td>
<td>29700000</td>
<td>0.011580083</td>
<td>0.02254</td>
<td>0.053868</td>
<td>0.077235</td>
</tr>
<tr>
<td>0.21</td>
<td>56700000</td>
<td>0.008381049</td>
<td>0.016313</td>
<td>0.038987</td>
<td>0.055899</td>
</tr>
<tr>
<td>0.31</td>
<td>83700000</td>
<td>0.006898063</td>
<td>0.013427</td>
<td>0.032088</td>
<td>0.046008</td>
</tr>
<tr>
<td>0.41</td>
<td>110700000</td>
<td>0.005998133</td>
<td>0.011675</td>
<td>0.027902</td>
<td>0.040005</td>
</tr>
<tr>
<td>0.51</td>
<td>137700000</td>
<td>0.005378026</td>
<td>0.010468</td>
<td>0.025017</td>
<td>0.03587</td>
</tr>
<tr>
<td>0.61</td>
<td>164700000</td>
<td>0.004917485</td>
<td>0.009572</td>
<td>0.022875</td>
<td>0.032798</td>
</tr>
<tr>
<td>0.71</td>
<td>191700000</td>
<td>0.004558047</td>
<td>0.008872</td>
<td>0.021203</td>
<td>0.030401</td>
</tr>
<tr>
<td>0.81</td>
<td>218700000</td>
<td>0.004267421</td>
<td>0.008306</td>
<td>0.019851</td>
<td>0.028462</td>
</tr>
<tr>
<td>0.91</td>
<td>245700000</td>
<td>0.004026126</td>
<td>0.007837</td>
<td>0.018729</td>
<td>0.026853</td>
</tr>
<tr>
<td>1.01</td>
<td>272700000</td>
<td>0.003821618</td>
<td>0.007439</td>
<td>0.017777</td>
<td>0.025489</td>
</tr>
</tbody>
</table>

However, one point should be considered. Because a reduced Deff by a factor of 100 would yield a larger Thiele Modulus by a factor of ten, the resulting concentration profile for this system would be largely unchanged. The actual quantitative value of the Thiele Modulus is still considerably lower than unity. The system is so severely reaction limited (as a result of the very low [E]₀ parameter and the very small internal diffusion distances on the order of 10⁻⁹ meters) that even very extreme cases of reduced diffusivity have almost no impact on the reaction profile. The substrate diffusion is so rapid in comparison to the reaction kinetics that the observed rates are very similar to the rate that would ensue due to reaction at surface conditions, without diffusion consideration (an
internal effectiveness factor, if so defined as a measurement of observed reaction divided by reaction under the conditions of no internal mass transfer limitations, would be unity).

In the above discussions, as indicated by equation 4-4, a 100-fold decrease in Deff corresponds equivalently mathematically to a 100-fold increase in the reaction constant. In other words, similar arguments can be made if the sensitivity to the concentration profiles regarding the reaction term was considered. Even if the reaction term had been 100 times larger, the Thiele Modulus would be increased likewise by a factor of ten and the actual system under study would not change to any appreciable degree.

In order to increase the Thiele Modulus such that the internal factor would begin to be much less than unity, either the Deff would need to be reduced by a factor of \(10^4\), the effective particle radius increased by a factor of \(10^2\) (possible if large degrees of particle aggregation) or some combination thereof.

The other factor in the equation, the reaction term (a function of enzyme loading and activity, \([E]_0 \cdot V_{\text{max}}\)) would need be \(10^4\) times larger. This case can be analyzed using equation 4-5. For \(V_{\text{max}}\) to increase by a factor of \(10^4\), the efficiency factor \(f\), as defined in equation 4-5 and evaluated in figure 3-6, would need to be much greater than unity. If the efficiency factor is greater than unity, then the immobilized enzymes are more reactive than are the free enzymes. This scenario does not make sense because it is hard to imagine the reactivity of an enzyme being so magnified, especially after considering the activity loss discussed in figure 3-6.
The concentration profiles for both substrates (glucose and hydrogen peroxide) were calculated using the experimentally determined GOx loading and are not included for brevity. However, in general the profiles are represented by figure 4-3. The change in concentration between the center of the particle and the particle surface are almost identical, due to very limited reaction kinetics. Furthermore, it can be concluded that the concentration of hydrogen peroxide must be low, because very little glucose undergoes reaction to form hydrogen peroxide. Therefore, the reaction system would be more efficient if the reaction limitations were overcome.
4.4 External Mass Transfer Discussions

In the discussion of observed kinetics, external mass transfer resistances must also be considered. Classically, the significance of a system’s external mass transfer limitations is considered by the Damköhler Number.

\[
Da = \frac{Rxn}{ExternalDiffusion} = \frac{krxn * C_s}{k_s * a * C_b}
\]  

(4-6)

\[krxn = \text{as defined in equation 4-5}\]

\[k_s = \text{mass transfer coefficient of the glucose solution to the surface of the particle}\]

\[C_s = \text{glucose concentration at particle surface}\]

\[C_b = \text{glucose concentration in bulk solution}\]

\[a = \text{dimensional factor, defined as the particle surface area/volume}\]

In equation 4-6, the surface reaction term is based on Michaelis-Menten kinetics which can be approximated as a first order reaction, as discussed earlier (chapter IV, section 4.3, equation 4-5). The diffusion term is a result of the mass flux equation through a thin film, where the mass flux is proportional to concentration difference.

In reaction engineering, it is common to define an external effectiveness factor, defined in equation 4-7. The subscripts s and b indicate surface and bulk conditions, respectively (69).

\[
\eta_E = \frac{r_{xns}}{r_{xnb}} = \frac{C_s}{C_b}
\]  

(4-7)
At steady state, the mass flux of substrate due to reaction must be equal to the flux due to mass transfer:

\[ ks \cdot a \cdot (C_b - C_s) = krxn \cdot C_s \]  

(4-8)

This equation can be solved to determine the surface glucose concentration in terms of measurable parameters. Equation 4-9 results when the dimensionless variables discussed in equation 4-6 are included and combining equations 4-7 and 4-9 yields 4-10.

\[ C_s = \frac{ks \cdot a \cdot C_b}{a \cdot ks + krxn} \]  

(4-9)

\[ \eta_E = \frac{rxn_s}{rxn_b} = \frac{a \cdot ks}{a \cdot ks + krxn} \]  

(4-10)

Combining equations 4-9 and 4-6 results in the Damköhler Number in equation 4-11

\[ Da = \frac{krxn \cdot C_s}{ks \cdot a \cdot C_b} = \frac{1}{a \cdot ks} \]  

(4-11)

All of the above parameters and terms are defined for the system, except for the mass transfer coefficient, which was not measured. In order to discuss the effect of external mass transfer, a semi-quantitative approach can be taken for extreme cases of mass transfer coefficient, just as was done in section 4-3 with the case of effective diffusion coefficient, as a function of particle porosity factors.

In equation 4-6 the only unknown parameter is \( k_s \), the mass transfer coefficient of glucose across the stagnant thin film around the particle is the bulk diffusion coefficient.
divided by the boundary layer thickness. The boundary layer thickness is unknown. However, some insight can be obtained based on extreme cases, from very large film thickness (order of $10^6$ nm) to very short thickness (order $10^0$ nm). 

In the following discussion, the surface concentration of glucose on the particle is held constant, and the ensuing Da number represents the value which would be required for the surface concentration to remain at this level. If the Da number were lower, the surface concentration would be greater and vice-versa. For the following calculations in table 4-2, the particle diameter was 750 nm. The reaction term was as defined previously. Glucose does not participate in any reaction outside of the pores. $C_b$ was known at the time of experimentation (80 mM). Finally, the diffusion coefficient, D, of glucose in water is known ($4.5 \times 10^{-7}$ cm$^2$s$^{-1}$) (7).

Table 4-2 The Damköhler Number and the External Effectiveness Factor as Functions of Film Thickness

<table>
<thead>
<tr>
<th>Film Thickness (nm)</th>
<th>Da</th>
<th>$\eta_E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.E+00</td>
<td>5.E-08</td>
<td>1.00</td>
</tr>
<tr>
<td>1.E+01</td>
<td>5.E-07</td>
<td>1.00</td>
</tr>
<tr>
<td>1.E+02</td>
<td>5.E-06</td>
<td>1.00</td>
</tr>
<tr>
<td>1.E+03</td>
<td>5.E-05</td>
<td>1.00</td>
</tr>
<tr>
<td>1.E+04</td>
<td>5.E-04</td>
<td>0.99</td>
</tr>
<tr>
<td>1.E+05</td>
<td>5.E-03</td>
<td>0.94</td>
</tr>
<tr>
<td>1.E+06</td>
<td>5.E-02</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Even in the extreme case of a film thickness on the order of 1 mm ($10^3$ times the particle diameter), Da is less than unity, and $\eta_E$. Consequently, it can be concluded that the particle system is also reaction limited with respect to external mass transfer.
CHAPTER V

FURTHER DISCUSSIONS

5.1 Oxygen Limited Reaction

Because it was shown that the enzymatic reaction is, in this case, severely kinetics limited, it is prudent at this point to discuss the reaction itself. In scheme 4-1, in the first reaction, one must consider that the reaction requires molecular oxygen (O$_2$), in the ratio of one mole of oxygen for one mole of glucose. Throughout the kinetic studies, the concentration of glucose in bulk solution was held constant at 80 mM (see 3.2.5) and the solubility of oxygen in water at room temperature is on the order of 0.3 mM. Furthermore, as noted in figure 4-3, the internal concentration of glucose does not vary much from 80 mM in the current state of reaction limitation. Consequently, it must be concluded that at least some of the reaction limitation could be a result of low oxygen concentration, which is the limiting reactant in this process.

However, it should be noted that the reaction kinetics were studied based on a specifically defined standard assay, which provides for oxygen limitation. Moreover, because it can be observed in free enzyme and free glucose reaction conditions show an effect of glucose concentration of reaction rate (figure 3-5), there must be more to the picture than oxygen limitation. If the reaction was exclusively oxygen limited, then
further increasing the glucose concentration above the oxygen concentration should have no impact on the observed reaction velocity.

One possible explanation is the localized concentrations in or around the enzyme active site. If GOx is extremely efficient at capturing dissolved oxygen and only moderately successful with glucose molecules, then it is possible that glucose could still be the limiting reactant. For example, if GOx is 100% efficient at collecting oxygen but can only collect one of every 1,000 glucose molecule then the real reaction concentrations of the respective species would be 0.3mM and 0.08mM. Increasing the glucose concentration in bulk by a factor of 2 would result in 0.16mM glucose reaction concentration, and it would still be the limiting reactant. The authors have not proven this concept, but the observed reaction kinetics as a function of glucose concentration of this and other studies, including the widely accepted Sigma Aldrich GOx kinetics assay (5, 11), leads one to believe that oxygen is indeed not the limiting reactant.

5.2 Discussions Relating the Experimental and Theoretical Sections

One assumption that was made in designing the experiments described in chapter III was that the particles reached equilibrium with the modified enzyme in toluene solution in three hours. Enzyme will only be entrapped within the polymer matrix only if the toluene sufficiently diffuses into the polymer matrix; unsteady state diffusion of toluene in the nanoparticle was considered. The results of these considerations suggest that the amount of time necessary for 99% equilibrium swelling of toluene in the particle matrix is on the order of $10^{-3}$ s. The amount of time given ($10^4$ s) is more than adequate,
even qualitatively allowing for possible phenomenon like non-Fickian diffusion and dilution overshoot (1), which might increase the time required to reach final equilibrium.

After the toluene brings the enzyme inside the polymer matrix, hexane is used to shrink the particles. At this point, there are two possible fates for the entrapped GOx. The first case is that hexane seeps in, displaces the toluene and thus the enzyme. Even though hexane will swell the polystyrene to a certain degree, the equilibrium swelling fraction for alkanes is much lower in polystyrene than that of aromatic solvents (1), so it is unlikely that toluene would be replaced to any appreciable extent. The second, more likely case, is that as toluene is forced out of the shrinking particle, it would take some GOx with it. In either case, some of the entrapped enzyme is leeched out. The externalized enzyme would then be washed away in subsequent washing cycles, resulting in lower enzyme loading than would be theoretically expected.

In the analysis of the results, a couple of points became apparent. First, the highest rates observed for the entrapped enzyme system are on the order equivalent to the same reaction conditions with a free enzyme concentration in the nM range. Furthermore, it was extremely difficult to experimentally determine the enzyme loading ([E]₀) via materials balance. The mass of modified enzyme recovered from all washes subtracted from the mass of modified enzyme originally in toluene. Unfortunately, with such small enzyme concentrations to measure, the error was too large to be able to draw conclusions regarding any correlation between the theoretical model and experimental observations regarding enzyme mass loading.

Finally, preliminary light scattering data and visual observation of the particles show that at some step between particle synthesis and entrapment, some of the particles
aggregate and the consequent effective diameter is ten to one-hundred times the expected size. However, comparisons of reaction results with the entrapped enzyme system to that of blank tests (i.e. same reaction system, only using particles containing no enzyme) show that the observed reaction is significant. In other words, after surfactant modification, dissolving in organic phase, and entrapment into a polymer matrix, the aqueous phase enzymes retain some level of activity albeit a very small level.

One outcome of particle agglomeration is a marked increase in the Thiele Modulus, and thus the consideration of the internal effectiveness factor (i.e. diffusion compared to reaction limitation). For example, an increase in effective particle size even by a factor of ten has the same impact on the Thiele Modulus as would a decrease in the Deff by a factor of 100. As described in the theoretical considerations, the system would no longer be severely reaction limited with a 100-fold increase in effective particle size. It will be exciting to see the results of future studies where particle aggregation can be better controlled, keeping in mind that the use of surface stabilizers would lead to even lower enzyme loading (3, 13).
Glucose oxidase was successfully entrapped inside the polymer matrix of cross-linked polystyrene co divinyl-benzene nanoparticles, as shown by observed catalytic activity of the thoroughly washed enzyme-particle systems. The enzyme mass loading was experimentally measured using materials balances; the theoretical mass loading was calculated using polymer swelling theory. The reaction rate constant was calculated as follows: a reciprocal plot of the Michaelis-Menten enzyme reaction rate data for free enzyme was used to find the enzyme’s $V_{\text{max}}$ (i.e. $k_{\text{cat}} \times [E]_0$) in free solution.

A reaction constant for the first order reaction kinetics was approximated as $V_{\text{max}}$ for the free enzyme system multiplied by a factor, which in this case was 0.1, as it was determined that the modified enzyme was only 10% as active as the natural enzyme (figure 3-6). First order rate kinetics were used as an approximate, based on the fact that the glucose concentration was in all cases well below $k_M$, which was evaluated in the same graph as $V_{\text{max}}$ (the reciprocal plot of $V_{\text{obs}}^{-1}$ and $[\text{Glucose}]^{-1}$).

To evaluate mass transfer resistances, the effective substrate diffusivities were considered as a function of particle porosity and the respective diffusion coefficient of free substrate in bulk solution. The particle porosity was not directly measured and was assumed to be a function of cross-linking and was approximated in theoretical
calculations as a function of the product of three parameters: particle porosity, pore tortuosity and an additional constriction factor, as defined in reaction engineering textbooks (6) and discussed in section 4.3.

The reactivity losses were explored and can, in part, be attributed to loss of enzyme activity from the organic-solubilization step. As described in chapter III, experiments were run to compare the activity of glucose oxidase free in water: one batch was free enzyme, as provided by the manufacturer; the other batch had gone through the solubilization process and subsequently re-dissolved into water instead of immobilization within polystyrene particles. The activity of the natural free enzyme was found to be roughly ten times that of the organic-solubilized free enzyme, thus the 0.1 factor in calculating the reaction rate constant from $V_{\text{max}}$.

Finally, from a process point of view, experiments were run to see how the reaction velocities behaved with changing particle size and degree of cross-linking. The effect of cross-linking, a physical parameter that dictates how much the matrix can swell, was examined in addition to the effect of varied nanoparticle size on the immobilized enzyme reaction rates.

While the effect of particle size is as expected (smaller particles yield higher reaction velocities), the reason is not. It was expected that smaller particles would provide lower diffusion resistance and thus higher reaction velocities; this is indeed the case, but it was found that the reaction was already severely reaction limited, so a faster diffusion step does not bring any advantages. The effect of particle size can then be attributed to one of two possibilities (or a combination of two possibilities). First, with the same enzyme loading and same particle weight, smaller particles will have a higher
surface area for reaction. Second, smaller particles move faster in solution, resulting in faster reaction velocities, as discussed in more detail in other publications (5).

The effect of particle cross-linking was explored as an experimental measure of particle porosity. The expected effect of cross-linking from a diffusion point of view is that a looser particle would have lower diffusion resistance. However, as noted the observed kinetics must have not been a function of diffusion because the process is severely reaction limited. Thus, the observed effect of cross-linking can instead be attributed to mass enzyme loading ([E]₀), as this has a direct impact on V_{max}, which is used as the kinetic parameter. Experimental results indicate a point of cross-linking at which the observed velocity jumps to a relatively much higher value. At low particle cross-linking (a loose, porous particle), even though substrate can diffuse faster, less enzyme is entrapped as it is allowed to escape back out through the pores.

Finally, once a tight enough particle is formed, the entire quantity of enzyme which can thermodynamically be entrapped is loaded, thus the effect of loading on V_{max} is no longer a function of cross-linking. Furthermore, even though more cross-linking will ultimately result in slower substrate diffusion, the process is so severely reaction limited that this effect on diffusion is not observed. Indeed, to observe such an effect on diffusion, the diffusion resistance would have to be increased by a factor greater than ten-thousand. This would be a result of either an increase in diffusion distance by a factor of one-hundred, or a decrease in diffusion coefficient by a factor of ten-thousand. Alternatively, the activity of the enzyme would need to be enhanced due to immobilization by a factor of ten-thousand.
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