NANOPARTICLE BIOCONJUGATES AS “BOTTOM-UP” ASSEMBLIES OF ARTIFICAL MULTIENZYME COMPLEXES

A Dissertation in
Chemistry
by
Jacqueline D. Keighron

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The dissertation of Jacqueline D. Keighron was reviewed and approved* by the following:

Christine D. Keating  
Associate Professor of Chemistry  
Dissertation Advisor  
Chair of Committee

Philip C. Bevilaqua  
Professor of Chemistry

Scott Showalter  
Assistant Professor of Chemistry

Peter J. Butler  
Associate Professor of Bioengineering

Barbara Garrison  
Shapiro Professor of Chemistry  
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
ABSTRACT

The sequential enzymes of several metabolic pathways have been shown to exist in close proximity with each other in the living cell. Although not proven in all cases, colocalization may have several implications for the rate of metabolite formation.

Proximity between the sequential enzymes of a metabolic pathway has been proposed to have several benefits for the overall rate of metabolite formation. These include reduced diffusion distance for intermediates, sequestering of intermediates from competing pathways and the cytoplasm. Restricted diffusion in the vicinity of an enzyme can also cause the pooling of metabolites, which can alter reaction equilibria to control the rate of reaction through inhibition.

Associations of metabolic enzymes are difficult to isolate ex vivo due to the weak interactions believed to colocalize sequential enzymes within the cell. Therefore model systems in which the proximity and diffusion of intermediates within the experiment system are controlled are attractive alternatives to explore the effects of colocalization of sequential enzymes. To this end three model systems for multienzyme complexes have been constructed.

Direct adsorption enzyme:gold nanoparticle bioconjugates functionalized with malate dehydrogenase (MDH) and citrate synthase (CS) allow for proximity between to the enzymes to be controlled from the nanometer to micron range. Results show that while the enzymes present in the colocalized and non-colocalized systems compared here behaved differently overall the sequential activity of the pathway was improved by (1) decreasing the diffusion distance between active sites, (2) decreasing the diffusion coefficient of the reaction intermediate to prevent escape into the bulk solution, and (3) decreasing the overall amount of bioconjugate in the solution to prevent the pathway from being inhibited by the buildup of metabolite over time.

Layer-by-layer (LbL) assemblies of MDH and CS were used to examine the layering effect of sequential enzymes found in multienzyme complexes such as the pyruvate...
dehydrogenase complex (PDC). By controlling the orientation of enzymes in the complex (i.e. how deeply embedded each enzyme is) it was hypothesized that differences in sequential activity would determine an optimal orientation for a multienzyme complex. It was determined during the course of these experiments that the polyelectrolyte (PE) assembly itself served to slow diffusion of intermediates, leading to a buildup of oxaloacetate within the PE layers to form a pool of metabolite that equalized the rate of sequential reaction between the different orientations tested.

Hexahistidine tag – Ni(II) nitrilotriacetic acid (NTA) chemistry is an attractive method to control the proximity between sequential enzymes because each enzyme can be bound in a specific orientation, with minimal loss of activity, and the interaction is reversible. Modifying gold nanoparticles or large unilamellar vesicles with this functionality allows for another class of model to be constructed in which proximity between enzymes is dynamic. Some metabolic pathways (such as the de novo purine biosynthetic pathway), have demonstrated dynamic proximity of sequential enzymes in response to specific cellular stimuli. Results indicate that Ni(II)NTA scaffolds immobilize histidine-tagged enzymes non-destructively, with a near 100% reversibility. This model can be used to demonstrate the possible implications of dynamic proximity such as pathway regulation.

Insight into the benefits and mechanisms of sequential enzyme colocalization can enhance the general understanding of cellular processes, as well as allow for the development of new and innovative ways to modulate pathway activity. This may provide new designs for treatments of metabolic diseases and cancer, where metabolic pathways are altered.
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<td>ATPS</td>
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For my parents
Chapter 1

Introduction


The sequential enzymes of several metabolic pathways have been proposed to, or shown to exist in close proximity with each other in the living cell. Although not proven in all cases, colocalization may have several implications for the rate of metabolite formation.\(^1\) Fluorescence based techniques have demonstrated that two or more enzymes from the glycolytic pathway\(^2\), TCA cycle\(^3\) and de novo purine biosynthetic pathway\(^4\) can be found colocalized in vivo. Several reports suggest that these colocalized enzymes are associated into three dimensional complexes found within the cytoplasm or associated with cellular structures (Figure 1-1).\(^4,6\)

In these complexes, enzymes are theorized to be organized into specific structures by weak interactions (such as electrostatics and Van der Waals) between the surface residues of enzymes in the pathway.\(^1,5\) Factors of the intracellular environment such as macromolecular crowding, volume exclusion caused by the high macromolecule (proteins, nucleic acids, etc.) concentration in the cell, help to hold these complexes together, making them hard to isolate in vitro upon cell lysis. Due to the differences between common solutions used in protein expression and purification and the cellular matrix very few complexes have been isolated whole from the cell,\(^5\) but several pairs of enzymes or proteins have been co-eluted from purification columns\(^4,7\) or co-precipitated in viscous solution.\(^8,9\)
Due to the difficulty of isolating these complexes for study, model systems (both experimental and computational) are key to understanding the effects of colocalization and organization of sequential enzymes in a metabolic pathway.

**Advantages of Colocalization**

Several sources have detailed the possible advantages of sequential enzyme colocalization. Proximity between sequential enzymes reduces the diffusion distance for intermediates between the active sites, reducing the transit time. This can prevent the loss of reactants to diffusion and prevent scavenging by competing pathways. Short diffusion distances also prevent the buildup of intermediates which can have a negative effect on pathway activity and protect labile intermediates from reacting in the cytoplasm.\(^1,10-13\)

Several known multienzyme complexes have been established to be static associations of enzymes localized to various portions of the cell (Figure 1-1). Pathways such as glycolysis and the TCA cycle have been shown to associate with the plasma membrane\(^2\) or mitochondrial membrane,\(^5\) respectively. Many others are thought to assemble to cytoskeletal structures such as actin filaments, or exist free in the cytosol or in an aqueous compartment. Colocalization such as this is thought to promote sequential activity by reducing the diffusion distance for intermediates, or providing electrostatic channels (pathways along the surface of the enzymes which promote direct transfer of intermediates). This may also promote regulation of pathways through feedback inhibition of intermediates or products which would accumulate in the vicinity of the complex.\(^1\)

Pyruvate decarboxylation is carried out by the pyruvate dehydrogenase complex (PDC). This pathway consists of three enzymes, pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3), which have been isolated as a static complex with a regular structure. As seen in Figure 1-2, colocalization of these three enzymes
forms a complex in which intermediates are easily channeled, or transferred from one enzyme to another. Channeling is a proposed mechanism for transfer of intermediates from one active site to the next without diffusion into solution. In this process, intermediate are directed through electrostatic “channels” or pathways on the surface of the enzymes, or “tunneled” through cavities in between two enzymes. Colocalization of sequential enzymes alone reduces the diffusion distance for intermediates between reaction sites providing much of the same benefits.

Colocalization can also be achieved by sequestering sequential enzymes to a segregated phase of solution. This effectively increases the local concentration of each enzyme and reduces the distance between enzymes by constraining them to an unbound compartment. In 2009, Brangwynne et al. and Ge et al. both reported the occurrence of segregated liquid phases within the volume of a living cell.

Proximity as a Mechanism for Metabolic Control

Dynamic proximity between sequential enzymes has been proposed as a mechanism for controlling the rate of a sequential activity to effectively turn a pathway on or off by altering the efficiency of the sequential reaction. Selective association and dissociation of a multienzyme complex may alter the efficiency of a sequential reaction so that a pathway is only active when the end product is required.

One recent example of this is the purinosome. Benkovic and coworkers have demonstrated the reversible assembly of several enzymes in the de novo purine biosynthetic pathway. As seen in Figure 1-3, when the cell is not provided with purines through the media fluorescently tagged enzymes from this pathway are found in clusters within the cell that can be detected via fluorescence microscopy. When purines are then added to the media, these enzymes
disperse. This process was demonstrated to be reversible by depleting the media of purines, causing the clusters to reform.

**In vitro Enzyme Assemblies**

Dynamic multienzyme assemblies of metabolic enzymes such as the de novo purine biosynthetic pathway are difficult to isolate ex vivo because of the weak interactions that hold them together in vivo. Additionally, the cell may regulate these dynamic interactions by post translational modifications such as phosphorylation which are difficult to replicate. Therefore a model system where proximity between enzymes is modulated artificially is necessary to study the effect of colocalization on sequential activity.

**Fusion Proteins**

Fusion proteins have been prepared via genetic engineering which combine the genes for two sequential genes with a small chain of amino acids as a spacer between the two enzymes structures. Several reports have used fusion proteins as models for multienzyme complexes because they provide control the proximity between enzymes as well as control the stoichiometry of the enzymes in the fusion.

Colocalization of enzyme with this method has been proven to increase the activity of a sequential reaction and decrease the transient time before a steady state activity is achieved. Proximity also enhances the rate of sequential reaction as compared to a competing reaction. One example of this method was reported by Mao et al in 1995, the subunits of four enzymes from the glucose phosphotransferase system in *E. coli* were expressed as a fusion protein. They found that the activity of the fusion protein was 3-4x greater than the isolated subunits, and that a
competing pathway did not affect the activity of the fusion protein, but did affect the rate of the isolated subunits.\textsuperscript{21} Datta et al. found sequential activity of fusion proteins was enhanced by the presence of poly(ethylene) glycol (PEG), a macromolecule which reduces the rate of diffusion in solution. These results support the hypothesis that proximity between sequential enzyme plays a dramatic role in the overall activity of a metabolic pathway.\textsuperscript{8}

Fusion proteins are not without limitations. Although gene fusion orients the C and N termini of the proteins, the orientation of the active sites cannot be easily controlled, and aggregation is a common occurrence for multi-subunit proteins. The individual enzymes in a fusion protein can have reduced activity, as well as differences in $K_M$, and stability from native enzymes.\textsuperscript{17} In some cases, it is uncertain if proximity alone or electrostatic channeling of intermediates along the protein structure are responsible for the increases in activity found.\textsuperscript{22, 23}

**Enzymes Attached to Artificial Scaffolds**

An alternative method to control the proximity between sequential enzymes is adsorption or immobilization of enzymes to a solid support. Within the cell, intracellular surfaces such as membranes and the cytoskeleton are thought to serve as scaffolds for the assembly of some multiprotein complexes\textsuperscript{2-15} In vitro, supports such as metallic, polymeric, or silica particles can serve as a simple model system for these types of assemblies.\textsuperscript{24} Methods for the preparation and analysis of protein-coated scaffold particles are relatively advanced due to the long history of use of protein gold nanoparticle bioconjugates from biospecific stains for electron microscopy,\textsuperscript{25} to more recent bioanalytical and biotechnological applications.\textsuperscript{26} Unfortunately, most of this work has not focused on quantification or characterization of protein bound. There are a growing number of studies that have compared activity of bound vs. free enzymes and for colocalized vs. non-colocalized sequential reactions\textsuperscript{27-33}
Adsorption to a scaffold can alter enzymatic activity for several reasons, such as denaturation of the protein structure, steric hindrance due to the adjacent enzymes, and orientation of the active site after adsorption. It is therefore important to have a suitable control, such as the characterization of enzymes in solution and on particle, to determine the effect of conjugation on enzyme activity. Specific activity, a useful measurement in determining enzyme denaturation, requires the quantification of the number of enzymes bound per particle which can be challenging to determine for irreversibly bound molecules. While the specific activity is not consistently reported in the literature it can be determined with two methods. Several authors have determined stoichiometry indirectly by subtractive methods where the amount of protein bound is determined based on what is left in solution after adsorption. Alternatively, some scaffolds such as gold nanoparticles can be dissolved to release adsorbed protein back into solution for quantification directly.

Enzymes can also be attached to scaffolds using directed methods that result in a single layer of enzyme becoming associated with the surface of the particle, either covalently or non-covalently. Reversible approaches are particularly attractive because they enable removal of bound enzyme molecules for quantification and characterization. For example, horseradish peroxidase (HRP) has been expressed with a hexahistidine tag, which was then bound to Ni(II)-nitrilotriacetic acid groups on a gold nanoparticle. The activity of HRP bound, as a single layer, to AuNP was essentially the same as free enzyme in solution. This general method has been used to prepare surfaces coated with a mixture of two sequential glycolytic enzymes, hexokinase and glucose-6-phosphate isomerase. Sequential activity of the surface-tethered enzyme pair could then be measured to determine the rate enhancement due to colocalization.

Biological multienzyme assemblies can have protein cores, and in some cases the interior proteins are enzymes responsible for catalysis of one of the reaction steps, such that layers of
enzyme exist on top of each other (Figure 1-2). An attractive model system for controlled fabrication of enzyme multilayer is the incorporation of sequential enzymes into a layer-by-layer (LbL) deposition of polyelectrolytes (PEs). LbL assemblies are constructed by the sequential adsorption of polycations and polyanions on a latex bead or other support. Enzymes can be incorporated in desired layers during assembly. Although most efforts in this area have been directed towards biotechnological or sensor applications, these types of assemblies are also interesting as models for intracellular organization. Pescador et al. recently used this approach to immobilize two enzymes onto a scaffold particle with controlled geometry: the two enzymes were assembled either into the same or different layers of the PE coating. They not only confirmed that the sequential reaction was more efficient for the artificial complex than enzymes free in solution, but also showed that even within the complex, enzymes work more efficiently when they are closer together and fewer barriers for diffusion of intermediates exist.

The properties of the scaffold itself can be an important factor in the colocalization of sequential enzymes. When Koch-Schmidt et al. compared the activities of separate and colocalized malate dehydrogenase (MDH) and citrate synthase (CS), where colocalization was achieved either using a fusion protein, or by adsorption to a Sepharose bead scaffold, they found that fusion had no effect on the efficiency of the pathway in dilute solution, and the activity of the immobilized enzymes were 10-20% higher than that of enzymes in solution. Sepharose beads provided a unique support for this enzyme system, because of the large pores that enabled diffusion and concentration buildup of the intermediate, oxaloacetate, into the bead and unstirred layer of solution associated with the surface of the bead. Restricting the diffusion of oxaloacetate in this study served to increase the sequential rate of activity by improving the efficiency of intermediate transfer.
MDH and CS as a model for Sequential Activity

MDH and CS are two sequential enzymes found in the TCA cycle, which has been demonstrated to exist as multienzyme complex associated with the inner mitochondrial membrane within the cell. Independently, MDH performs a reversible redox reaction (Figure 1-4) which oxidizes malate to form oxaloacetate, and CS performs a Claisen condensation reaction (Figure 1-5) transferring an acetyl group to oxaloacetate to produce citrate. As seen below when working sequentially, MDH and CS catalyze the conversion of malate to oxaloacetate and finally to citrate with the aid of NAD$^+$ and acetyl-CoA as coenzymes. Both of these enzymes exist as a dimer of two identical monomers, each with an independent active site. Additionally, there are known interactions between CS and itself, MDH1 (mitochondrial MDH) and itself, and MDH1 and CS, while a second isoform, MDH2 (cytoplasmic MDH) has been shown not to interact with CS.

\[
(1) \quad \text{Malate} + \text{NAD}^+ \xleftrightarrow{\text{MDH}} \text{Oxaloacetate} + \text{NADH} \\
(2) \quad \text{Oxaloacetate} + \text{Acetyl} - \text{CoA} \xrightarrow{\text{CS}} \text{Citrate} + \text{CoA}
\]

The many previous studies of these two enzymes make them an attractive model system for the study of sequential reaction of colocalized enzymes. Srere and coworkers immobilized these two enzymes as well as a third, lactate dehydrogenase (LDH), to polyacrylamide gel and found an enhancement in the sequential rate of reaction when the enzymes were colocalized within the gel versus native enzymes in solution. Stemming from this work Srere and several others continued to study the sequential reaction of MDH and CS using solid state aggregates, fusion proteins, and Sepharose beads to control the localization of enzymes. Detailed information is also available about how the two enzymes interact, and the possible mechanisms
for intermediate transfer in the multienzyme complex.\textsuperscript{8, 42} Additionally, computational models have been created to characterize and explore the interactions found.\textsuperscript{42, 43}

### Scaffolds for Artificial Multienzyme Complex Formation

In each of the experimental studies above some information about the advantages of co-localization of a sequential pair of enzymes was determined, but few of the initial studies contained proper control systems to account for differences in enzyme activity or structure between native enzymes and aggregates, fusion proteins, or covalently modified enzymes. To fully understand the kinetic consequences of colocalization of sequential enzymes the colocalized and non-colocalized enzymes must be in similar, well understood environments. To this end I have developed and characterized three multienzyme complex models to further identify the implications of proximity between sequential enzymes.

Direct adsorption-gold nanoparticle bioconjugates, L\textsubscript{B}L assemblies, and Ni(II)NTA functionalized scaffolds each have the ability to immobilize multiple sequential enzymes to the surface of a single scaffold in various stoichiometries and geometries. Additionally, non-colocalized controls can be easily produced on identical scaffolds to provide the best available controls for the study of proximity between sequential enzymes.

**AuNP Bioconjugates**

Enzymes can be directly adsorbed to the surface of gold nanoparticles non-specifically, via hydrogen bonding, hydrophobic, van der Waals, and/or electrostatic interactions.\textsuperscript{44} Multiple enzymes can be adsorbed to a single scaffold to produce a colocalized model of sequential kinetics, while an equivalent non-colocalized control can be created by immobilizing each
enzyme in the pathway to a different nanoparticle. In this way the proximity between sequential enzymes can be modulated from nanometer when immobilized to the same particle to micrometers when immobilized to separate particles. The distance between nanoparticles can be modulated by the solution concentration of bioconjugates, allowing the effect of proximity on a sequential reaction to be observed.

Adsorption of multiple types of enzymes to a single AuNP surface also allows for the study of enzyme activity and pathway efficiency for different arrangements of enzymes. This allows for the comparison of specific and random arrangements of enzymes to determine optimal pathway efficiency, and optimal arrangement for the activity of individual enzymes.

**L_{BL} Assemblies**

Electrostatic interactions between proteins and PEs allows for the controlled deposition of enzymes into specific layers in an assembly. Layers are formed by sequential addition of oppositely charged PEs and proteins or enzymes to form a defined coating on the outside of a nanoparticle. This allows for the study of not only proximity between enzymes but also orientation between enzymes in single complex, because the addition of each enzyme can be can be controlled by how and when it is deposited on the surface. As seen in Figure 1-2, multienzyme complexes can have specific orientations of enzymes to create an essentially layered assembly of enzymes, L_{BL} assemblies provide an attractive method to test the effects of the arrangement enzymes in relation to each other.
**Ni(II)NTA Scaffolds**

Histidine-tag – Ni(II)NTA interactions may provide the most promising model for dynamic proximity between sequential enzymes. If each enzyme in the pathway is cloned with a his-tag, the affinity of immobilization for each enzyme can be controlled to allow precise control over the stoichiometry of the assembly. Additionally, this interaction is reversible and non-destructive to the enzyme which allows for the co-localized enzymes to become non-colocalized upon stimulus, with little effect on the individual catalysis. Ni(II)NTA can be used with multiple types of scaffolds; solid supports can be functionalized with these moieties, while it can be used to decorate more fluid supports such as large unilamellar vesicles (LUVs). The reversibility of this interaction allows for colocalized enzymes to be released from the scaffold surface to model the suspected interactions found in Figure 1-3.

**Summary and Objectives**

In summary, many examples of the colocalization of sequential metabolic enzymes into multienzyme complexes exist, and the advantages of colocalization on the diffusion of intermediates and rate of sequential reaction have been proposed. While some complexes, or partial complexes have been isolated ex vivo, this is generally difficult to accomplish due to the vast differences between cytoplasm and bulk dilute solutions. Therefore, model systems are necessary to explore and understand how and why sequential enzymes are so often found in association in vivo.

The goal of this work is to study, characterize, and understand the effect of proximity between sequential enzymes and how it may relate to the formation and activity of multienzyme
complexes of metabolic enzymes in vivo. To this end, three models for multienzyme complexes have been developed and characterized in order to establish the effects of colocalization.

Chapter 2 presents a detailed analysis of single activity (one type of enzyme) and dual activity (two types of enzymes) enzyme AuNP bioconjugates. Here, the stoichiometry, specific activity, substrate saturation, and turnover rate were measured in order to fully understand the effects of direct adsorption on MDH and CS. This information was then used to evaluate the best method for colocalizing the enzymes in a dual activity bioconjugate. The use of AuNP bioconjugates as a model for proximity between two sequential enzymes is presented in chapter 3. In this section, the effects of diffusion distance, and limitations on the rate of diffusion of reaction intermediates were evaluated to determine the effect they may have on sequential reactions.

An evaluation of the effects of orientation of MDH and CS within a LbL assembly are presented in chapter 4. Here, the sequential enzymes were deposited into LbL layers in different arrangements to determine the effect of enzyme layering on the rate of a sequential reaction. Each assembly was also characterized in terms of their specific activity of each enzyme when immobilized to different layers of the complex. Chapter 5 describes two additional scaffolds in which his-tag – Ni(II)NTA interactions are used to reversibly immobilize enzymes to the surface of solid supports such as AuNP and fluid supports such as LUVs.

While the motivation for this work is to understand the associations of sequential enzymes within the cell, the results found have further implications for nanotechnology designs. In this work, each model system described was characterized as completely as possible before the overall hypothesis could be tested. This provides a large pool of information about the functionality and usability of immobilized enzymes that is not commonly obtained for the types of assemblies created, allowing this work to give new insight into better designs and characterization for nanotechnological applications such as nanoreactors.45
References


Figure 1-1. Localization of multienzyme complexes within the cell. Pathways such as glycolysis (plasma membrane/cytoplasm), TCA cycle (mitochondrial membrane), de novo purine biosynthesis (cytoskeleton or cytoplasm), and P granules (aqueous partitions) can be found as associations or complexes of colocalized enzymes within the cell.
Figure 1-2. The pyruvate dehydrogenase complex. The PDC has been isolated ex vivo from the cell and examined using methods such as cryo EM (A). The complex was found to have a regular structure in which up to 60 copies of E2 provide a scaffold for assembly of E1 and E3 (B). This pathway carries out the reaction to produce Acetyl-CoA from pyruvate within the mitochondria (C).
Figure 1-3. Dynamic localization of the enzymes of the de novo purine biosynthetic pathway. Differences in localization can be visualized using fluorescently tagged enzymes in mammalian cells. When cells are starved for purines, these enzymes can be seen localizing into clusters containing the enzymes of the pathway. In purine rich media the clusters disperse into the cytoplasm, and only reform when purines are removed from the media. Figure provided by Dr. Songon An.
Figure 1-4. Reaction mechanism of MDH for the conversion of malate to oxaloacetate. (Left) malate (pink) and NAD$^+$ (blue) bind to the active site of MDH noted by the amino acid residues present (black). His 195 then aids in the oxidation of malate. (Right) this forms oxaloacetate (pink) and NADH (blue). 47
Figure 1-5. Reaction mechanism for CS for the conversion of oxaloacetate to citrate. Acetyl-CoA (blue) initially binds to the active site of CS (black residues) followed by the binding of oxaloacetate (pink). His 274 then donates a proton to acetyl-CoA which allows Asp 375 to remove a methyl proton from acetyl-CoA. Oxaloacetate then gains a proton from His 320 while the enol of acetyl-CoA attacks the carbonyl of oxaloacetate to form a bond between the acetyl group and oxaloacetate. Water (purple) then attacks the CoA to break the bond between citrate and CoA.48
Chapter 2

Enzyme: nanoparticle bioconjugates with two sequential enzymes:
Stoichiometry and activity of malate dehydrogenase and citrate synthase on 
Au nanoparticles

(Reproduced in part from Keighron, J. D.; Keating, C. D. Enzyme: nanoparticle bioconjugates 
with two sequential enzymes: Stoichiometry and activity of malate dehydrogenase and citrate 

Biomolecule-nanoparticle conjugates are increasingly important in a wide range of 
applications including bioanalysis, imaging, and medicine. In particular protein-nanoparticle 
bioconjugates have been used as electron-dense biospecific stains in electron microscopy for 
many years and more recently are showing promise for applications such as bioanalysis, 
biocatalysis and nanomedicine.¹⁻⁵ For example, antibody-coated particles have been used as 
affinity labels to detect antigens via changes in solution optical absorbance or scattering, surface 
plasmon resonance, surface enhanced Raman scattering, or electrical conductivity.³ Enzyme-
nanoparticle conjugates that take advantage of the catalytic ability of bound enzymes have been 
reported for bioanalytical and biotechnological applications.¹⁻²,⁶,⁷ Increasingly complex 
multienzyme conjugates are being developed. For example the sequential enzymes horseradish 
peroxidase and glucose oxidase have been co-immobilized in polyelectrolyte multilayers on silica 
microparticles.⁸ A set of three sequential enzymes was immobilized on phospholipid polymer 
particles to facilitate the overall reaction.⁹ Multiactivity bioconjugates such as these are attractive 
as model systems for biological enzyme assemblies, in bioanalytical sensors, and for industrial 
processes.⁸⁻¹² For any of these applications the performance of the final constructs is impacted by
a number of different factors and consequently characterization of the conjugates, and in particular their enzymatic activity, is essential.

Many methods have been reported for preparation of protein-nanoparticle conjugates.\textsuperscript{4} Electrostatic attraction can be used to assemble proteins in polyelectrolyte multilayers.\textsuperscript{13,14} Reactive functional groups such as primary amines, thiols, or carboxylates present on the protein can be coupled to molecules on the particle surface using any number of crosslinking chemistries.\textsuperscript{15,16} Depending on the presence and distribution of the reactive groups, chemical coupling can result in excellent to very poor retention of bioactivity for bound proteins. Alternatively, the techniques of molecular biology can be used to insert unique functional groups (e.g. cysteine residues, C- or N-terminal polyhistidine tags) at predetermined sites to enable oriented attachment. This approach requires greater effort, but provides excellent control over the properties of the resulting bioconjugates.\textsuperscript{17} For example, Abad and coworkers showed nearly 100\% activity retention for histidine-tagged horseradish peroxidase conjugation to Co(II)-nitrilotriacetic acid functionalized gold particles.\textsuperscript{18} Despite these successes, the most commonly used means of bioconjugation is direct adsorption, where nanoparticles are simply exposed to the protein in solution and the resulting conjugates are separated by centrifugation. Proteins adhere non-specifically to the particle surface through a combination of hydrogen bonding, hydrophobic, van der Waals, and/or electrostatic interactions.\textsuperscript{19,20} Enzyme adsorption often results in a significant loss of catalytic activity due to denaturation and/or blockage of the active site.\textsuperscript{19-25} After an initial activity loss, however, particle-bound enzymes have in several cases been shown to resist further activity loss upon aging, pH changes or exposure to high temperatures better than free enzyme.\textsuperscript{25-27}

Because enzyme structure, active site accessibility, and/or catalytic activity have been shown to change upon adsorption for many enzyme-nanoparticle systems, it is important to characterize nanobioconjugates. Enzymatic activity and Michaelis-Menten kinetics parameters for
bioconjugates vary widely depending on the enzyme, the size and type of nanoparticle, and the conjugation method. \cite{25,26,28,29} Circular dichroism spectroscopy and tryptophan fluorescence have provided information on enzyme secondary structure \cite{19,25,30-32} and when coupled with activity measurements can lend insight into whether conformational changes or active site orientation are responsible for observed changes in activity as compared to free enzymes. \cite{22-24} In many cases, the number of enzyme molecules bound to each nanoparticle, and/or the total number of nanoparticles in solution are not known. Consequently reports of specific activity are relatively uncommon, \cite{21} particularly for multienzyme conjugates. Specific activity, measured as U/mg of enzyme, is a standard measurement for determining enzyme purity. \cite{33} For enzyme-nanoparticle conjugates it can give the extent of inactivation due to the combined effects of denaturation, orientation, and steric hindrance.

In chapter, the preparation and characterization of enzyme:Au nanoparticle bioconjugates incorporating two different enzymes, malate dehydrogenase (MDH) and citrate synthase (CS) is described. Bioconjugates were prepared by direct adsorption of one or both enzymes to 30-nm diameter gold nanospheres as illustrated in Figure 2-1. The two enzymes were labeled with different fluorescent dyes, which made it possible to quantify the number of copies of each enzyme adsorbed per nanoparticle and determine specific activities for bound vs. free enzyme. Substrate saturation (K_M), turnover number (k_cat), and per-particle individual and sequential activities were also determined. By varying the order in which enzymes were adsorbed, we were able to investigate the effect of each enzyme on the adsorption and activity of the other. In all cases we observed a decrease in specific activity upon adsorption for both enzymes. However, both the number of copies each enzyme bound and their specific activities depended on the order of addition of the enzymes to the nanoparticles. The specific activity of MDH and CS varied by nearly an order of magnitude in different configurations on the surface.
Experimental

Materials

Colloidal gold particles (mean diameter 30.7 nm, $2 \times 10^{11}$ particles/ml) were purchased from Ted Pella, Inc. (Redding, CA). AlexaFluor 488 and AlexaFluor 633 protein labeling kits were purchased from Invitrogen (Carlsbad, CA). Citrate synthase (CS) from porcine heart (EC 2.3.3.1) ammonium sulfate suspension, malic dehydrogenase (MDH) from porcine heart (EC 1.1.1.37) ammonium sulfate suspension solution, L-(-)-malic acid, oxaloacetic acid, acetyl coenzyme A trilithium salt, β-nicotinamide adenine dinucleotide hydrate (NAD’), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), Trisma® hydrochloride (Tris[hydroxymethyl]aminomethane hydrochloride), Trisma® base (Tris[hydroxymethyl]aminomethane), sodium bicarbonate, and Ellman’s Reagent (5,5’-dithiobis[2-nitro-benzoic acid]) (DTNB) were purchased from Sigma-Aldrich (St Louis, MO). Pall Life Sciences Nanosep 10k MWCO centrifugal devices and Whatman 20 nm pore diameter syringe filters were purchased from VWR. Deionized water with a resistivity of ≥18.2 MΩ from a Barnstead NANOpure Diamond water purification system (Van Nuys, CA) was used in all experiments.

Enzyme Labeling

A 10 kDa MWCO centrifuge filter was used to remove ammonium sulfate, which can reduce labeling efficiency by outcompeting primary amines in the protein structure for the coupling reaction. CS and MDH were resuspended in 5 mM sodium bicarbonate (pH 8.3) at a 2 mg/ml concentration prior to fluorescent labeling. CS and MDH were labeled with AlexaFluor 633 and AlexaFluor 488 respectively, according to the protocol provided by Invitrogen.
Flocculation Assays

Flocculation assays are a traditional means of determining how much protein must be added to produce a stable bioconjugate with gold nanoparticles. The Au nanoparticles used in bioconjugation are negatively charged, and prevented from aggregating by electrostatic repulsions. Addition of NaCl to screen these electrostatic repulsions results in aggregation or flocculation, which is observable as a color change from red to blue or purple. Adsorption of protein to the nanoparticle surface creates a steric barrier that prevents salt-induced flocculation. Here, we performed flocculation assays to determine the minimum amount of fluorescently labeled CS or MDH necessary to prevent single enzyme bioconjugates from salt-induced aggregation. Samples with mole ratios of enzyme:Au of 25:1 to 500:1 were prepared by diluting Au particles (3.75 x 10^{-14} mol) in buffer then adding enzyme from stock prepared in 5 mM sodium bicarbonate (pH 10) for a total volume of 150 µl. Samples were incubated for 1 hour at room temperature protected from light followed by addition of 150 µl of buffer to dilute the solution for absorbance measurements and 17 µl of 2 M NaCl, to induce aggregation of unprotected nanoparticles. After 60 min incubation samples were analyzed by absorbance spectroscopy using a Hewlett-Packard 8453 diode-array UV-visible spectrometer with Agilent ChemStation Software.

Enzyme Conjugation and Bioconjugate Purification

Single enzyme bioconjugates were prepared by first concentrating 6.0 x 10^{11} Au nanoparticles (9.96 x 10^{-13} mol) to a volume of 1 ml and washing with 5 mM sodium bicarbonate (pH 8.3) in 20 nm filtered water to remove any possible contaminants. CS or MDH was then added from a stock solution in the same buffer to create the desired enzyme:Au ratio, and buffer
was added to a final volume of 1.5 ml. Enzymes were allowed to adsorb to the surface of the nanoparticle for 1 hour at 4°C protected from light. Excess enzyme was subsequently removed by centrifugation of the bioconjugates at 5000 x g for 15 min at 4°C, the supernatant was removed and bioconjugates were resuspended in 1.5 ml of fresh buffer. This process was repeated 3 times to ensure all excess enzyme was removed from the bioconjugate solution. Multienzyme bioconjugates were prepared by adsorbing CS and MDH to the surface of the same nanoparticle either sequentially or simultaneously (Figure 2-1). Bioconjugates were formed in sequential steps by adsorbing CS or MDH onto the surface of a preexisting bioconjugate containing the other enzyme, or by simultaneously adding both enzymes to bare Au nanoparticles. All incubation and washing procedures follow those laid out for single enzyme bioconjugates.

**Enzyme Activity Assays**

To measure the rate of reaction for the conversion of malate to oxaloacetate ($v_{m-o}$) for MDH in single and multienzyme bioconjugates of 100 mM Tris (pH 8.1), 25 µl of 36 mM malate and 25 µl of 60 mM NAD$^+$ (25 µl of 60 mM oxaloacetate and 25 µl of 12 mM NADH for the conversion of oxaloacetate to malate by MDH or $v_{o-m}$), for a final concentration of 0.3 mM malate and 0.1 mM NAD$^+$ (0.5 mM oxaloacetate and 0.1 mM NADH) were mixed in a cuvette and equilibrated to room temperature, in accordance with previous reports. A volume of 200 µl of bioconjugate was then added to the solution, which was mixed again to ensure homogeneity and the absorbance of NADH was monitored at 340 nm for 5 min (Figure 2-2) $\varepsilon = 6220 \text{ M}^{-1}\text{cm}^{-1}$. For each assay the volume of bioconjugate added (200 µl) remained constant and the concentration of bioconjugate was measured by the absorbance of the gold plasmon at 524 nm ($\varepsilon = 3.585 \times 10^9 \text{ M}^{-1}\text{cm}^{-1}$). Each assay was repeated at least 3 times and had a total volume of 3 ml. The activity of CS for conversion of oxaloacetate to citrate ($v_{o-c}$) in single and multienzyme bioconjugates was
assayed in similar fashion to MDH with the exception that 25 µl of 12 mM Acetyl-coenzyme A and 25 µl of 60 mM oxaloacetate were used as substrates, 25 µl of 18 mM DTNB, for a final concentration of 0.5 mM oxaloacetate, 0.1 mM Acetyl-CoA, and 0.15 mM DTNB. DTNB was added to monitor the production of coenzyme A by CS by adsorption at 412 nm $\varepsilon = 13,600 \text{ M}^{-1}\text{cm}^{-1}$.

Determination of kinetic constants was carried out in the same fashion, varying the amount of either malate or oxaloacetate while the concentration of the coenzymes remained constant. $K_M$, $V_{\text{max}}$, and $k_{\text{cat}}$ were determined by nonlinear regression using equation 1 for CS and the forward reaction of MDH, and equation 2 for the reverse reaction of MDH, this was necessary because high concentrations of oxaloacetate are known to inhibit MDH activity.

\begin{align}
(1) \quad & v = \frac{V_{\text{max}} [S]}{K_M + [S]} \\
(2) \quad & v = \frac{V_{\text{max}} [S]}{[S] + K_M (1 + \frac{S}{k_i})}
\end{align}

Sequential activity of MDH and CS in multienzyme bioconjugates was measured by monitoring the production of coenzyme A in the presence of 25 µl of 36 mM malate, 25 µl of 60 mM NAD$^+$, 25 µl of 12 mM Acetyl-Co A, and 25 µl of DTNB in a total volume of 3 ml, for a final substrate concentration of 0.3 mM malate, 0.5 mM NAD$^+$, and 0.1 mM Acetyl-Co A, with 0.15 mM DTNB to monitor Co-A production. Kinetic assays were run for 300 s time periods with a full spectrum collected every 30 s.
**Determination of Enzyme: Nanoparticle Stoichiometry**

The number of enzymes adsorbed per nanoparticle was determined by two complementary methods: (1) indirectly, by monitoring the enzyme remaining in solution after conjugation to the nanoparticles. The concentration of enzyme found in solution after conjugation was subtracted from the initial enzyme concentration and divided by the initial Au concentration; measurements of enzyme concentration were corrected to account for enzyme lost to adsorption to the ependorf tube.\(^{21}\) (2) Directly, by washing away excess enzyme and dissolving the Au core to release the adsorbed enzymes into solution for quantification. This was done by first determining the concentration of bioconjugates based on the absorbance of the gold nanoparticles at 524 nm, \(\varepsilon = 3.585 \times 10^7 \text{M}^{-1} \text{cm}^{-1}\).\(^{39}\) 100 µl of 50 mM potassium cyanide was then added to 100 µl of bioconjugate, mixed, and allowed to react for 12 hrs at 4ºC. A Horiba Jobin Yvon Fluorolog 3-21 was then used to find the concentration of each fluorescently labeled enzyme in solution. Stoichiometry is expressed as the ratio of enzyme to Au present after conjugation and washing steps.

**Results and Discussion**

We chose for this study two well-characterized, readily available enzymes that carry out a sequential reaction (Figure 2-2) as part of the TCA cycle. Malate dehydrogenase (MDH) catalyzes the reversible conversion of malate to oxaloacetate using the cofactor NAD(H),\(^{45}\) and citrate synthase (CS) carries out a Claisen condensation to catalyze formation of citrate from oxaloacetate using acetyl-CoA.\(^{44}\) Mitochondrial MDH and CS are found physically associated with each other in a multienzyme complex thought to enhance the overall kinetics of this pathway.\(^{44,46-48}\) We used cytoplasmic MDH, which has been reported not to interact with CS, for
the experiments described here in order to reduce protein-protein interactions. Since we are relying on direct adsorption of the enzymes to the gold nanoparticles, differences in the relative affinity of the two enzymes for the particles (and any propensity for binding each other) will affect the bioconjugates formed.

Single-activity enzyme:Au nanoparticle (Au) bioconjugates will be described first, followed by those in which both CS and MDH were adsorbed to the same particles. The bioconjugates were named systematically based on the order in which enzymes were adsorbed to the nanoparticle surface (Figure 2-1). Our notation lists the enzymes adsorbed from last to first, followed by the core Au separated by colons. For example, MDH:Au, CS:Au, and MDH/CS:Au were created using a single adsorption step while MDH:CS:Au was made by adsorbing MDH to a preexisting CS:Au bioconjugate. In all of the experiments described here, MDH has been fluorescently labeled with AlexaFluor 488 and CS with AlexaFluor 633.

**MDH:Au and CS:Au bioconjugates**

Single-activity bioconjugates were formed through direct adsorption of either MDH or CS onto 30 nm Au nanoparticles. Several ratios of enzyme to Au nanoparticle were tested to determine the effects of ratio on bioconjugate stoichiometry (i.e., the number of enzyme molecules adsorbed per particle) and enzyme activity.

**Stoichiometry**

We first determined the number of enzyme molecules adsorbed per particle as a function of the enzyme:Au ratio added to solution. After incubating enzymes with nanoparticles, the resulting bioconjugates were spun down and any free enzyme remaining in solution was removed by
rinsing. The Au cores of the bioconjugates were then dissolved in KCN solution, after which the released enzymes were quantified via fluorescence. This approach had been demonstrated previously for DNA oligonucleotide functionalized Au nanospheres and for HRP:Au bioconjugates and was extended here to the two-enzyme CS/MDH system by using a spectrally distinct dye on each enzyme (Alexa488 on MDH and Alexa633 on CS). We favor this method for determining the number of adsorbed enzymes over more common subtraction-based approaches because it enables us to account for any loss of Au nanoparticles during rinsing due, e.g., to adsorption to the centrifuge tube walls, incomplete pelleting, and/or incomplete resuspension. Additionally, this approach is applicable regardless of the relative amounts of bound vs. unbound enzyme; subtraction-based measurements are most accurate when the amount of adsorbed enzyme is a substantial fraction of the total. For the bioconjugates prepared here, the amount of adsorbed enzyme was generally 30% or more of the total enzyme, and subtraction gave comparable results to particle dissolution. Figure 2-3 compares these two quantification methods for each of the types of bioconjugates studied in this work. We used the values obtained from the direct measurement in our calculations throughout the paper.

Figure 2-4A shows our experimental results for enzyme:Au bioconjugate stoichiometry based on direct detection of fluorescently-labeled MDH or CS. Based on crystal structure data, it was estimated that 73-106 MDH or 56-91 CS molecules would be required to form a monolayer on each 30.7-nm diameter Au nanoparticle, depending on adsorption orientation. Because no effort was made to control adsorption orientation, we have assumed that enzymes adsorb randomly with an averaged molecular footprint in all estimations of how many layers a given number of enzyme molecules corresponds to (i.e. we assume a monolayer of MDH corresponds to roughly 90 molecules, and a monolayer of CS to ca. 74 molecules). Our data indicate that the Au NP did not become saturated at monolayer enzyme coverage, instead beginning to form multilayers at high enzyme:Au ratios. This type of adsorption behavior,
including multilayer formation, has been observed for several other protein:Au bioconjugates, including protein A, concavalin A, bovine serum albumin, and HRP.\textsuperscript{21,33,54}

At MDH:Au ratios 100:1 and lower, nearly all the enzyme present adsorbed to the Au surface. At higher MDH:Au ratios less than 60\% of the total enzyme was associated with the bioconjugate. MDH:Au ratios of 100:1 or higher were also required to prevent salt induced flocculation (Figure 2-5). This is consistent with ca. 90 molecules needed to form the first layer on the nanoparticle surface. Incubation of CS with Au also led to enzyme adsorption, with multilayer formation at high enzyme:Au ratios. In contrast to MDH:Au bioconjugates, however, a greater percentage of the CS added to solution remained unbound. At a solution ratio of 300:1, less than one third of the CS molecules were associated with the nanoparticles. CS also required greater concentrations to prevent salt-induced flocculation of the Au nanoparticles as compared to MDH (Figure 2-5). The lower affinity of CS for Au nanoparticles as compared to MDH is consistent with their net charge at the adsorption pH and with their molecular weights. MDH (pI 10) is positively charged during adsorption, while the net charge on CS (pI 6-6.5) is negative.\textsuperscript{55,56} The relationship between pI and adsorption pH is often invoked in attempts to control binding, but is complicated by the nonuniform spatial distribution of charged groups and by other types of adsorption interactions with the Au surface.\textsuperscript{19,20,36} Using radiolabeled proteins, De Roe et al. found an inverse relationship between protein molecular weight and the amount of protein adsorbed per Au nanoparticle, suggesting that any specific surface interactions particular to a given protein were less important than overall size of the adsorbing protein.\textsuperscript{36}

\textit{Specific Activity}

Adsorption of proteins to solid surfaces can lead to conformational changes\textsuperscript{22-24} and/or reduced active site accessibility, decreasing the biological activity for the adsorbed proteins.\textsuperscript{21,25,32} The
effect of adsorption on activity varies with the identity of the enzyme, the type of solid support, and the way in which the enzymes are attached. For example, Gole et al.\textsuperscript{32} found that fungal protease retained approximately 90% of its native activity upon adsorption to Au NPs, while Cans et al. determined that the specific activity of adsorbed HRP was as high as 50% that of native HRP with low surface coverages, but retained only 5% the native specific activity with higher surface coverages.\textsuperscript{21} Even lower retention of 1% native activity have been reported for lysozyme on silica particles.\textsuperscript{57}

We quantified the activity of the adsorbed CS and MDH enzymes as compared to the same number of molecules free in solution. MDH performs a reversible reaction to convert malate to oxaloacetate ($v_{m-o}$) or oxaloacetate to malate ($v_{o-m}$). The conversion of oxaloacetate to malate is more favorable and is routinely used in characterization of this enzyme,\textsuperscript{58} however the $v_{m-o}$ is relevant to its biological function in the citric acid cycle,\textsuperscript{37} which includes the sequential reaction between MDH and CS studied here; hence, we performed activity assays for both reactions. We also measured the rate of conversion of oxaloacetate to citrate for CS, and determined specific activities for each enzyme from these data and the stoichiometry measurements described above.

\textbf{Figure 2-4B} shows the specific activity of adsorbed enzyme as a function of the amount of enzyme adsorbed per Au. For both MDH and CS the specific activity of bound enzyme at all of the enzyme:Au ratios was greatly reduced as compared to free enzyme in solution. Specific activity for MDH:Au and CS:Au nanoparticle bioconjugates depended on the ratio of enzyme molecules to nanoparticles present in solution during adsorption. For CS:Au when more enzyme was associated with the bioconjugate, the specific activity increased. For MDH:Au the highest specific activities were found for intermediate enzyme:Au ratios. These results can be rationalized by considering that (a) submonolayer coverages may have allowed greater denaturation on the surface and (b) multilayered assemblies could experience less conformational
change but also limited substrate diffusion or steric hindrance due to neighboring enzyme molecules. These types of surface coverage-dependent changes in activity have been observed in other systems. For example, Aubin-Tam et al. found lower alpha helical content for lower coverages of yeast cytochrome c bound to magnetic nanoparticles, and Vertagel et al. found similar results for lysozyme on glass beads. For multilayered enzyme assemblies, Cans et al. reported a similar trend with HRP-Au NP and linked it to decreased accessibility of the active site as the lateral packing of enzymes increases within a layer.

\[ K_M \text{ and } k_{cat} \]

For further characterization a solution ratio of 300:1 was used to prepare MDH:Au and CS:Au bioconjugates; this ratio was chosen because it was the lowest that provided bioconjugates stabilized from salt-induced flocculation (Figure 2-5). The \( K_M \), the substrate concentration at which half the active sites are filled, for free enzyme and MDH:Au and CS:Au are presented in Table 2. The observed changes in \( K_M \) suggest differences in the secondary and tertiary structure of enzyme upon binding the particles. Such changes are expected. Pandey et al. determined the \( K_M \) value for immobilized glucose oxidase on Au nanoparticles to be slightly lower than the free enzyme, similar results were found for lipase on \( \text{Fe}_3\text{O}_4 \) particles by Huang et al. where the \( K_M \) value was over three times lower for immobilized vs. free enzyme. Jia et al. also found a decrease in \( K_M \) value for immobilized \( \alpha \)-chymotrypsin to 110 nm diameter particles. They also measured \( K_M \) as a function of scaffold size in the 100 to 1000 nm range, and found increasing \( K_M \) value with larger polystyrene bead sizes.

The turnover number, \( k_{cat} \), is the number of substrate molecules converted to product per enzyme in a given amount of time. For MDH:Au and CS:Au the rate of substrate turnover is significantly diminished from that of free enzyme (Table 2-2), following the same trend as
specific activity which is not surprising considering the similarities between the measurements. These data complement the results seen for specific activity and $K_M$, representative of a change in the enzyme structure upon adsorption to Au nanoparticles that decreases the catalytic ability of each enzyme.

**Dual Activity Bioconjugates**

Having characterized the stoichiometry and kinetic parameters for MDH:Au and CS:Au bioconjugates, we next prepared dual-activity bioconjugates that contained both MDH and CS on the same nanoparticle (Figure 2-1). MDH and CS were adsorbed either by incubating one of the enzymes with a preexisting bioconjugate of the other enzyme (e.g., MDH added to CS:Au to form MDH:CS:Au), or by simultaneous adsorption of both enzymes (MDH/CS:Au) as described in the Methods section. A solution ratio of 300:300:1 MDH:CS:Au was used in each case because it was the minimum ratio of each enzyme necessary to prevent flocculation in single-activity bioconjugates and provided the maximum specific activity of MDH:Au.

**Stoichiometry**

As for the single activity bioconjugates described above, the number of fluorescently labeled enzymes adsorbed per Au was determined for each of the three types of dual-activity bioconjugates (Table 2-1 and Figure 2-3). The three differed in the number of each enzyme adsorbed per particle.

CS:MDH:Au was made by adsorbing CS onto an existing MDH:Au bioconjugate. During the adsorption of CS, the amount of MDH adsorbed decreased from $180 \pm 25$ to $102 \pm 19$ per particle, consistent with loss of a second layer of MDH to leave behind roughly a monolayer of
the enzyme. MDH loss was apparent not only in the reduced number of enzyme found associated with each particle, but also by the appearance of free MDH in the supernatant after centrifugation to remove the bioconjugates. Exchange between protein in solution and adsorbed protein has been observed previously for multilayers of BSA and concavalin A. The amount of CS in the CS:MDH:Au bioconjugates was much larger than in the single-activity CS:Au bioconjugates (186 ± 30 as compared with 79 ± 15). In part, this could be due to the larger diameter of the MDH-coated as compared to bare Au nanoparticles requiring a greater number of CS molecules to coat this surface with a monolayer. However, it could indicate the formation of partial multilayers of CS and/or a change in the average CS adsorption orientation to yield a smaller molecular footprint.

MDH:CS:Au was made by adsorbing MDH to existing CS:Au bioconjugates; here the amount of CS present remained constant while MDH was adsorbed (79 ± 15 and 80 ± 4 for CS:Au and MDH:CS:Au, respectively). However, far less MDH adsorbed to the CS:Au bioconjugate than adsorbed directly to Au in the single-activity bioconjugate (30 ± 14 for MDH:CS:Au as compared with 180 ± 25 for MDH:Au). Thus, MDH cannot make multilayers on an underlying CS support, and most likely the amino acid residues involved in binding to CS-coated nanoparticles differ from those used in binding directly to the Au nanoparticle surface. Additionally, the fact that CS adsorbs to the MDH:Au bioconjugates in greater numbers than MDH adsorbs to the CS:Au bioconjugates suggests that the interacting portions of the enzymes are different in the two cases, either due to different orientations of adsorption or conformational changes that differ in the two cases.

When MDH and CS are adsorbed simultaneously (MDH/CS:Au), the resulting stoichiometry was unlike either of the sequentially formed bioconjugates. The amount of both MDH and CS adsorbed per particle was far lower, suggesting possible binding interactions in solution prior to adsorption of either enzyme to the particles, and that the two enzymes adsorbed into a mixed
arrangement on the Au surface. This correlates with the data presented in Figure 2-4, which shows that more MDH (70 kDa) molecules are adsorbed to Au than CS (85 kDa) using the same E:Au ratios.

Specific Activity

Unlike the single-activity MDH:Au and CS:Au bioconjugates, several of the dual-activity bioconjugates exhibited greater than 10% of the specific activity seen for free MDH or CS in solution (Table 2-1), indicating that the enzyme conformation and/or environment in the these bioconjugates was more favorable than for the single-activity bioconjugates. For MDH the highest specific activity was found in MDH:CS:Au. The increase in specific activity over MDH:Au (10 for the forward reaction and 8 for the reverse reaction) is likely due to the fact that MDH was interacting with a layer of adsorbed CS instead of directly with the Au surface in the MDH:CS:Au. This is consistent with a greater retention of native conformation and/or a more favorable orientation of the MDH active site when it adsorbs atop the CS layer.

For CS the highest specific activity was found for CS:Au alone, this value decreased when MDH was adsorbed onto the CS:Au bioconjugate, but was still more active than CS:MDH:Au. These results suggest that the interaction between MDH and CS was detrimental to CS activity. Although we had selected the cytoplasmic MDH rather than the mitochondrial MDH in order to reduce binding interactions with the CS, a computational study conducted at Virginia Tech has predicted that CS also binds to cytoplasmic MDH. The model suggests the same residues are used as in binding to mitochondrial MDH, but that the active site of cytoplasmic MDH is rotated away from the active site of CS, as compared to the alignment of the mitochondrial MDH and CS active sites. In our experiments, this interaction could orient the CS active site away from solution, reducing the activity as compared to when the CS was adsorbed directly onto Au.
MDH activity in MDH/CS:Au was found to lie between that of MDH:CS:Au and CS:MDH:Au, consistent with the hypothesis that the enzyme adsorbs in multiple arrangements, both directly to Au and onto other adsorbed enzymes. CS activity was determined to be the same for MDH/CS:Au and MDH:CS:Au suggesting that most of the CS present is adsorbed directly to Au.

For each enzyme reaction, MDH:CS:Au had the highest specific activity, suggesting the structure of MDH and CS are less denatured and/or more favorably oriented in this arrangement than the other two multi-enzyme bioconjugates. Additionally, far less MDH adsorbs (<< 1 layer) to CS:Au than CS adsorbs (> 1 layer) to MDH:Au, which may allow for more interaction between solution and the bottom enzyme layer of MDH:CS:Au than CS:MDH:Au and increase activity of the bottom layer of enzyme.

\( K_M \) and \( k_{cat} \)

\( K_M \) and \( k_{cat} \) values for multi-activity bioconjugates are listed in Table 2-2. For MDH the \( K_M \) for malate in MDH:CS:Au and CS:MDH:Au was lower than in MDH:Au or free MDH. For MDH/CS:Au, where the two enzymes are adsorbed simultaneously, the \( K_M \) was within error of the value for free MDH, suggesting that there is less change in the structure of the active site of MDH in this bioconjugate; this could be due to interactions between the two enzymes during the adsorption process. \( K_M \) determinations for MDH with oxaloacetate show a similar trend. The binding site affinity for MDH/CS:Au is equivalent to free MDH, suggesting little change in the structure of the active site upon adsorption. Comparison of the \( K_M \) values for CS:MDH:Au and MDH:CS:Au show that the \( K_M \) for oxaloacetate is closer to that of free MDH when it is adsorbed to a pre-existing bioconjugate; this may indicate less enzyme denaturation upon adsorption. For all three bioconjugates the \( K_M \) of oxaloacetate for CS was approximately the same. Furthermore,
the values were lower than that for free CS, indicating a change in active site structure. This suggests that adsorption to Au and MDH:Au affected the structure of CS to the same extent.

Similar to the trends found for specific activity, the turnover number for MDH is higher when adsorbed to existing CS:Au bioconjugates. For MDH $\nu_{m-o}$ is at least 6 times faster than MDH:Au, this increase is due to the interaction between MDH and CS, which is less destructive to MDH activity than the interaction of MDH with Au nanoparticle based on enzyme activity. For $\nu_{o-m}$ the turnover rate is 1.7 times the rate for free enzyme, however due to the large standard deviation in $\nu_{o-m}$ for the MDH:CS:Au, the two values are not statistically different according to a student’s t-test. Rate enhancements have been seen before for bioconjugates and are usually reported as $V_{\text{max}}$ (equal to $k_{\text{cat}} \cdot E_{\text{total}}$). For example, Huang et al. found that lipase immobilized to magnetic nanoparticles had a greater $V_{\text{max}}$ than free lipase while Pandey et al. found that immobilized glucose oxidase was an order of magnitude more active than free glucose oxidase. Both sets of authors attributed this increase to structural changes in the enzyme upon adsorption that allowed for a better orientation of the active site. In the case of MDH:CS:Au the interaction between MDH and CS may have a similar effect on active site orientation.

Similarly the turnover number for CS was greater for MDH:CS:Au than CS:MDH:Au, suggesting that there is a more favorable interaction between CS and Au than MDH:Au. As seen in Table 2-1, there is less MDH present than necessary to form a monolayer, increasing the likelihood of substrate diffusing to the CS active site. This corresponds with $K_M$ data (Table 2-2), where the substrate specificity was greater for CS adsorbed to Au than CS adsorbed to MDH:Au. Again, this could be due to a specific interaction between MDH and CS causing an unfavorable orientation of CS when it adsorbs.

For MDH/CS:Au the MDH $k_{\text{cat}}$ of each reaction was approximately 2x greater than for MDH:Au, suggesting that co-immobilization led to less denaturation than when adsorbed directly to Au nanoparticles, but the turnover number for each reaction was lower than MDH:CS:Au. The
CS turnover rate was lower for MDH/CS:Au than CS:Au. Taken together these data imply that the active sites for some of the MDH and CS molecules were blocked by other enzymes in this mixed arrangement.

This data suggests that both enzymes interact with the Au surface in similar ways to the first enzyme adsorbed in the sequential adsorption bioconjugates. \( K_M \) data for this bioconjugate indicate that MDH is in a near native conformation, while specific activity and turnover number suggest that catalysis may be limited by the surrounding adsorbed enzyme.

**Activity per Particle**

An important measure for characterizing enzyme bioconjugates is activity per particle, which is often easier to determine than specific activity for the adsorbed enzymes and is relevant for biotechnological applications. High enzymatic activities on a per particle basis can result from a large number of moderately-active molecules on each particle or a smaller number of highly-active molecules. In previous reports the per particle activity has been used to show the increased stability of adsorbed enzymes over time and to increased pH\(^{26,27}\) here we report activity per particle (Table 2-3) to compare the catalytic ability of the different bioconjugates produced. We found similar trends in the activity per Au as for specific activity (Table 2-1). Interestingly, the reverse MDH reaction was consistent and independent of the type of bioconjugate, indicating that the large number of enzymes adsorbed was able to offset the effect of enzyme denaturation. This underscores the idea that activity measurements without knowledge of the amount of enzyme present are incomplete and cannot speak to the amount of enzyme denaturation.
**Sequential Activity**

Because the product of MDH (oxaloacetate) is a substrate for CS, it is also possible to evaluate the overall activity of the dual enzyme bioconjugates for conversion of malate to citrate. Sequential activity of MDH and CS in multi-activity bioconjugates was measured as activity per particle (Table 2-3). The highest sequential activity of MDH and CS was found for MDH:CS:Au. Examination of the individual activity per Au rates indicates that the individual reaction rates are limiting factors for CS:MDH:Au (CS rate) and MDH:CS:Au (MDH rate) but not for MDH/CS:Au. For MDH:CS:Au the sequential rate of reaction is very close to the rate of oxaloacetate production by MDH, suggesting that the intermediate is efficiently transferred from MDH to CS. For CS:MDH:Au the rate of oxaloacetate production by MDH is faster than the rate of consumption by CS this allows oxaloacetate time to build up or diffuse away from the bioconjugate leading to a less efficient reaction. The individual MDH and CS rates per particle are 10x faster than the rate of the sequential reaction which may be due to inefficient diffusion of oxaloacetate from the MDH active site to the CS active site. These data suggest that the organization and ratio of activity of sequential enzymes is important for the sequential rate of reaction.

Previous reports have found that the rate of sequential activity for a multiple enzyme system is improved by colocalization. For example, Watanabe and Ishihara found that a higher local concentration of each enzyme lead to higher sequential activity rates when a three enzyme system was immobilized to polymer nanoparticles, due to effective diffusion of intermediates. Similar results were reported by Pescador et al. for L₃L assemblies of GOx and HRP. Pescador et al. also found that an optimized configuration of GOx and HRP in L₃L assemblies lead to a 2.5 x increase in the sequential reaction rate. Here, the individual MDH (forward reaction) and CS rates are nearly an order of magnitude faster than the sequential reaction rate, suggesting that
reaction is limited instead by an inability for the intermediate, oxaloacetate, to diffuse from one active site to the other efficiently.

Conclusions

The work presented here demonstrates how activity measurements combined with detailed knowledge of bioconjugate stoichiometry can be used to further the characterization of enzyme adsorbed to nanoparticulate surfaces. In particular it can be noted that (1) MDH adsorbed to CS:Au bioconjugates has superior kinetic parameters and ability to catalyze substrate as compared to CS adsorbed to MDH:Au and more closely approaches the characteristics of free enzyme. Additionally, CS orientation when adsorbed to MDH:Au appears to be dictated by a specific protein-protein interaction. While every set of enzymes will behave differently, this suggests that near native activity and/or specific orientation on particle can be achieved with a direct adsorption strategy even for proteins not known for strong binding in solution. (2) Kinetic characterization can be used to help understand how multiple types of enzymes are adsorbed single nanoparticle, and the effect on each enzymes structure and activity. Furthermore, common measurements of activity without knowledge of stoichiometry may be misleading when comparing methods for bioconjugate formation since the number of enzymes present can obscure differences in activity. (3) The sequential activity of two types of enzyme adsorbed to the same nanoparticle is dependent not only on the individual reaction rates, but also on the ability of intermediates to move between active sites. Although the bioconjugates described here differ substantially from the enzyme complexes found in biological cells, multienzyme complexes are known to form intracellularly. Our findings underscore the importance of control over enzyme position in biological or artificial multienzyme complexes to enable effective transfer of intermediates between active sites.
References


31. Priyam, A.; Chatterjee, A.; Bhattacharya, S. C.; Saha, A. Conformation and activity dependent interactions of glucose oxidase with CdTe quantum dots: towards


34. MDH (light spheres) and CS (dark spheres) were adsorbed either sequentially or simultaneously to Au nanoparticles. The amount of each enzyme measured for each bioconjugate is depicted as accurately as possible by estimating the number of layers of each enzyme present from their sizes. The diameter of each enzyme was determined by averaging measurements made in the x, y, and z dimensions of each enzymes crystal structure. This diameter was then used to calculate the area a single enzyme molecule would occupy on the surface area of the 30.7 nm diameter Au nanoparticle. The number of MDH or CS molecules necessary to cover the entire surface area of the nanoparticle was considered “monolayer coverage”. To determine the number of MDH or CS necessary to form a complete layer on an existing MDH:Au or CS:Au bioconjugate 2x the average diameter of the previously immobilized enzyme was added to the diameter of the nanoparticle and the surface area was recalculated.


53. Considering the average diameter of the enzymes measured from crystal structures, 6.5 nm diameter for MDH, 7.2 nm diameter for CS, and the 30.7 nm diameter of Au each MDH molecule would occupy at least 33 nm$^2$, and each CS molecule would occupy at least 40 nm$^2$ on the 2960 nm$^2$ surface area of Au.


59. While the specificity constant or catalytic efficiency \( k_{cat}/K_M \) can be calculated from the data presented in this section we have chosen to present the two values separately. Considering the effects of adsorption on enzyme structure and activity the differences between free and bound enzymes may limit the usefulness of the specificity constant for the bioconjugates presented here.\(^{60,61}\)


Figure 2-1. Multienzyme bioconjugates prepared by sequential and simultaneous addition of enzymes. Multienzyme bioconjugates were prepared by sequential or simultaneous addition of MDH (light) and CS (dark). The sequential addition of enzymes creates bioconjugates with two layers of enzyme on adsorbed on top of the other. Simultaneous addition of enzymes may form bioconjugates with distinctive or mixed layers of enzymes. \(^{35}\)
Figure 2-2. The enzymatic reactions carried out by MDH and CS individually and in sequentially. MDH performs a reversible reaction to convert malate to oxaloacetate or oxaloacetate to malate with NAD(H) as a cofactor, while CS utilizes oxaloacetate to produce citrate
Figure 2-3. Comparison of two methods for quantifying the number of enzyme molecules bound to each AuNP. The “direct” method quantified fluorescently-labeled proteins after removing unbound enzyme in the supernatant and dissolving the AuNP to release the bound enzyme. The “indirect” method quantified bound protein by measuring the unbound enzyme in solution and subtracting from the total enzyme added.
Figure 2-4. Single activity bioconjugate characterization. (A) The number of enzymes adsorbed per Au was determined as a function of the Enzyme:Au ratio present in solution during conjugation for MDH (filled circles, solid line) and CS (open circles, dotted line). (B) Once the number of adsorbed enzymes was known the specific activity for MDH (filled circles, solid line) and CS (open circles, dotted line) could be calculated.
Figure 2-5. Flocculation assays of MDH:Au and CS:Au. Absorbance spectra of conjugates created with various ratio of E:Au can be compared to the spectra of bare AuNP (black trend) to determine the point at which no spectral shift can be detected due to salt induced flocculation. 25:1 red trend, 50:1 green trend, 100:1 blue trend, 300:1 purple trend, 500:1 grey trend.
Table 2-1: Specific Activity of Unbound and Bound Enzymes prepared at 300:1 solution enzyme:AuNP ratios.

<table>
<thead>
<tr>
<th>Sample</th>
<th>enzyme molecules per particle</th>
<th>MDH (v&lt;sub&gt;o-m&lt;/sub&gt;)</th>
<th>MDH (v&lt;sub&gt;o-m&lt;/sub&gt;)</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>U/mg</td>
<td>%</td>
<td>U/mg</td>
</tr>
<tr>
<td>MDH</td>
<td>n.a.</td>
<td>182 ± 3</td>
<td>100</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td>MDH:Au</td>
<td>180 ± 25</td>
<td>n.a.</td>
<td>7 ± 3</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>CS</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CS:Au</td>
<td>n.a.</td>
<td>79 ± 15</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CS:MDH:Au</td>
<td>102 ± 19</td>
<td>186 ± 30</td>
<td>10 ± 2</td>
<td>5.5 ±0.3</td>
</tr>
<tr>
<td>MDH:CS:Au</td>
<td>30 ± 14</td>
<td>80 ± 4</td>
<td>56 ± 15</td>
<td>31 ± 9</td>
</tr>
<tr>
<td>MDH/CS:Au</td>
<td>64 ± 5</td>
<td>34 ± 11</td>
<td>30 ± 6</td>
<td>16 ± 4</td>
</tr>
</tbody>
</table>

*Not applicable. Values for CS activity were not measured for MDH or MDH:Au, and values of MDH activity were not measured for CS or CS:Au.*
<table>
<thead>
<tr>
<th>Sample</th>
<th>MDH (ν₀-m)</th>
<th>MDH (νₘ-₀)</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>k&lt;sub&gt;cat&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>(s⁻¹)</td>
<td>(mM OAA)</td>
<td>(s⁻¹)</td>
</tr>
<tr>
<td>MDH</td>
<td>377 ± 46</td>
<td>0.020 ± 0.005</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>MDH:Au</td>
<td>19 ± 5</td>
<td>0.04 ± 0.02</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>CS</td>
<td>n.a.⁺⁺⁺⁺⁺⁺</td>
<td>n.a.⁺⁺⁺⁺⁺⁺</td>
<td>n.a.⁺⁺⁺⁺⁺⁺</td>
</tr>
<tr>
<td>CS:Au</td>
<td>n.a.⁺⁺⁺⁺⁺⁺</td>
<td>n.a.⁺⁺⁺⁺⁺⁺</td>
<td>n.a.⁺⁺⁺⁺⁺⁺</td>
</tr>
<tr>
<td>CS:MDH:Au</td>
<td>119 ± 82</td>
<td>0.11 ± 0.09</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>MDH:CS:Au</td>
<td>642 ± 334</td>
<td>0.05 ± 0.02</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>MDH/CS:Au</td>
<td>37 ± 8</td>
<td>0.019 ± 0.009</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

*⁺⁺⁺⁺⁺: Not applicable
### Table 2-3: Activity per particle

<table>
<thead>
<tr>
<th></th>
<th>MDH Reverse (x $10^{-14}$ U/Au)</th>
<th>MDH Forward (x $10^{-14}$ U/Au)</th>
<th>CS (x $10^{-14}$ U/Au)</th>
<th>Sequential (x $10^{-14}$ U/Au)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDH:Au</td>
<td>16 ± 6</td>
<td>1.1 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS:Au</td>
<td></td>
<td></td>
<td>3.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>CS:MDH:Au</td>
<td>21 ± 4</td>
<td>1.4 ± 0.1</td>
<td>0.9 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>MDH:CS:Au</td>
<td>20 ± 5</td>
<td>1.9 ± 0.1</td>
<td>3.1 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>MDH/CS:Au</td>
<td>22 ± 4</td>
<td>1.8 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>0.18 ± 0.03</td>
</tr>
</tbody>
</table>
Chapter 3

Kinetic Consequences of Restricted Diffusion and Proximity on Sequential Enzyme-Au Bioconjugates

(The computational model present in this chapter was developed by Francisco G. Vital-Lopez, a graduate student in Chemical Engineering, using data collected in this chapter)

Several metabolic pathways such as glycolysis, pyruvate decarboxylation, the TCA cycle, and purine and pyrimidine biosynthesis have been demonstrated to exist as associations of metabolic enzymes within the cell.\textsuperscript{1-5} Organization of enzymes has been proposed to have several advantages for overall metabolism such as sequestering intermediates from competing pathways, preventing unfavorable reaction equilibria, and decreasing transit time for intermediates, and regulating pathway activity through product inhibition or dynamic proximity.\textsuperscript{6}

Several key experiments have shown some of the kinetic consequences of colocalizing sequential enzymes. Srere et al. in 1973 showed how immobilization of sequential enzymes into a polyacrylamide gel served to colocalize enzymes and pool intermediates, leading to a four-fold increase in the sequential rate of reaction over enzymes free in solution.\textsuperscript{7} Several others have used fusion proteins to colocalize sequential enzymes in solution.\textsuperscript{8-10} In each of the reports noted above, proximity was only found to have an effect on the sequential reaction rate if an electrostatic channel between the active sites of the enzymes existed,\textsuperscript{8} or an agent such as PEG, was added to reduce the rate of diffusion of intermediates in solution, and mimic a cytoplasmic environment.\textsuperscript{10} Within the cell, it is hypothesized that restricted diffusion as well as short diffusion differences promotes the efficiency of reactions between sequential enzymes.
While the results of these experiments have demonstrated the importance of proximity in sequential kinetics, the colocalized and non colocalized systems presented in each had significant differences in the structure and environment of the enzymes in each system. Enzymes were either chemically coupled to a gel support, leading to loss of activity through binding and immobilization, or expressed as fusion proteins, which commonly leads to misfolding of protein structures. In contrast, the work presented here compares colocalized enzymes, which still suffer from denaturation, to a comparable control where the non-colocalized enzymes should be similarly denatured, to better account for the differences in activity between the enzyme systems.

More comparable systems were used by Koch-Schmidt et al in 1977, fusion proteins of MDH and CS, or the native enzymes were coupled to Sepharose beads. Here, researchers found an increase in reaction rate for enzymes immobilized to Sepharose beads, but not for colocalized enzymes versus non-colocalized enzymes. From these results it was concluded that proximity alone did could not enhance the rate of reaction in dilute solution, the presence of Sepharose beads which provide an unstirred layer around the beads in which intermediates can accumulate and be effectively transferred between enzymes.11

In this chapter the effects of proximity between sequential enzymes and diffusion of intermediates is studied using bioconjugates of AuNP and MDH and CS. This specific enzyme pair was used in several of the experiments discussed above7-9,11-20 and are involved in the enzyme complex that carries out the TCA cycle in mitochondria.2

Directly adsorbing each enzyme either to the same AuNP to form a dual activity bioconjugate MDH:CS:Au, or alone to form MDH:Au and CS:Au provides a colocalized and non-colocalized systems in which the enzymes were initially in the same conformation. As noted by Koch-Schmidt et al. unstirred layers of solution associated with the scaffold have an effect on the rate of reaction. By immobilizing all enzymes to AuNP scaffolds this effect is accounted for. While Chapter 2 discusses how the activity of these bioconjugates varies after adsorption, the
detailed characterization performed allows for a more accurate comparison of the activity in each system.

In this chapter the sequential activity of MDH and CS was determined as a function of the diffusion coefficient of oxaloacetate, the intermediate of the sequential reaction, and distance between sequential reactions. In addition to this, a diffusion based computational model of the sequential reaction was constructed which allows for further analysis and evaluation of the possible roles of diffusion and proximity for sequential enzyme reactions.

The enzyme: AuNP bioconjugates characterized in chapter 2 were used to examine the effects of macromolecular crowding, restricted diffusion and proximity between sequential enzymes. MDH and CS in particular are a sequential pair of enzymes found in the mitochondria as part of the Krebs cycle with have been extensively studied to examine metabolite pooling, channeling and diffusion.7, 8, 12-20

**Experimental**

**Materials**

Colloidal gold particles (mean diameter 30.7 nm, (2 x 10\(^{11}\) particles/ml) were purchased from Ted Pella, Inc. (Redding, CA). AlexaFluor 488 and AlexaFluor 633 protein labeling kits were purchased from Invitrogen (Carlsbad, CA). Citrate synthase (CS) from porcine heart (EC 2.3.3.1) ammonium sulfate suspension, malic dehydrogenase (MDH) from porcine heart (EC 1.1.1.37) ammonium sulfate suspension solution, L-(-)-malic acid, oxalacetic acid, acetyl coenzyme A trilithium salt, β-nicotinamide adenine dinucleotide hydrate (NAD\(^+\)), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), Trisma® hydrochloride (Tris[hydroxymethyl]aminomethane hydrochloride), Trisma® base
(Tris[hydroxymethyl]aminomethane), sodium bicarbonate, Ellman’s Reagent (5,5’-dithiobis[2-nitro-benzoic acid]) (DTNB) and poly(ethylene) glycol (PEG) MW 4.6kDa were purchased from Sigma-Aldrich (St Louis, MO). Pall Life Sciences Nanosep 10k MWCO centrifugal devices, Whatman 20 nm pore diameter syringe filters, and glycerol were purchased from VWR. Deionized water with a resistivity of $\geq 18.2 \, \text{M} \Omega$ from a Barnstead NANOpure Diamond water purification system (Van Nuys, CA) was used in all experiments.

**Enzyme Labeling**

A 10 kDa MWCO centrifuge filter was used to remove ammonium sulfate, which can reduce labeling efficiency by out competing primary amines in the protein structure for the coupling reaction. CS and MDH were resuspended in 5 mM sodium bicarbonate (pH 8.3) at a 2 mg/ml concentration prior to fluorescent labeling. CS and MDH were labeled with AlexaFluor 633 and AlexaFluor 488 respectively, according to the protocol provided by Invitrogen.

**Enzyme Conjugation and Bioconjugate Purification**

Single enzyme bioconjugates were prepared by first concentrating $6.0 \times 10^{11}$ Au nanoparticles (9.96 $\times 10^{13}$ mol) to a volume of 1 ml and washing with 5 mM sodium bicarbonate (pH 8.3) in 20 nm filtered water to remove any possible contaminants. CS or MDH was then added from a stock solution in the same buffer to create the desired enzyme:Au ratio, and buffer was added to a final volume of 1.5 ml. Enzymes were allowed to adsorb to the surface of the nanoparticle for 1 hour at 4°C protected from light. Excess enzyme was subsequently removed by centrifugation of the conjugates at 5000 x g for 15 min at 4°C, the supernatant was removed and bioconjugates were resuspended in 1.5 ml of fresh buffer. This process was repeated 3 times to
ensure all excess enzyme was removed from the bioconjugate solution. Multienzyme bioconjugates were prepared by adsorbing CS and MDH to the surface of the same nanoparticle either sequentially or simultaneously (Figure 2-1). Bioconjugates were formed in sequential steps by adsorbing CS or MDH onto the surface of a preexisting bioconjugate containing the other enzyme, or by simultaneously adding both enzymes to bare Au nanoparticles. All incubation and washing procedures follow those laid out for single enzyme bioconjugates. The bioconjugates studied here were named systematically based on the order in which enzymes were adsorbed to the AuNP surface. The notation lists the enzymes adsorbed from last to first, followed by the core AuNP separated by colons. For example, MDH:Au, CS:Au, and MDH/CS:Au were created using a single adsorption step while MDH:CS:Au was made by adsorbing MDH to a preexisting CS:Au conjugate.

**Viscosity Measurements**

Viscosity measurements were made using Ostwald Viscometer. Each concentration of PEG used was measured three times independently and the viscosity was calculated from the average time required for solution to flow through a capillary in the viscometer. The diffusion coefficient for oxaloacetate was then calculated using the Stokes-Einstein relation for diffusion of a hard sphere.

**Activity Assays**

MDH performs a reversible reaction to convert malate to oxaloacetate or oxaloacetate to malate. To measure the activity of MDH in single and multienzyme bioconjugates of 100 mM Tris (pH 8.1), 25 µl of 36 mM malate and 25 µl of 60 mM NAD⁺, for a final concentration of 0.3
mM malate and 0.1 mM NAD$^+$ were mixed in a cuvette and equilibrated to room temperature. A volume of 200 µl of bioconjugate was then added to the solution, which was mixed again to ensure homogeneity and the absorbance of NADH was monitored at 340 nm for 5 min (**Figure 2**-2). For each assay the volume of conjugate added (200 µl) remained constant and the concentration of bioconjugate was measured by the absorbance of the gold plasmon at 524 nm. Each assay was repeated at least 3 times and had a total volume of 3 ml. The activity of CS ($v_{o-c}$) in single and multienzyme bioconjugates was assayed in similar fashion to MDH with the exception that 25 µl of 12 mM Acetyl-coenzyme A and 25 µl of 60 mM oxalacetate were used as substrates, 25 µl of 18 mM DTNB, for a final concentration of 0.5 mM oxalacetate, 0.1 mM Acetyl-CoA, and 0.15 mM DTNB, was added to monitor the production of coenzyme A$^7$ which adsorbs at 412 nm $\varepsilon = 13,600$ M$^{-1}$cm$^{-1}$.23,24

**Mathematical Modeling**

A reaction-diffusion model was developed to analyze the kinetics of the MDH-CS sequential reaction. The model was described by a set of partial differential equations (PDEs) that account for the effect of diffusive phenomena on the observed sequential activities for both double and single enzyme bioconjugates. The kinetic expressions were developed based on Michaelis-Menten like kinetics modified to account for the inhibitory effect of oxaloacetate on MDH and malate on CS, based on experimental observations. The diffusion coefficients for malate, oxaloacetate and citrate were estimated from viscosity measurements. Parameter estimation was performed in MATLAB (the MathWorks, Inc., Natick, MA.) using COSMOL (COSMOL AB. 2007: Burlington, MA.) for solving the PDEs.
Results and Discussion

To study the effects colocalization between sequential enzymes on the rate of sequential reaction proximity was measured using two methods. First, the activity of colocalized and non-colocalized enzymes was measured. PEG and glycerol were added to these reactions to reduce the rate of diffusion of intermediates in each system. Second, the activity non-colocalized enzymes was measured as a function of the distance between the sequential enzymes. For each of these experiments a diffusion based computational model of activity was developed to further examine the effect of diffusion and proximity on a sequential reaction.

Sequential Activity of MDH and CS with Limited Diffusion

The diffusion of intermediates between sequential enzymes is key to the overall rate of reaction for a pathway. Within the cell the diffusion coefficient of small molecules such as oxaloacetete is about 25% of what it is found in dilute solution. Due to this, measurements made in dilute solution may not truly reflect what happens in the cell. Koch-Schmidt et al. stated that the sequential rate of reaction for fusion proteins of MDH and CS, and the rates of the native enzymes were identical to each other in dilute solution, Datta et al. found that hindrances to diffusion, such as the addition of PEG improved the rate of the same sequential reaction. To this end, rate of sequential reactions for colocalized and non-colocalized enzymes in solutions where the diffusion coefficient of the intermediate is controlled are of interest.

Diffusion Limited by PEG

To directly measure the effect of PEG as an agent to reduce the rate of diffusion of intermediates on colocalized and non-colocalized enzymes, MDH and CS AuNP bioconjugates
described in chapter 2 were used. The double enzyme bioconjugate MDH:CS:Au functioned as a colocalized system and single enzyme bioconjugates, MDH:Au and CS:Au, as a non-colocalized control. In this way both the proximal and non-proximal sequential enzyme systems are in more closely matched environments than previous experiments. The rate of sequential activity of MDH:CS:Au double enzyme bioconjugates was measured in increasing concentrations of PEG and compared to controls containing MDH:Au and CS:Au which were matched by enzymatic units (µmol/min) to the MDH and CS rates of activity of double enzyme bioconjugates in dilute solution. This was done in order to prevent enzyme concentration from altering the rate of sequential activity.

**Figure 3-1** shows the sequential activity of colocalized and non-colocalized MDH and CS in increasing amounts of PEG. For double bioconjugates, an estimate of the distance the intermediate must travel is equal to half the circumference of the bioconjugate, or 0.078 µm, while the distance traveled in single conjugates is 23.5 µm. The distance between bioconjugates was calculated from the concentration of bioconjugates in solution. The (w/w) % PEG, the common method for reporting PEG concentration is shown in **Figure 3-1A**, while the resulting diffusion coefficient of the PEG solution is shown in **Figure 3-1B**. **Table 3-1** displays the relationship between PEG concentration, solution viscosity, and diffusion coefficient. The data shows that with increasing concentration of PEG (lower diffusion coefficient) the activity of the non-colocalized system becomes very close to the rate of the colocalized system. The sequential rates of colocalized and non-colocalized enzyme differ at low concentrations of PEG [under 15 (w/w)%] due to the difference in diffusion distance between sequential enzymes. At higher concentrations of PEG these rates become indistinguishable from each other, since these results are counterintuitive the rate of individual reactions were studied.

The individual enzyme activity of MDH and CS in double enzyme bioconjugates and single enzyme bioconjugates is presented in **Figure 3-2A** (MDH) and C (CS). The rate of MDH
catalysis in MDH:Au and MDH:CS:Au varies significantly with increasing concentration of PEG (decreasing diffusion coefficient). For MDH:CS:Au the rate of reaction decreases at low diffusion rates, while in MDH:Au the rate increases. This may have a significant impact on the rate of sequential catalysis. Recently, it has come to light that PEG may have a greater impact on the structure and activity of enzymes than other agents used to alter the viscosity and diffusion rate in solution. As seen in the previous chapter, MDH:Au and MDH:CS:Au contain different amounts of MDH per AuNP and the specific activity and kinetic constants of MDH:Au show that it is more denatured than MDH:CS:Au, therefore PEG may have a bigger effect on the activity of MDH:Au.

For CS activity it is evident that while PEG has little effect on the CS:Au rate, while for MDH:CS:Au the rate of catalysis drops off with decreasing rate of diffusion (increasing concentration of PEG). This difference could be due to the presence of MDH in the double conjugate and the effect PEG has on its structure.

**Diffusion Limited by Glycerol**

In light of this, another viscosity agent was tested. Glycerol, in contrast to PEG is a small molecule which is less likely to cause volume exclusion effects the way PEG does. The activity of individual reactions of double and single bioconjugates is presented in Figure 3-2B (MDH) and D (CS). For MDH, the effect of glycerol is the same on both bioconjugates, although the magnitude of the effect is greater for double conjugates. This is likely due to the different ratios of MDH to AuNP present in each bioconjugate. For both single and double bioconjugates CS activity increases as diffusion coefficient decreases (increasing glycerol concentration), this effect is relatively consistent, and can be predicted based on previous literature, Srere et al. found that glycerol is a renaturing agent for CS. Since it was determined in the previous chapter that
conjugation to AuNP results in activity loss and denaturation of CS, an increase in activity with increasing concentrations of glycerol is reasonable.

The effect of restricted diffusion on the rate of production for MDH:Au and CS:Au can be seen in Figure 3-3. MDH performs a reversible reaction to convert malate and NAD$^+$ to oxaloacetate and NADH (Figure 2-2).$^{21}$ When malate and NAD$^+$ are added to MDH:Au the production of NADH is monitored for the first several seconds of the reaction. When enough oxaloacetate and NADH have been formed the reaction is reversed to decrease the amount of product in the vicinity of the active site. At low concentrations of glycerol the reaction then moves forward at a slower rate, while at higher concentrations a longer period of time is necessary for diffusion of NADH and oxaloacetate away from the active site. Because of this, it takes progressively longer to return the forward reaction. Since the reaction is now proceeding at a slower rate, products of the reaction diffuse away from the bioconjugate quickly enough to prevent inhibition from occurring again.

Alternatively, CS performs an irreversible reaction to catalyze oxaloacetate to citrate. As seen in Figure 3-3B the initial rate of catalysis increases with decreasing diffusion coefficient until $1.2 \times 10^{-10}$ m$^2$/s is reached. At this point the rate enhancement from glycerol is outweighed by the limited diffusion of substrate and reaction rate decreases. These rates appear to be the steady state reaction rates that occur after an initial faster rate of reaction, at lower diffusion coefficients this initial rate is harder to monitor due to time required to prepare, start, begin monitoring the reactions experimentally.

Figure 3-4 shows the rate of sequential reaction of double bioconjugates and single bioconjugates in glycerol. As the concentration of glycerol increases the rate of activity for both colocalized and non-colocalized enzymes increases, but the differences between rates is statistically different. The difference between the colocalized and non-colocalized rates are due to
the differences in diffusion of oxaloacetate between the active sites of MDH and CS. The relatively large error associated with data collected at higher concentrations of glycerol are due to the difficulty in mixing very viscous solutions (up to 70 % glycerol).

**Computational Analysis of Sequential Activity in Viscous Solution**

The characterization data presented in chapter 2 for each type of bioconjugate as well as the sequential reaction rates in glycerol were used to construct a computational model to predict the sequential activity of two bioconjugates with a range of different parameters determined by Latin Hypercube Sampling. This statistical method generates a distribution of plausible parameter values from a multidimensional distribution of values.39,30 This sampling method allowed for a wide variety of parameters to be tested so that any parameter differences between the two sets of enzymes (colocalized and non-colocalized) can be seen in the mathematical model if they cannot be seen in the experimental system. **Figure 3-5** shows the activity of MDH (upper panels) and CS, or sequential activity (lower panels) in low viscosity solution (diffusion coefficient = 2.5 x 10^-9 m^2/s), left panels, and high viscosity solution (1.2 x 10^-10 m^2/s), right panels. Each point in the graph represents a different set of parameters that converge to produce the activity shown for double bioconjugates (green) and single bioconjugates (blue).

From the MDH activity analysis it is evident that the rate of oxaloacetate production is consistent both between MDH:Au and MDH:CS:Au, and solution viscosity. This result is expected because of the abundance of malate and NAD^+, the substrates for MDH, distributed uniformly in solution before the reaction begins. Since substrate is readily available any limitations on diffusion would have little effect on activity.

In contrast the activity of CS in the sequential reaction is limited both by the rate of formation (MDH activity) and diffusion of the intermediate to the CS catalytic center. **Figure 3-
5C shows the CS activity in solution without the presence of viscous agents such as PEG or glycerol, the activity of double bioconjugates and single bioconjugates is clearly differs. In comparison the CS activity for each bioconjugate changes in viscous solution (Figure 3-5D). For double bioconjugates the activity increases slightly (cannot be visualized with the axes range on this plot), while for single bioconjugates the activity decreases significantly. Since the model is designed to model the diffusion of oxaloacetate between MDH and CS the distance between active sites has a large effect on the rate found. For double bioconjugates, where the diffusion distance is very small (0.078 µm) the change in activity with diffusion coefficient is very small, but for single bioconjugates where the distance between bioconjugates is significantly larger (23.5 µm) the change in activity is much greater.

In all, this model confirms the results found for sequential activity of colocalized and non-colocalized enzymes in dilute solution and high concentrations of glycerol. The difference in activity for CS between the two systems can be linked through the model to the limited diffusion of oxaloacetate between the sequential enzymes.

**Effect of Proximity on a Sequential Two Enzyme Reaction**

In the previous section the proximity of enzymes was controlled by using two different types of bioconjugates. The activity of MDH and CS in viscous solution have proven to be different for each bioconjugate. To account for this difference, the sequential reactions occurring in MDH:CS:Au and between MDH:Au and CS:Au were examined independently. First, the rate of MDH and sequential activity was monitored independently to determine the rates of oxaloacetate production and utilization within the system. Second, the concentrations of the bioconjugates MDH:Au and CS:Au was varied in the sequential activity assay (the ratio of
MDH:Au to CS:Au remained constant), to modulate the distance between sequential enzymes, was measured experimentally and modeled using a similar model to the one above.

**Diffusion of Intermediates Between Catalytic Sites**

To determine how much oxaloacetate is present in an activity assay at a given time the production (MDH activity) and utilization (sequential activity) was monitored over the course of a 3 min reaction. Figure 3-6 shows the activity of MDH and CS as a function of time for dual and single activity conjugates in buffer or viscous solution (70.2% glycerol). By comparing the traces between the four plots it is evident that rate of oxaloacetate production, MDH activity (red trend) and the rate of oxaloacetate consumption, CS activity (black trend), match closely for dual activity conjugates in viscous solution (Figure 3-6C) but not for the other cases. When the trend lines fall on top of each other the bulk concentration of oxaloacetate is very low because it is used as quickly as it is made. The differences in the initial concentrations of oxaloacetate can be linked to the effect of glycerol on the individual rates of reaction, and the preparation time of the sample which changes with increasing concentration of glycerol.

The differences in oxaloacetate concentration in each case demonstrates that a sequential reaction is more efficient when the enzymes are in close proximity (colocalized to the same AuNP) in viscous solution, when the intermediate, oxaloacetate, pools at the surface of the conjugate. Further evidence of this observation can be seen from the average concentration of oxaloacetate in solution as a function of time. From the data presented in Figure 3-6, the rate of oxaloacetate production and utilization can be calculated in activity units (µmol/min). If the rate of consumption of oxaloacetate is subtracted from the rate of production the rate of oxaloacetate build up in bulk solution can be calculated. Figure 3-7 shows how oxaloacetate concentration builds up in the bulk solution when the molecule can freely diffuse (grey and green trends) and
when the molecule must diffuse over several microns to complete the sequential reaction (green and blue trends).

These results agree with a model of the oxaloacetate concentration in solution during the course of a sequential activity assay. Figure 3-8A shows a periodic box model where the enzymatic activity of nine bioconjugates, either all double enzyme bioconjugates or five MDH:Au and four CS:Au was calculated. The concentration of oxaloacetate in solution was determined in viscous solution (diffusion coefficient $1.2 \times 10^{-10} \text{ m}^2/\text{s}$) for double enzyme bioconjugates (B) and single enzyme bioconjugates (C). The results show that the concentration of intermediate remains consistent throughout the solution at approximately 4 µM for double bioconjugates. Within the mitochondria the concentration of oxaloacetate is approximately 5 µM. For single bioconjugates the intermediate concentration is high in the vicinity of MDH:Au and is depleted in the vicinity of CS:Au, while the bulk concentration of intermediate is higher than found in bulk solution.

**Sequential Activity with Increasing Diffusion Distance**

To determine the effect of diffusion distance on the rate of a sequential reaction the distance between sequential enzymes, MDH:Au and CS:Au, was controlled by changing the concentration of bioconjugates. As a control the effect of concentration of MDH:Au or CS:Au on the individual enzyme reactions was measured. As seen in Figure 3-9A, the rate of product formation in each solution decreases with decreasing bioconjugate concentration as would be expected when less catalytic centers are present in the assay. Figure 3-9B shows how the activity per bioconjugate changes with bioconjugate concentration. For each enzyme the rate of activity per bioconjugate is not statistically different for decreasing concentrations of bioconjugate (increased distance between bioconjugates) until very low concentrations are reached.
The effect of distance between MDH:Au and CS:Au on sequential activity can be seen in Figure 3-10. In these experiments the concentration of each bioconjugate was matched by enzymatic unit to what was found for sequential reactions of MDH:CS:Au dual activity bioconjugates in PEG (circles) or glycerol (squares), for comparison these values are included in the figure as filled symbols where the distance between MDH and CS is estimated to be half the circumference of the bioconjugate. For each reaction substrates for MDH (malate and NAD$^+$) were added to the reaction container and equilibrium between reactants and products for this reversible reaction was allowed form to prevent inhibition and the rate changes seen in Figure 3-3. Here, the reaction equilibria drive the production of a large pool of malate and a much smaller pool of oxaloacetate, which would not build up significantly in the reaction volume and would be localized in the vicinity of MDH:Au by the decreased rate of diffusion caused by the presence of PEG or glycerol in solution. The sequential reaction was then monitored by the addition of acetyl-CoA to the assay and the sequential activity (3-10A) and sequential activity per bioconjugate (3-10B) was measured. Similarly to Figure 3-9, the rate of sequential reaction decreases with increasing distance between bioconjugates while the activity per bioconjugate increases with increasing distance. This is likely due to the same effects seen in the single activity reactions. When compared to the reaction rate for dual activity bioconjugates in which the distance between sequential enzymes is 0.078 µm the rate of sequential activity is higher when the distance between MDH and CS is reduced significantly in both PEG and glycerol. Additionally, the rate of sequential activity per bioconjugate is lower for the dual activity conjugates. Since it was demonstrated in Figure 3-7 that the concentration of oxaloacetate is not building up around the bioconjugate it is likely that a buildup of the final product, citrate is regulating the reaction by inhibiting MDH.$^{21}$

Figure 3-11 shows a computational model similar to the one presented in Figure 3-5 in which the concentration of bioconjugates was varied in a sequential reaction measurement. The
open circles represent data collected experimentally in solution without viscous agents present while the blue and green dots represent the results of two models constructed to match the data presented. The degree of agreement between the experimental and computational data demonstrates how the rates of sequential activity found can be accounted for by the diffusion of oxaloacetate and its affect on the rate of activity MDH and CS.

**Conclusions**

Together the computational and experimental results presented in this chapter demonstrate that when diffusion is limited, similarly to what is found in the cell, has several implications for the sequential reaction between two enzymes. First, close proximity between sequential enzymes can results in a more efficient transfer of intermediates between sequential enzymes in a pathway, as evident from the increased rate of reaction and lower bulk concentration of intermediate for colocalized enzymes. Secondly, the rate of diffusion can have a significant effect on the sequestering of reaction intermediate to protect, as demonstrated by the differences in sequential activity of colocalized enzymes when the diffusion coefficient was altered by the addition of glycerol. Lastly, the concentration of intermediate can have a regulating effect on the rate of a pathway, by inhibiting enzyme activity when concentrations reach a certain point, as evident by the reversible inhibition of MDH by oxaloacetate found here.

While these results concur with previous reports for fusion proteins and the proposed benefits for colocalization in the cell, the analysis can be taken further in light of the range of conditions tested and computational models constructed. By studying not only proximity between sequential enzymes but also the rate of diffusion for intermediates between sequential enzymes it can be established that the distance that an intermediate must traverse between active sites alone does not control the rate of the sequential reaction. Pooling of intermediates is also important. As
reported previously by Koch-Schmidt et al., enzymes immobilized to solid particulate supports are surrounded by an unstirred layer of solution. This serves to trap intermediates in the vicinity of the particle and improve activity. When the rate of diffusion of the intermediate is reduced, as it was with glycerol in these experiments, the activity is improved further and the reaction becomes more efficient.

References


Figure 3-1. Sequential reactions of MDH and CS in the presence of PEG. Sequential reactions of MDH and CS from MDH:CS:Au double conjugates (filled circles) and activity matched MDH:Au and CS:Au (open circles), with PEG as a function of PEG concentration (A), or the resulting diffusion coefficient for oxaloacetate (B).
Table 3-1: Diffusion coefficient of small metabolites

<table>
<thead>
<tr>
<th>(w/w)% PEG</th>
<th>c % Glycerol</th>
<th>a Viscosity (Pa*s)</th>
<th>b Diffusion Coefficient (m²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>8.9 x 10⁻⁴ ± 1%</td>
<td>2.5 x 10⁻⁹</td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>1.9 x 10⁻³ ± 0.3%</td>
<td>1.2 x 10⁻⁹</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
<td>2.9 x 10⁻³ ± 0.2%</td>
<td>7.5 x 10⁻¹⁰</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>5.1 x 10⁻³ ± 0.4%</td>
<td>4.3 x 10⁻¹⁰</td>
</tr>
<tr>
<td>20</td>
<td>58</td>
<td>8.0 x 10⁻³ ± 0.1%</td>
<td>2.7 x 10⁻¹⁰</td>
</tr>
<tr>
<td>25</td>
<td>64.5</td>
<td>1.2 x 10⁻² ± 0.8%</td>
<td>1.8 x 10⁻¹⁰</td>
</tr>
<tr>
<td>30</td>
<td>70.2</td>
<td>1.8 x 10⁻² ± 3%</td>
<td>1.2 x 10⁻¹⁰</td>
</tr>
</tbody>
</table>

aMeasured viscosity of PEG solutions, bCalculated using 2 Å diameter for oxaloacetate,

glycerol used was chosen to match the viscosity of PEG samples.
Figure 3-2. Individual enzyme activity of dual enzyme bioconjugates. Individual enzyme activity of double activity bioconjugates, MDH:CS:Au (filled symbols) and single activity bioconjugates, MDH:Au and CS:Au (open symbols). Panel (A) MDH activity in PEG, (B) MDH activity in glycerol, (C) CS activity in PEG, and (D) CS activity in glycerol.
**Figure 3-3.** Product accumulation over time for MDH: Au and CS: Au. 
Product accumulation over time for MDH: Au (A) and CS: Au (B), in viscous solution. Diffusion coefficient for metabolites = $2.5 \times 10^{-9}$ m$^2$/s (black), $7.5 \times 10^{-10}$ m$^2$/s (red), $2.7 \times 10^{-10}$ m$^2$/s (green), and $1.2 \times 10^{-10}$ m$^2$/s (blue).
**Figure 3-4.** Sequential reactions of MDH and CS in the presence of glycerol. Sequential reactions of MDH and CS from MDH:CS:Au double conjugates (filled circles) and activity matched MDH:Au and CS:Au (open circles), with glycerol as a function of glycerol concentration (A), or the resulting diffusion coefficient for oxaloacetate (B).
Figure 3-5. Predicted MDH and sequential activity of single and double bioconjugates. Predicted MDH and sequential CS activity of single and double bioconjugates. MDH activity of double and single bioconjugates in buffer (diffusion coefficient = $2.5 \times 10^{-9}$ m$^2$/s) (A) high viscosity solution ($1.2 \times 10^{-10}$ m$^2$/s). And CS activity of double and single bioconjugates in sequences in buffer (C) and high viscosity solution (D). Blue points represent single bioconjugate activities and green dots represent double bioconjugate activity. Simulations were conducted by Francisco Vital-Lopez, a graduate student in Chemical Engineering.
Figure 3-6. Enzymatic activity of MDH and CS as a function of time. Enzymatic activity of MDH (red) and CS (black) as a function of time for sequential reactions of (A) double bioconjugates in buffer, (B) single bioconjugates in buffer (diffusion coefficient = $2.5 \times 10^{-9}$ m$^2$/s), (C) double bioconjugates in viscous glycerol solution, and (D) single bioconjugates in viscous glycerol solution ($1.2 \times 10^{10}$ m$^2$/s)
Figure 3-7. Concentration of oxaloacetate over the course of a sequential reaction. The concentration of intermediate, oxaloacetate, estimated from the rate of MDH and CS with double bioconjugates in buffer (grey), single bioconjugates in buffer (green) (diffusion coefficient = 2.5 x 10^{-9} m^2/s), single bioconjugates in viscous solution (blue), and double bioconjugates in viscous solution (red) (1.2 x 10^{-10} m^2/s). Concentrations were estimated from the rate of production and consumption of oxaloacetate over the course of a 5 min reaction. Associated errors were calculated from the error in enzyme activity over a minimum of 3 separate trials.
Figure 3-8. Oxaloacetate concentration gradients for single and double bioconjugates. A nine bioconjugate periodic box model constructed as a model of sequential activity of double (9 MDH:CS:Au) and single activity bioconjugates, 5 MDH:Au (red) and 4 CS:Au (green). Oxaloacetate concentration gradient for double bioconjugates (B) and single bioconjugates (C).
Figure 3-9. Individual activity of MDH:Au and CS:Au as a function of bioconjugate concentration. Individual activity of MDH:Au (filled circles) and CS:Au (open circles) as the concentration of bioconjugate decreased to increase the distance between AuNPs. (A) Activity of the solution as a function of distance between bioconjugates, (B) Activity per bioconjugate as a function of distance between bioconjugates in buffer.
Figure 3-10. Sequential activity of MDH:Au and CS:Au with increasing distance between bioconjugates. Sequential activity of MDH:Au and CS:Au with increasing distance between bioconjugates in 30 (w/w)% PEG (circles) or 70.2 % glycerol (squares) both of which have a viscosity of $1.8 \times 10^{-2}$ Pa*s. The activity of MDH:CS:Au dual activity conjugates is included as filled symbols, the distance between enzymes was calculated as half the circumference of the bioconjugate. (A) Activity of the solution as a function of distance between bioconjugates, (B) Activity per bioconjugate as a function of distance between bioconjugates.
Figure 3-11. Computational model of activity of bioconjugates as a function of the distance between bioconjugates. Computational model of activity of bioconjugates as a function of the amount of conjugate present (distance between bioconjugates). Markers with error bars represent experimental data, blue and green dots denote results from two different experimental models.
Chapter 4

Layer-by-Layer Assemblies of Enzymes and Polyelectrolytes on Nanoparticle Scaffolds

All data presented in this chapter was collected by the author in collaboration with Stacey Dean, a graduate student in the Keating group.

Layer-by-Layer (LbL) assemblies of alternately charged polyelectrolytes onto micro and nano-scaled scaffolds have become an attractive approach for fabricating controlled, highly ordered molecular assemblies that can be self assembled into specific designs for use in bioseparations,\(^1^,\(^2\) catalysts,\(^3^,\(^5\) and biosensors.\(^6^,\(^8\) LbL assemblies containing inorganic nanoparticles,\(^9\) dyes,\(^10\) dendrimers,\(^11\) viruses,\(^12\) nucleic acids,\(^13\) and proteins\(^14\) have been previously reported. LbL assembly is a result of electrostatic interactions between polyelectrolytes in solution and an oppositely charged surface. Anionic and cationic polyelectrolytes are then added stepwise to create layers which build upon each other. Salt concentration, type of polyelectrolye (PE) and identity and deposition time can be used to control the thickness of each layer, and charged constituents can be embedded in a PE layer, or as a PE layer to impart specific functionalities to the assembly, such as fluorescence, biomolecule capture, and enzymatic activity to the assembly.\(^15^,\(^22\)

LbL deposition of biomolecules, such as enzymes, have several advantages over other immobilization techniques such as direct adsorption (discussed in chapter 2) and chemical modification of the enzyme.\(^25\) It is a simple method to create multilayer structures with precise control over assembly size, layer composition and thickness. For these reasons LbL assemblies of polyelectrolytes and sequential enzymes can be used as a model to explore the kinetic advantages of proximity and orientation between sequential enzymes in a metabolic pathway. The pyruvate
dehydrogenase complex (PDC) (Figure 1-2) is an example of a static, irreversible assembly of three enzymes that are arranged in a specific orientation in which the second enzyme in the pathway makes up the core of the complex while enzymes 1 and 3 are found on the outermost part of the complex. Immobilizing sequential enzymes in different spatial arrangements within LbL assemblies will create an in vitro model of this type of complex, and allow for characterization and manipulation that is not possible in vivo.

Polymers –enzyme assemblies have been previously characterized to determine the particle diameter\(^1,4\) and mass change\(^17,23\) as layers are added. The rate of activity per particle, but not specific activity, has been determined for enzymes such as β-galactosidase, GOx, and HRP and other enzymes embedded in different PE environments.\(^1,23\) Lvov and Caruso found that an increase in urease activity per particle could be obtained by increasing the number of layers of urease embedded in the assembly, creating a more efficient biocatalyst by increasing the amount of enzyme present.\(^5\) Embedded enzymes are more thermally stable, less susceptible to inhibition and degradation, and allow for greater control over enzymatic activity than enzymes in solution, and assemblies may also be reusable.\(^23,24\)

This chapter focuses on the formation and characterization of several LbL assemblies on submicron latex scaffolds with embedded enzymes of one or more type. The enzyme:scaffold stoichiometry, the ratio of enzyme deposited per latex bead, both when the enzyme is embedded as part of a PE layer with an oppositely charged polyelectrolyte, or when it is treated as a PE layer, are determined for several enzymes. The specific activity of each enzyme is measured both as a function of the deposition method and the depth in which the enzyme is embedded in the assembly.

To study how orientation and depth within a complex affect the sequential rate of activity of two enzymes a three dimensional assembly of two sequential enzymes, MDH and CS, were co-immobilized in four different arrangements (Figure 4-1). In these arrangements the orientation of
the enzymes, and the distance between them (in layers of PE) was probed. The rate of sequential activity was measured to determine if the orientation in the complex, or distance (in layers) between the enzymes have a significant effect on the rate of formation for the final product.

**Experimental**

**Materials**

Poly(sodium 4-styrene sulfonate (PSS), poly(allylamine hydrochloride) (PAH), poly(acrylic acid) (PAA), 2-mercaptoethanesulfonic acid (MESA), lactic acid, β-nicotinamide adenine dinucleotide (β-NAD'), β-nicotinamide adenine dinucleotide hydrate (β-NADH), oxalacetate, acetyl-coenzyme A, Ellman’s reagent, malate, β-(D)-glucose, urea, Sigma FAST o-phenylenediamine dihydrochloride tablet sets, peroxidase Type VI from horseradish, malic dehydrogenase from porcine heart (cytoplasmic), lactic dehydrogenase from bovine heart, citrate synthase from porcine heart, glucose oxidase Type X-S from *Aspergillus niger*, urease, and 0.5 μm and 1 μm carboxylate modified fluorescent polystyrene beads were purchased from Sigma-Aldrich. 0.4 μm carboxylate modified fluorescent polystyrene beads were purchased from Bangs Laboratories. 2,7′-dichlorodihydrofluorescein diacetate (DCF) and AlexaFluor™ protein labeling kits (AlexaFluor™ 488, 555, 633) were purchased from Invitrogen Molecular Probes. Buffers were prepared using sodium phosphate monobasic, sodium phosphate dibasic, from Fluka, HEPES Free Acid (4-(2-hydroxyethyl)-1-piperaznieethanesulfonic acid), from J.T. Baker, Trizma Base (Tris[hydroxymethyl]aminomethane), sodium acetate, acetic acid, hydrochloric acid, from Sigma, and sodium chloride, 99+% from Aldrich. All water used was 18.2 MΩ-cm Nanopure water from a Barnstead system. All reagents were used as purchased without any
modification. Silanes were aliquoted out in a nitrogen glove box to prevent hydrolysis of the chemical.

**Fluorescent Labeling of Enzymes and Polyelectrolytes**

Malic dehydrogenase (MDH) was dialyzed against deionized water to remove any free amines, which would interfere with conjugation of the fluorescent dyes. CS, MDH, GOx, and HRP were labeled with AlexaFluor™ 633, AlexaFluor™ 555 (both MDH and GOx), and AlexaFluor™ 488, respectively, and PAH was labeled with AlexaFluor™ 647 according to the provided protocol. All fluorescent polymer conjugates were then further purified by dialysis against deionized water to remove any free dye remaining in the solution, while protein conjugates were purified by column chromatography according the protocol provided by Invitrogen.

**Polyelectrolyte Coated Beads**

Negatively charged beads were functionalized with PAH/PSS, PAH/PAA, Enzyme/PAH, or Enzyme/PAA multilayers by alternating deposition of positively and negatively charged solutions. Polyelectrolytes were deposited from a 1 mg/ml solution for 30 min with 3 buffer rinses between each step. Enzyme layers were deposited from 1 mg/ml enzyme solutions (of which a portion was fluorescently labeled) for 30 min with 3 buffer rinses between steps. MDH (pI 10) was deposited as the positive layer alternating with PSS, or as a component of the PSS layer from a 1 mg/ml PSS, 1mg/ml MDH solution; and CS (pI ~ 6-6.5) was deposited as the negative layer altering with PAH, or as a component of the PAH layer from a 1 mg/ml PAH, 1 mg/ml CS solution in 50 mM pH 8.1 Tris buffer, 0.5 M NaCl; HRP was either embedded in the
negatively charged PAA layer or deposited as the positive layer altering with PAA in 50 mM pH 6.8 HEPES buffer, 0.1 M NaCl.

**Enzyme Quantification**

Polyelectrolyte coated beads which included PAA as the negatively charged layer were used to determine enzyme stoichiometry by (1) disassociation of PAA by neutralizing the charge on the PAA carboxylic acid by overnight incubation while vortexing in 50 mM pH 5 acetate buffer, 0.75 M NaCl. This was followed by 3 washes with deionized water, enzyme and particle concentrations were determined through fluorescence, or by (2) subtraction of what remained in solution after deposition from the starting concentration of the solution for each enzyme layer deposited.

**Fluorescence Measurements**

Fluorescence measurements of polyelectrolyte coated beads were carried out on a Horiba Jobin Yvon Fluorolog 3-21 using right angle detection for uncoated beads and unembedded enzyme/polyelectrolyte, and front face detection for polyelectrolyte coated beads and embedded polyelectrolyte. All measurements were taken using a 0.1 sec integration time for bead fluorescence and 0.5 sec integration time for enzyme fluorescence, 5 nm excitation and emission slits and using the excitation/emission wavelengths provided with the fluorescent molecule.
Dynamic Light Scattering

DLS was used to obtain the hydrodynamic diameter of polyelectrolyte coated beads on a Malvern Nanosizer ZS using a 173° detection angle. Each measurement comprised at least 11 ten second runs and was repeated a minimum of 5 times.

Activity Assays

Activity assays were conducted colorimetrically using a Hewlett-Packard 8453 UV/Vis spectrophotometer. To measure the forward activity of MDH in single and multienzyme bioconjugates of 100 mM Tris (pH 8.1), 25 µl of 36 mM malate and 25 µl of 60 mM NAD⁺ (25 µl of 60 mM oxaloacetate and 25 µl of 12 mM NADH for the reverse reaction), for a final concentration of 0.3 mM malate and 0.1 mM NADH (0.5 mM oxalacetate and 0.1 mM NADH) were mixed in a cuvette and equilibrated to room temperature. A volume of 200 µl of bioconjugate was then added to the solution, which was mixed again to ensure homogeneity and the absorbance of NADH was monitored at 340 nm for 5 min \( \varepsilon = 6220 \text{ M}^{-1}\text{cm}^{-1} \). Each assay was repeated at least 3 times and had a total volume of 3 ml. The activity of CS assayed in similar fashion to MDH with the exception that 25 µl of 12 mM Acetyl-coenzyme A and 25 µl of 60 mM oxalacetate were used as substrates, 25 µl of 18 mM DTNB, for a final concentration of 0.5 mM oxalacetate, 0.1 mM Acetyl-CoA, and 0.15 mM DTNB, was added to monitor the production of coenzyme A which adsorbs at 412 nm \( \varepsilon = 13,600 \text{ M}^{-1}\text{cm}^{-1} \). HRP activity was determined using the SigmaFast® OPD kit as described previously.
Results and Discussion

LbL assemblies of alternating positively and negatively charged polyelectrolytes and enzymes have been constructed on submicron sized fluorescent latex beads. Here, several bead assemblies of various enzymes and PEs were characterized in terms of enzyme:bead stoichiometry, specific activity of each enzyme present, and the sequential activity of assemblies containing more than one type of enzyme.

Polyelectrolyte-Microparticle Assemblies

Polyelectrolyte coated 1 µm polystyrene beads similar to those used by Caruso et al. were used to characterize polyelectrolyte deposition and the behavior of enzymes in polyelectrolyte layers. In this report, bead assemblies are composed of three bilayers of positively and negatively charged PEs, for a total of six deposition steps per assembly.

The deposition of PAH/PSS layers onto beads is shown in Figure 4-2. Fluorescently labeled PAH and beads were used to determine a fluorescence ratio as polyelectrolyte layers were deposited onto the bead surface. An increase in the fluorescence ratio is detected each time a PAH layer is added (layers 1, 3, and 5), while no increase should be detected after PSS deposition (layers 2, 4, and 6). According to Invitrogen, the emission wavelength for AlexaFluor647 is 668 nm, when PAH-647 is immobilized onto PE assemblies the emission shifts to 680 nm, this value was used to determine the ratio of PAH to particle fluorescence. From this trend it is evident that solution phase PAH adsorbs to the bead surface, and that the amount of PAH deposited increases as subsequent layers are added.
**HRP Deposition into Polyelectrolyte Bead Assemblies**

We have immobilized several enzymes onto latex beads ranging in diameter from hundreds of nanometers up to 1 µm. Horseradish peroxidase (HRP), malate dehydrogenase (MDH), citrate synthase (CS), and urease have all been successfully deposited into polyelectrolyte assemblies and proved to retain at least part of their native activity.

The deposition and activity of HRP in polyelectrolyte coated beads was characterized using a variety of techniques and deposition methods. Fluorescently labeled HRP was deposited as either (1) as the positive layer of an L_bL assembly with poly(acrylic acid) (PAA), a negatively charged polyelectrolyte, or (2) embedded in the PAA layer with PAH as the positive layer. The number of HRP molecules deposited per bead was determined for each deposition method (Figure 4-3). Briefly, after bead scaffolds were exposed to a PE or enzyme solution they were centrifuged and washed with buffer. The wash solutions were analyzed for HRP content and this value was subtracted from the initial enzyme concentration to determine the amount of enzyme immobilized per bead. An alternative method of direct quantification by removal of the PE layers was found to be inefficient. Control experiments were conducted to determine the amount of enzyme lost to nonspecific adsorption to the reaction vessel by carrying out the experiment without beads present. The hydrodynamic radius of HRP and bare beads were measured to be 4.0 ± 0.1 nm and 245 ± 2 nm, respectively, from this the approximate area HRP would occupy on the surface of the bare bead, and the number of HRP needed to form a monolayer on the particle (~15,400) was estimated, which would provide a coverage of $2.04 \times 10^{12}$ HRP/cm$^2$. The coverage found for HRP/PAA beads is 98% ($2.00 \times 10^{12}$ HRP/cm$^2$) of this theoretical value, suggesting that when HRP, which a pI near 9 and would positively charged in solution, was deposited into the L_bL assembly as the positive layer it formed a monolayer. Alternatively, when HRP was embedded in the PAA layer in PAH/PAA-HRP, approximately 2.4x more enzyme (4.84
x 10^{12} \text{ HRP/cm}^2 was deposited into the assembly, suggesting that enzyme deposition was more efficient when the oppositely charged polyelectrolyte was being deposited at the same time. Due to the opposite charge of the PE and HRP it is likely that more HRP was attracted to the assembly as the layer grew since electrostatic repulsions between HRP molecules would be limited.

**Specific Activity of HRP in Polyelectrolyte Bead Assemblies**

The specific activity of HRP in each of the two assemblies was determined through activity measurements using hydrogen peroxide and o-phenylene diamine as reactants. As seen in Figure 4-4, although less HRP was deposited in the HRP/PAA beads than PAH/PAA-HRP more activity and a significantly higher specific activity was determined for HRP in the HRP/PAA assembly. As compared to free HRP, HRP in HRP/PAA retained 51% of its native activity as compared to 13% found for the PAH/PAA-HRP assembly. This could be due to unfavorable interactions between the oppositely charged PAA and HRP during deposition.

**Deposition of MDH and CS in Polyelectrolyte Bead Assemblies**

To understand the effect of layer position on the activity of an embedded enzyme or MDH and CS was immobilized using both as an independent layer, MDH/PSS and PAH/CS, or as an embedded component of the polyelectrolyte with the opposite charge, PAH/MDH-PSS and PAH-CS/PSS. The deposition of PE and enzymes were monitored by zeta potential of assemblies after each layer was added and the assemblies rinsed into filtered water (Figure 4-5). As oppositely charged layers are adsorbed the surface charge of the assemblies alternates between positive and negative, with a magnitude that is dependent on the species being deposited. Originally the carboxylated latex beads have a negative zeta potential, when PAH is adsorbed the
surface the potential becomes positive, and PSS causes the surface to become negative again. The first two panels of Figure 4-5 show the change in zeta potential as L$_b$L assemblies including MDH are created. MDH has a pI of 10, and at the deposition pH of 8 will be positively charged. In the red trend layer 4 (the negative half of bilayer 2) is composed of PSS with embedded MDH while in the blue trend layer 3 (the positive half of bilayer 2) is composed of purely of MDH. For each of these two layers the potential does not vary significantly from the average potential of a PE layer, and the potential of the next layer is not affected by the MDH.

The lower two panels of Figure 4-5 show the immobilization of CS as a component of the PAH in layer 3 (purple trend) or as layer 4 of the assembly (green trend). CS has a reported pI around 6, and will be negatively charged in the pH deposition buffer. Here, there is a clear difference in potential when CS is present. When CS was adsorbed on its own (green trend) the surface potential was less negative than when PSS is adsorbed. Similarly, when CS is co-immobilized with PAH the surface has a near neutral charge.

This data demonstrates that each PE or enzyme layer adsorbs to the surface predictably. This type of measurement has become standard to report the successful assembly of L$_b$L assemblies.$^{1,5,23}$

The amount of enzyme adsorbed per particle layer was measured as a function of the method and PE layer it was deposited in. As seen in Figure 4-6, no matter which layer MDH or CS were immobilized in the amount of enzyme embedded was consistent for each layer. Similarly to what was found for HRP, when MDH is embedded as a component of the PAH layer a significantly larger portion of enzyme is immobilized than when MDH is immobilized as an independent layer. Conversely, the same amount of CS was immobilized independent of the method used to embed it. This may be related to the PE it was embedded in, both MDH and HRP were embedded in the positive PAH, while CS was embedded in the negative PSS layer.
Specific Activity of MDH and CS in Polyelectrolyte Bead Assemblies

Figure 4-7 shows the specific activity of immobilized MDH and CS. As the activity of MDH is reversible the reverse activity, converting oxaloacetate to malate, was measured in this experiment as it is the more favorable reaction. In each case the specific activity generally increases as the enzyme layer occurs farther to the surface of the particle. This is likely due to a reduced diffusion of substrate through the PE layers, reducing the possible activity by limiting the concentration of substrate available to the enzyme.\(^4\) For CS, much like HRP, the activity was greater when CS was immobilized as an independent layer as opposed to embedded in the PAH layer, while the two methods for immobilizing MDH gave similar specific activities. This could be due to interactions between the PE and enzyme which cause reduced activities in the case of PAA and PAH but not PSS, which has a different structure (\textit{Table 4-1}) from the other PEs which may cause different interactions with the enzyme.\(^1,4\)

The percent activity of these enzymes in PE assemblies represents only a very small fraction (less than 1%) of the native enzyme activity. As compared to free enzyme, the embedded enzyme displays a markedly reduced activity; MDH was determined to be 182 ± 3 U/mg, while CS was 73.6 ± 0.6 U/mg. HRP was found to be much more active in PE assemblies, retaining 13 - 50% of its native activity when immobilized. Up to 90% activity loss compared to enzyme in solution has been noted in previous reports, with enzymes in deeper layers being 2-3x less active than enzymes closer to the surface,\(^4\) and successive loss of activity upon deposition of additional PE/enzyme bilayers due to lower diffusion and/or blocked active sites.\(^1\) The differences in activity may be linked to the structure of the enzymes, and the resuspension methods necessary during assembly preparation. HRP is a monomeric protein with a molecular weight of 44 kDa and one active site per enzyme,\(^4\) while MDH and CS exist natively as dimers with two active sites per enzyme.\(^4\) While one active site is found per monomer the activity of each site can be
severely reduced when the enzyme exists as a monomer. During the preparation of LbL assemblies the beads are centrifuged several times to remove them from solution in order to both wash the complexes and introduce PE, to resuspend the beads sonication was found to be a necessary step to dislodge assemblies tightly stuck to the walls of the centrifuge tube they were prepared in. Sonication can have a great impact on enzyme activity by denaturing the protein secondary and tertiary structure which would result in a greater loss of activity for a dimeric enzyme than a monomeric enzyme.

**Sequential Enzymes Embedded in Polyelectrolyte Layers**

Multiple enzymes have also been deposited into LbL assemblies, the sequential enzyme pair MDH and CS were immobilized to the same bead scaffold by embedding them as PSS-MDH and PAH-CS in one of 3 PE bilayers. Four different LbL bead types were constructed by immobilizing MDH and CS to different layers of PE. Organizations in which either MDH or CS were more deeply embedded in adjoining PE layers as seen in Figure 4-8, (1) PAH-CS and PSS-MDH were the positive and negative PEs of layer 2, (2) PAH-CS was the positive PE of layer 2, while PSS-MDH was the negative PE of layer 3, (3) PAH-CS was the positive PE of layer 3 and PSS-MDH was the negative PE of layer 1, and (4) PAH-CS was the positive PE of layer 2 and PSS-MDH was the negative PE of layer 3. Immobilization of MDH and CS without the presence of PE has been discussed previously in chapter 2.

As seen in Figure 4-9 and Table 4-2, the amount of enzyme embedded in each type of bead was consistent, as would be expected from the characterization discussed in the previous section. The specific activity of embedded MDH followed the trends found when MDH is immobilized to different layers as well, when the enzyme is present in the first layer of PE it shows the least activity, while it shows a much greater activity when present in the outer most
layer. The placement of CS in the assembly did not vary as much as the placement of MDH but the same trend applies, when CS was embedded in the third PE layer it is more active than when it is embedded in the second PE layer.

The rate of sequential activity for the two enzymes was also measured, in this reaction MDH uses malate to produce oxaloacetate, oxaloacetate is then converted to citrate by CS. Table 4-3 shows the sequential activity of each assembly measured as units/particle. This measurement is used to compare the activity of the assemblies because specific activity is not relevant for a sequential reaction. No significant difference was found in the rate of sequential activity per particle even though there were differences in the activity rates for individual enzymes. This could be due to the sequential activity of each assembly being limited by diffusion through the PE layers. While the activity of CS is low and very similar between assemblies, this is not the limiting factor for the sequential activity since the independent CS activity per bead is at least 5x greater than the rate of sequential activity in each case. The impression that sequential activity may be greater when MDH is embedded deeper into the assembly may be due to a buildup of intermediate. These results imply that while sequential enzyme activity is possible in LbL assemblies the organization of the enzymes in relation to each other may not have a noticeable effect on the reaction rate.

Conclusions

A variety of fluorescently labeled enzymes have been embedded in LbL assemblies on submicron sized latex beads. While enzyme:scaffold stoichiometry could not be determined directly by desorption of the assembly, it could be determined indirectly by determining the concentration of enzyme present in solution after enzyme adsorption and washing away of excess
enzyme. These determinations showed the variability in amount of enzyme embedded when it is treated as a PE layer or as a component in an oppositely charged PE layer.

Knowledge of the amount of enzyme present allowed for the specific activity of embedded enzymes to be determined. Specific activity measurements demonstrate the extent of activity loss due to restricted substrate diffusion through PE layers, as well as interactions between the PE and enzyme. In all cases a higher rate of activity was found for enzymes immobilized closer to the surface of the assembly, while for most enzymes tested specific activity was greater when the enzyme was treated as an independent layer and not complexed with a PE. Determinations such as these could be useful in the design of future assemblies to maximize the number of enzymes embedded, or the activity of each enzyme.

Embedding multiple enzymes to the same assembly has been reported previously\(^1\) presented here was a comparison of different strategies in which two sequential enzymes could be introduced to the same assembly, varying which enzyme is found deeper within the assembly, and how many PE layers were present between enzyme layers. The results above show that a significant difference between the sequential activities of the different assemblies created could not be found. This may be due to the low activity of CS in the complex or the low rate of substrate diffusion through the assembly.

Unlike direct adsorption bioconjugates, L\(_4\)L assemblies allow for the organization of enzymes into a three dimensional model of a multienzyme complex. The limited rate of diffusion in the PE layers allowed for the buildup of metabolites within the assembly which proved to severely reduced enzyme activity, and equalize the sequential reaction rate of each multienzyme assembly presented here, limiting the effectiveness of the model.
References


42. Radtchenko, I. L.; Sukhorukov, G. B.; Leporatti, S.; Khomutov, G. B.; Donath, E.; Mohwald, H. Assembly of alternated multivalent ion/polyelectrolyte layers on colloidal particles. Stability of the multilayers and encapsulation of macromolecules


45. To estimate the approximate amount of enzyme necessary to completely coat the surface of a bead the diameter of each enzyme was determined by averaging measurements made in the x, y, and z dimensions of each enzymes crystal structure. This diameter was then used to calculate the area a single enzyme molecule would occupy on the surface area of the latex bead. The number enzyme molecules necessary to cover the entire surface area of the bead was considered “monolayer coverage”. The estimated amount of enzyme present for subsequent layers of enzyme was determined using the original diameter of the bead because the increase in diameter due to previously deposited PE layers and embedded enzymes was unknown.


### Table 4-1: Structure of Polyelectrolytes

<table>
<thead>
<tr>
<th></th>
<th>PAH</th>
<th>PSS</th>
<th>PAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poly(allylamine) hydrochloride</td>
<td>Poly(sodium 4-styrenesulfonate)</td>
<td>Poly(acrylic acid)</td>
</tr>
<tr>
<td></td>
<td>Poly(allylamine) hydrochloride</td>
<td>Poly(sodium 4-styrenesulfonate)</td>
<td>Poly(acrylic acid)</td>
</tr>
<tr>
<td></td>
<td>Positively charged</td>
<td>Negatively charged</td>
<td>Negatively charged</td>
</tr>
<tr>
<td></td>
<td>56 kDa</td>
<td>70 kDa</td>
<td>450 kDa</td>
</tr>
<tr>
<td></td>
<td>Positively charged</td>
<td>Negatively charged</td>
<td>Negatively charged</td>
</tr>
</tbody>
</table>
Figure 4-1. Schematic of layer-by-layer assembly. Oppositely charged polyelectrolytes or proteins can be adsorbed to the surface of a nanoparticle through electrostatic interactions. In this way PAH (+) and PSS(-) can be assembled by alternating deposition to form bilayers on the surface of the scaffold.
Figure 4-2. Adsorption of PAH/PSS onto 1 μm beads. Adsorption of PAH/PSS onto 1 μm beads was monitored by the ratio of PAH-AlexaFluor 647 and yellow-green bead fluorescence as a function of layer addition. PAH was added as the 1st, 3rd, and 5th layers. Release of the polyelectrolyte was attempted by incubation of the coated beads with 2 M NaCl for 2 hrs at 70°C while sonicating.
Figure 4-3. Deposition stoichiometry of HRP assemblies. The number of HRP molecules deposited in one layer per 0.5 µm bead determined by subtraction, when HRP was deposited as a layer (HRP/PAA), or as a component in a layer (PAH/PAA-HRP). Monolayer denotes the theoretical prediction of a monolayer of enzyme deposited onto the surface of the bead as described in the text.
Figure 4-4. Activity of HRP deposited on PE coated beads. Activity of HRP deposited on PE coated beads where HRP is added to the assembly as a layer (HRP/PAA) and as a component of a layer (PAH/PAA-HRP). HRP denotes the activity of HRP free in solution.
Figure 4-5. Zeta potential of L$_{\beta}$L assemblies as a function of layer. Zeta potential of LBL assemblies with MDH added as a component of PSS layer 4 (red) and as an independent layer 3 (blue), and CS added as component of PAH layer 3 (purple) and as an independent layer (green).
**Figure 4-6.** MDH and CS assembly stoichiometry. Number of MDH (A) and CS (B) molecules embedded in PAH/PSS coated 0.4 µm latex beads. Enzymes were embedded as independent layers (closed symbols), or as part of a PE layer as PAH-MDH or PSS-CS (open symbols).
Figure 4-7. Activity of embedded MDH and CS. Activity of MDH (A) and CS (B) molecules embedded in PAH/PSS coated 0.4 μm latex beads. Enzymes were embedded as independent layers (closed symbols), or as part of a PE layer as PAH-MDH or PSS-CS (open symbols).
Figure 4-8. Assemblies of MDH and CS in L_{aL} assemblies. In each assembly MDH is denoted by green dots and CS by blue circles. Each enzyme was deposited separately to produce assemblies in which MDH and CS were present in different orientations and had different distances between enzyme layers.
Figure 4-9. Stoichiometry of MDH and CS embedded in the same \( L_B L \) assembly. (A) Number of MDH and CS embedded in PAH/PSS layers when both enzymes are embedded in PE layers of the same bead. Assemblies measured are described in the Sequential Enzymes Embedded in Polyelectrolyte Layers portion of the text. Assembly 1-4 appear in order from left to right in the figure.
Table 4-2: Stoichiometry of MDH and CS embedded in the same L_{bL} assembly

<table>
<thead>
<tr>
<th>Assembly</th>
<th>MDH per bead</th>
<th>CS per bead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assembly 1</td>
<td>$(7 \pm 2) \times 10^7$</td>
<td>$(7.0 \pm 0.6) \times 10^7$</td>
</tr>
<tr>
<td>Assembly 2</td>
<td>$(8 \pm 1) \times 10^7$</td>
<td>$(6.2 \pm 0.7) \times 10^7$</td>
</tr>
<tr>
<td>Assembly 3</td>
<td>$(7.4 \pm 0.5) \times 10^7$</td>
<td>$(6.6 \pm 0.4) \times 10^7$</td>
</tr>
<tr>
<td>Assembly 4</td>
<td>$(7.9 \pm 0.2) \times 10^7$</td>
<td>$(5.8 \pm 0.5) \times 10^7$</td>
</tr>
<tr>
<td>Assembly</td>
<td>MDH</td>
<td>CS</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Assembly 1</td>
<td>$(1.9 \pm 0.3) \times 10^{-12}$</td>
<td>$(3 \pm 1) \times 10^{-13}$</td>
</tr>
<tr>
<td>Assembly 2</td>
<td>$(5 \pm 2) \times 10^{-12}$</td>
<td>$(4 \pm 2) \times 10^{-13}$</td>
</tr>
<tr>
<td>Assembly 3</td>
<td>$(1.3 \pm 0.3) \times 10^{-12}$</td>
<td>$(6 \pm 3) \times 10^{-13}$</td>
</tr>
<tr>
<td>Assembly 4</td>
<td>$(3 \pm 1) \times 10^{-12}$</td>
<td>$(1.1 \pm 0.3) \times 10^{-12}$</td>
</tr>
</tbody>
</table>

Table 4-3: Activity per bead for MDH/CS functionalized Assemblies (U/bead).
Chapter 5
Ni(II)NTA Functionalized Scaffolds for the Immobilization of His-Tagged Enzymes

Introduction

Multienzyme pathways such as protein biosynthesis and glycolysis are assemblies of sequential enzymes, where the proximity between reaction sites has consequences for the overall activity of a metabolic pathway. The purinosome, which contains the six proteins of the de novo purine biosynthetic pathway, has been observed to reversibly form and dissociate dependent on the need for purine formation (Figure 1-3). This suggests that dynamic proximity between sequential enzymes may act as a metabolic switch to turn on and off as needed.

Enzyme-nanoparticle bioconjugates have a variety of industrial and research applications. Here, the focus is on using nanoparticle bioconjugates as a method for controlling enzyme proximity and orientation. Each of the strategies discussed thus far have their limitations; direct adsorption, discussed in chapter 2, can result in substantial activity loss due to denaturation of the enzyme structure, active site blockage through unfavorable orientation, and/or steric hindrance from neighboring enzymes. Layer-by-layer (LbL) assemblies, discussed in chapter 4, have also shown activity loss due to interactions between the enzymes and polyelectrolytes and restricted diffusion into polyelectrolyte layers.

An alternative to these methods is to engineer specific, non-destructive attachments between enzyme and scaffold using non-covalent interactions. For example, nanoparticles have been functionalized with complimentary DNA strands or Ni(II) nitrilotriacetic acid (NTA) and imidazole functionalities for programmed assembly. Fusion proteins of enzymes and receptor proteins have been created to immobilize choline decorated surfaces, and recombinant proteins have been expressed with leucine zippers for electrostatic assembly onto lipoic acid
functionalized surfaces, and histidine mutations or tags to coordinate to Ni(II)NTA assemblies.

His-tag – Ni(II)NTA chemistry is an attractive method to control the proximity between sequential enzymes because each enzyme can be bound in a specific orientation, with minimal loss of activity. Two or more enzymes can be co-immobilized to a single nano- or micro-scaled particle to hold enzymes in close proximity to each other. Since the immobilization is reversible with changes in pH or addition of imidazole, enzymes can be released from the scaffold so that sequential activity can be measured as a function of proximity alone. This would allow us to investigate the ability of dynamic enzyme proximity as a mechanism to regulate a metabolic pathway, without the effect of other environmental factors.

Abad et al. compared the activity of his-tag – NTA immobilized enzymes to that those free in solution. By comparing the activity for His-HRP immobilized to NTA functionalized nanoparticles to the activity of His-HRP solution before particles were added they determined that nearly all solution phase enzyme adsorbed to their scaffold with minimal activity loss. Similar chemistry was used to immobilize mutant Ferredoxin:NADP+ reductase to gold electrodes, by characterizing the turnover rate of free and immobilized enzyme they found similar kinetic behavior when the enzyme was immobilized in a favorable orientation.

Because Ni(NTA) is a functionality covalently linked to the scaffold, different types of scaffolds can be functionalized. As seen in previous chapters, nanoparticles can be used as solid scaffold for enzyme assembly where the enzymes are immobilized into static arrangement. Large unilamellar vesicles (LUVs) provide another potential scaffold for the assembly of sequential enzymes. LUVs are submicron sized assemblies of lipids in a continuous membrane bilayer where individual lipid molecules can diffuse in two dimensions. Ni(II)NTA functionalized lipids can be incorporated into lipid membranes to create a scaffold with in-plane diffusion of immobilized enzymes, which may enable enzymes to interact in preferential orientations (Figure
This scaffold can mimic membrane bound complexes in which lateral mobility may play a role in complex assembly and activity.

This chapter describes the creation and characterization of two Ni(NTA) functionalized scaffolds. First, LUVs were used to immobilize and characterize the activity of LDH, an enzyme chosen for its native histidine residues on the protein surface which can bind to Ni(II)NTA. Second, an AuNP based scaffold was characterized using commercially available his-tagged enzymes as well as proteins from the de novo biosynthetic pathway provided by the Benkovic group. TrifGART is a trifunctional enzyme which carries out reactions 2, 3, and 5 of purine biosynthetic pathway. The results of these experiments indicate that his-tagged enzymes can be non-destructively immobilized to Ni(II)NTA scaffolds and that this interaction can be efficiently reversed by addition of 0.5 M Imidazole.

Experimental

Materials

Colloidal gold particles (50 nm, 0.075 nM) were purchased from Ted Pella, Inc. (Redding, CA). NeutrAvidin and NHS-PEG12-Biotin were purchased from Thermo Scientific (Rockford, IL). Tissue transglutaminase, His-Tag®, Human Recombinant from S. frugiperda was purchased from Calbiochem (La Jolla, CA). Sodium phosphate monobasic, sodium phosphate dibasic, imidazole, sodium chloride, nickel (II) chloride, Nα-Nα-Bis(carboxymethyl) L-lysine hydrate, hydrogen tetrachloroaurate, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH), Trisma® hydrochloride (Tris[hydroxymethyl]aminomethane hydrochloride), Trisma® base (Tris[hydroxymethyl]aminomethane), pyruvate, sodium citrate, dextran (MW 500 kDa) and PEG (MW 8 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). His-GFP, His-
C1THF-OFP and His-TrifGART-GFP were provided by Dr. Songon An of the Benkovic laboratory. L-α-phosphatidylcholine (Egg PC), 1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (sodium salt) (DOPG), 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DOGS-NTA(Ni), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (DOPE-rhodamine) were purchased from Avanti Polar Lipids (Alabaster, AL). Deionized water with a resistivity of ≥18.2 MΩ from a Barnstead NANOpure Diamond water purification system (Van Nuys, CA) was used in all experiments.

12 nm Au synthesis

Colloidal gold nanoparticle suspensions (13 nM) were prepared by citrate reduction of HAuCl₄ according to established protocols. Nanoparticle size was measured by Julie Brennan, a graduate student in the Keating lab, and determined to be 11.5 nm using a JEOL JIM 1200 EXII transmission electron microscope.

Flocculation assays

Flocculation assays were performed to determine the amount of NeutrAvidin (NA) necessary to prevent single enzyme bioconjugates from aggregation in high ionic strength solution. Samples with mole ratios of enzyme:Au of 10:1 to 5000:1 were prepared by diluting Au particles (3.75 x 10⁻¹⁴ mol) in water or buffer then adding enzyme from stock in the same solution for a total volume of 150 µl. Samples were incubated for 1 hour at room temperature protected from light followed by addition of 150 µl of buffer to dilute the solution for absorbance measurements and 17 µl of 2 M NaCl, to induce aggregation of unprotected nanoparticles. After 60 min incubation
samples were analyzed by absorbance spectroscopy using a Hewlett-Packard 8453 diode-array UV-visible spectrometer with Agilent ChemStation Software.

**Preparation of NTA functionalized PEG**

A 5:1 mol ratio of N\textsubscript{α}-N\textsubscript{α}-Bis(carboxymethyl) L-lysine hydrate :NHS-PEG-Biotin was dissolved in 100 mM phosphate, 150 mM NaCl (pH 7.2), and the reaction monitored by absorbance at 260 nm for 30 min at 25 °C. Reaction products were purified by dialysis using a molecular weight cut off lower than the molecular weight of the PEG molecule.

**Scaffold functionalization**

NA was adsorbed to gold nanoparticles of various size by incubating a ratio of 500:1 Avidin:Au in 5 mM phosphate pH 7.5, for 30 min at 4 °C protected from light. Excess protein was subsequently removed by centrifugation of the conjugates at 5,000 x g for 15 min at 4 °C, the supernatant was removed and conjugates were resuspended in fresh buffer, this process was repeated three times to ensure all excess protein was removed. Conjugates were then incubated with a 500:1 excess of NTA-PEG for 1 hr at 4 °C protected from light. Excess NTA-PEG was removed by centrifugation of conjugates, the supernatant was removed and replaced three times with fresh buffer. NiCl\textsubscript{2} (100 nmol) in water was added to NTA-PEG functionalized conjugates and incubated for 1 hr at 4 °C protected from light. Conjugates were washed three times and NiCl\textsubscript{2} the process was repeated to ensure that all NTA sites were functionalized.
**LUV preparation**

Giant vesicles containing 3:7 egg PC:DOPG with 10% DOGS-NTA(Ni) and 1% DOPE-rhodamine prepared through the gentle hydration method.\(^4\) Briefly, lipids (1 mg/ml) were dissolved in chloroform and evaporated under argon into a thin film in glass test tubes, which were then put under vacuum for no less than 3 hours to ensure the removal of solvent. Films were hydrated with 1 ml of deionized water at 37 °C for 3 days to form GVs (> 1 µm diameter). Vesicles were pushed through a 200 nm syringe filter five times to create large unilamellar vesicles with an average diameter similar to the pore size.\(^2\)

**His-tagged protein attachment**

His-tagged proteins were incubated with NTA scaffolds in various ratios in 5 mM phosphate pH 7.5, for a 1 hr period at 4 °C protected from light. Assemblies were washed three times with fresh buffer.

**Partitioning in an aqueous two phase system**

Nanoparticle based complexes at various states of complexity were partitioned into an ATPS composed of 4 (w/w)% Dextran 500 kDa and 4 (w/w)% PEG 8 kDa. Briefly, complexes were added to ATPS at room temperature where it existed as a single phase solution. The solution was then refrigerated overnight at 4 °C to phase separate the dextran and PEG rich phases. These phases were separated and partitioning was determined by measuring the nanoparticle concentration in each phase. Equation 1 was then used to determine the partitioning coefficient, K between the PEG rich and dextran rich phases.\(^5\)
His-tagged protein release

Conjugates were centrifuged (15 min, 5,000 x g, 4 °C) and incubated in 10 mM phosphate pH 7.5, 500 mM imidazole for 1 hr protected from light before being centrifuged to separate released protein from conjugate assemblies.

Determination of His-tagged enzyme:nanoparticle stoichiometry

Stoichiometry was determined by washing away excess enzyme and dissolving the Au core to release the adsorbed enzymes into solution for quantification. This was done by first determining the concentration of conjugates based on the absorbance of the 30 nm gold nanoparticles at 524 nm, $\varepsilon = 3.585 \times 10^9 \text{M}^{-1}\text{cm}^{-1}$. One hundred microliters of 50 mM potassium cyanide was then added to 100 µl of conjugate, mixed, and allowed to react for 12 hrs at 4 °C. A Horiba Jobin Yvon Fluorolog 3-21 was then used to find the concentration of each fluorescently labeled enzyme in solution. Stoichiometry is expressed as the ratio of enzyme to Au present after conjugation and washing steps.

Enzyme Activity assays

LDH activity assays were conducted by monitoring the absorbance of NADH at 340 nm ($\varepsilon = 6220 \text{M}^{-1}\text{cm}^{-1}$) in a 3 ml reaction volume with 25µl of 12 mM NADH and variable
concentrations of pyruvate in 100 mM Tris-HCl, pH 8.1. Transglutaminase was assayed in 1000 mM Tris, pH 6 according to a protocol provided by Sigma-Aldrich.

Results and Discussion

His-tag – Ni(II) NTA attachment is widely used for the purification of proteins expressed with a his-tag. This complexation provides a stable, reversible linkage for proteins to assemble on scaffolds that is superior to other immobilization strategies such as direct adsorption or chemical coupling in terms of enzyme denaturation and orientation.\textsuperscript{15,16} Immobilization and characterization of enzyme in LUV assemblies will be discussed first, followed by the characterization of AuNP scaffolds including particle coverage and release of immobilized protein.

NTA large unilamellar vesicles

Giant vesicles formed of 3:7 egg PC:DOPG with 10% DOGS-NTA(Ni) and 1% DOPE-rhodamine were filtered through a 0.2 µm syringe filter five times to produce LUVs with an average diameter of 166 ± 2 nm according to dynamic light scattering measurements. This produced an Ni(II)NTA functionalized scaffolds on which his-tagged proteins could be assembled. As seen in Figure 5-2, zeta potential measurements can be used to track the immobilization of LDH to the LUV surface by the change in surface charge that occurs when the Ni(II)NTA is chelated (top panel). As the ratio of LDH:LUV increases the zeta potential becomes less negative up to a ratio of 18,000:1 LDH:LUV is reached and then levels off. This data indicates that the LUV surface becomes saturated at 18,000:1 and additional LDH is not adsorbed to the surface. LUVs that should be unable to chelate LDH, either because no Ni\textsuperscript{2+} was present
(center) or the NTA lipid was not included (bottom panel), do not show the same leveling off effect. This indicates a specific interaction between LDH and Ni(II)NTA LUVs that allows for assembly of the enzyme on the membrane surface.

LDH:LUV assemblies can also be characterized by analyzing the enzymatic activity. Kinetic analysis of LDH in solution, immobilized to LUVs and directly adsorbed to Au nanoparticles is presented in Table 5-1. The $K_M$ value for a particular substrate reflects the substrate affinity of the enzyme and can be used to compare the behavior of LDH when immobilized, further characterization was limited by the uncertainty of number of vesicles present in the sample. When LDH is immobilized to the NTA-functionalized LUVs the value of $K_M$, which represents substrate affinity, is unchanged implying that none of the common limitations related to immobilization have affected LDH activity. Table 5-1 also shows the $K_M$ of LDH directly adsorbed to Au nanoparticles, this is a competing method that is sometimes favored for its ease of use (discussed in detail in chapter 2). Direct adsorption of LDH causes an increase in the value of $K_M$, demonstrating the effect of blocked active sites, steric hindrances and denaturation can have on enzyme activity.

Changes in the value of $K_M$ have been determined for a variety of enzymes immobilized to functionalized and unfunctionalized nanoparticle surfaces. In general the $K_M$ for a substrate decreases, demonstrating that the active site becomes saturated at lower concentrations of substrate and implying a change in enzyme structure.\textsuperscript{1,27,28} The increase found for LDH:Au is likely due to active site blockage which would make substrate – active site binding less likely.\textsuperscript{27}

NTA Particle functionalization

Co(II)NTA functionalized particle were previously reported by Abad et al\textsuperscript{1} using 4 nm Au as the scaffold and a lipoic acid based bifunctional linker to provide the NTA functionality.
They report the immobilization and activity of his-HRP bound to gold nanoparticle. Since molecules adsorbed to Au particles through thiol interactions can be displaced\textsuperscript{11,29,30} direct adsorption of an avidin protein and linkage to a biotin functionalized molecule was chosen. This approach has not previously been reported in the literature.

Ni(II)NTA functionalized particles (Figure 5-3) were assembled from several components. NeutrAvidin (NA) was non-specifically adsorbed onto gold scaffolds of various sizes. Flocculation assays were used to determine the amount of protein necessary to protect gold nanoparticles from salt induced aggregation. Figure 5-4 shows flocculation assays for 12, and 50 nm Avidin:Au. To determine when enough protein had adsorbed to the surface to allow for steric protection from aggregation various ratios of Avidin:Au were incubated with 0.15 M NaCl to screen electrostatic repulsions and the absorbance spectra were recorded. These spectra were compared to the spectrum of bare Au (solid black trend) to determine at what point the nanoparticles were completely protected. When unprotected nanoparticles are exposed to high salt solution the electrostatic repulsions that prevent aggregation are screened, allowing Van der Waals attractions between the particles to cause aggregation. The absorbance maximum of the Au plasmon is dependent on the size of the nanoparticle, when protected from aggregation the absorbance spectra remains almost identical to that of bare nanoparticles, if flocculation occurs the size of the particles increases and the plasmon absorbance’s is red shifted. From the data presented in Figure 5-4 Avidin:Au ratios of 250:1 and 3000:1 were used for scaffold construction with 12 nm and 50 nm Au, respectively.

Bifunctional PEG was used to link the NTA functional group to scaffold. Aminonitrilotriacetic acid was reacted with NHS-PEG-Biotin (Figure 5-5) in a 5:1 excess. This reaction was monitored by an absorbance change at 260 nm which indicates the leaving group, NHS, is released into solution to ensure the complete functionalization.\textsuperscript{31} The solution was subsequently dialyzed against water to remove excess reactants. The biotin functionality has a high affinity for
avidin proteins ($k_d = 10^{-15}$ M) and specifically binds to NA to introduce the NTA-PEG group to the assembly.\textsuperscript{31} PEG has been used as a flexible, neutral, hydrophilic linker that prevents non-specific binding to surfaces.\textsuperscript{32,33} Ni\textsuperscript{2+} was added to the assembly in a separate step to prevent NiCl\textsubscript{2} from causing charge-induced aggregation of Au.

To examine the change in surface chemistry as assemblies were functionalized they were partitioned into an aqueous two phase system (ATPS). PEG and dextran are dissimilar hydrophilic polymers that partition into separate aqueous phases at high enough wt\%. When nanoparticles are introduced into this system they partition preferentially based on their surface chemistry. As seen in figure 5-6 when each component is added to the assembly the partitioning alters. NA:Au partitions 19:1 to the PEG phase, proteins in general partition poorly, with a slight affinity for PEG.\textsuperscript{34} NTA-PEG functionalized particles have a greater affinity for the PEG phase, as would be expected due to the PEG linker. When transglutaminase, a his-tag enzyme, is adsorbed to the particles the surface chemistry is protein based again, and the affinity for the PEG phase is reduced. Transglutaminase catalyzes the amination of peptide sequences, therefore the ability of nanoparticle assemblies to catalyze this reaction (Table 5-2) is further evidence that transglutaminase has been immobilized successfully.

**NTA particle stoichiometry**

The number of proteins immobilized per assembly can be determined by using fluorescent his-tagged proteins such as green fluorescent protein (GFP), which has a native fluorescence due to an arrangement of amino acids in its structure.\textsuperscript{35} The surface coverage of his-tagged GFP, C1THF-GFP, and TrifGART-OFP are presented in Table 5-3. The coverage of His-GFP and C1THF-GFP are approximately equal even though their sizes are dramatically different. For other immobilization methods, such as direct adsorption, both protein size and protein-protein
interactions play a role in determining particle stoichiometries (chapter 2). This demonstrates that by using a flexible PEG linker and specific immobilization interactions size does not play a significant role in determining quantity of protein immobilized.

The coverage of TrifGART-OFP was about 20% of the other two proteins measured. Since it has a similar size to C1THF-GFP the difference in immobilization may be due to differences in the structures of the proteins, which is a common issue for recombinant proteins. This limitation could be remedied by redesigning the placement of the his-tag. 15

Multiple his-tagged species can be immobilized to the same assembly as seen in Table 5-4, TrifGART-OFP and C1THF-GFP were co-immobilized at different ratios on to 50 nm assemblies. As seen in Table 5-3, the two proteins immobilized do not have the same affinity for the NTA scaffold. Ratios of C1THF:TrifGART were therefore chosen to determine if increased concentrations of TrifGART could correct for the limitations in immobilization. The data shows that more C1THF is always immobilized even when 10-fold more TrifGART is present. The ratio of immobilized enzymes was somewhat error prone, suggesting that the best way to control the immobilization would be to insure identical affinity for the scaffold by reengineering the TrifGART his-tag location.

**Reversible immobilization of His-GFP**

Several methods have reported to either displace immobilized His-tagged proteins or regenerate Ni(II)NTA surfaces including addition of imidazole or pH change with NaOH. The presence of high concentrations of imidazole (up to 0.5 M) has been shown to reverse His-GFP binding from NTA functionalized surfaces, 17,18 and regenerate a surface that shows little to no degradation upon reuse. 10
Table 5 shows that immobilized His-GFP can be removed from the surface of assemblies by both methods. The structure of imidazole is similar to the portion of histidine that complexes to Ni\(^{2+}\). Since the orientation of imidazole is not limited like the orientation of histidine in a protein structure it has a stronger chelation with the metal ion and preferentially interacts to remove his-tagged proteins from the assembly surface. Similarly, the addition of NaOH introduces a shift in pH and an increase OH\(^-\) concentration in solution, OH\(^-\) preferentially complexes with Ni\(^{2+}\) leading to the removal of immobilized proteins. As seen in Table 5-4 imidazole and NaOH were both effective at releasing His-GFP from the assembly. While the concentration of NaOH necessary is far less than the concentration of imidazole used, buffered imidazole was deemed the preferred method because the solution pH is not affected. Changes in pH can lead to undesired denaturation of protein structures which would affect subsequent use of immobilized enzymes.

**Conclusions**

Both Ni(II)NTA functionalized LUVs and nanoparticles are promising scaffolds for investigating the effects of proximity on sequential enzyme reactions. \(K_M\) measurements show that enzymes immobilized to LUV scaffolds have catalytic properties very close to that of native enzyme. Studies with fluorescent enzymes show that multiple enzymes can be immobilized to the same scaffold, in predictable and possibly controllable ratios, and that immobilization is reversible upon addition of imidazole or increase in pH.

For these reasons the two scaffolds discussed here are promising tools for studying the effect of proximity on mulitenzyme kinetic pathways, such as the de novo purine biosynthetic pathway, to determine if proximity alone can be used as a mechanism to turn on and off a metabolic pathway. TrifGART is a trifunctional enzyme that carries out reactions 2, 3 and 5 of
the purine biosynthetic pathway. Its use here is a stepping stone for immobilizing all six proteins with his-tags to address the importance of dynamic proximity in vitro.

Further work with these scaffolds will include the reversible immobilization of the de novo purine biosynthetic pathway enzymes to determine the role of proximity in modulating the rate of sequential reaction.

References


36. Personal correspondence with Dr. Songon An
Figure 5-1. Ni(II)NTA LUV scaffold. Ni(II)NTA LUV scaffolds are composed of a lipid membrane containing Ni(II)NTA functionalized lipids (green) which can specifically immobilize his-tagged enzymes, and rhodamine functionalized lipids (red) which allow LUV concentration to be measured using fluorescence.
Figure 5-2. Zeta potential of LUVs with increasing concentrations of LDH. (Top) Ni(II)NTA functionalized LUVs, (middle) NTA functionalized LUVs, and (bottom) LUVS without NTA lipids
Table 5-1: Kinetic Characterization of Immobilized LDH

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<th>$K_M$ (pyruvate) (mM)</th>
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<tr>
<td>LDH</td>
<td>0.04 ± 0.01</td>
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<tr>
<td>aLDH:LUV</td>
<td>0.037 ± 0.009</td>
</tr>
<tr>
<td>bLDH:Au</td>
<td>0.107 ± 0.006</td>
</tr>
</tbody>
</table>

*a*LUV – large unilamellar vesicle scaffold  
*b*Au – gold nanoparticle scaffold
Figure 5-3. Schematic of Ni(II)NTA functionalized nanoparticles. Ni(II)NTA functionalized particles are assembled by directly adsorbing an NeutrAvidin onto a nanoparticle scaffold, bifunctional NTA-PEG-Biotin binds to the streptavidin to add a flexible linker for his-tagged protein immobilization.
Figure 5-4. Flocculation assays of NeutrAvidin with gold nanoparticles. (A) 12 nm Au (B) 50 nm Au to determine the point at which NeutrAvidin completely protects the surface of the gold nanoparticle. The black trend indicates the absorbance spectra of bare gold nanoparticles, while the following trends increase in NeutrAvidin:Au ratios according to the arrows.
Figure 5-5. Components of bifunctional PEG. Bifunctional PEG was synthesized by coupling NHS-PEG-Biotin with N$_{\alpha}$-N$_{\alpha}$-Bis(carboxymethyl) L-lysine to remove the NHS ester and create NTA-PEG-Biotin.
Figure 5-6. Partitioning of NTA functionalized AuNP assemblies in ATPS. Partitioning of NTA functionalized gold nanoparticles in PEG/Dextran ATPS. Bare Au partitioning coefficient from ref 34.
Table 5-2: Activity of Immobilized Transglutaminase

<table>
<thead>
<tr>
<th>Phase</th>
<th>Activity per Particle</th>
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<tbody>
<tr>
<td>PEG</td>
<td>$(3.2 \pm 0.9) \times 10^{11}$</td>
</tr>
<tr>
<td>Dextran</td>
<td>$(5 \pm 2) \times 10^{11}$</td>
</tr>
</tbody>
</table>
### Table 5-3: His-Tagged Protein Coverage

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight[^35]</th>
<th>Protein:50 nm Particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-EGFP</td>
<td>27 kDa</td>
<td>1180 ± 574</td>
</tr>
<tr>
<td>His-C1THF-EGFP</td>
<td>131 kDa</td>
<td>1154 ± 545</td>
</tr>
<tr>
<td>His-TrifGART-OFP</td>
<td>137 kDa</td>
<td>211 ± 136</td>
</tr>
</tbody>
</table>

[^35]: The molecular weights are estimated based on the protein sequences.
Table 5-4: Dual His-tagged Enzyme Immobilization

<table>
<thead>
<tr>
<th>C1THF:TrifGART added</th>
<th>C1THF:TrifGART bound</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:3</td>
<td>3.2:1</td>
<td>33%</td>
</tr>
<tr>
<td>1:6</td>
<td>2.4:1</td>
<td>37%</td>
</tr>
<tr>
<td>1:12</td>
<td>2.5:1</td>
<td>38%</td>
</tr>
</tbody>
</table>

*Error propagated from the concentrations of scaffolds and each enzyme
Table 5-5: Release of His-GFP from 12 nm Assemblies.

<table>
<thead>
<tr>
<th>Release Agent</th>
<th>GFP:Au Bound</th>
<th>GFP:Au after release</th>
<th>% Released</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM NaOH</td>
<td>70 ± 10</td>
<td>0.2 ± 0.1</td>
<td>99.7</td>
</tr>
<tr>
<td>0.5 M Imidazole</td>
<td>80 ± 10</td>
<td>0.00 ± 0.004</td>
<td>100</td>
</tr>
</tbody>
</table>
Chapter 6

Conclusions and Future Directions

Conclusions

This thesis describes the creation and characterization of four artificial multienzyme complexes designed to model one or more aspects of multienzyme complexes found in vivo. While the main goal of this work was to test the hypothesis that activity of a sequential enzymatic pathway is improved by the colocalization of the enzymes involved, the characterization of each bioconjugate has further implications. Through rigorous experimentation the limitations and assets of each model were determined, and the resulting analysis can provide insight not only to the design of future enzyme complex models but also biotechnological assemblies, such as nanoreactors, which use the same components.\(^1\)

To address the question of how proximity affects the rate of a sequential reaction for a two enzyme pathway, the distance between enzymes and orientation within a complex were determined using direct adsorption bioconjugates and L\(_{\alpha}\)L assemblies. In each model, the experimental (colocalized) and control (non-colocalized) systems were extensively studied to note the similarities and potential differences between the systems. This allowed for a more detailed comparison of proximal to non proximal activities that has not been noted previously in the literature.\(^2\)\(^-\)\(^8\)

Direct adsorption bioconjugates functionalized with one or two types of enzymes allowed proximity between MDH and CS to from the nanometer to micron range. Results show that while the enzymes present in the colocalized and non-colocalized systems compared here behaved differently in the presence of viscosity agents, overall the sequential activity of the pathway was
improved by (1) decreasing the diffusion distance between active sites, (2) decreasing the
diffusion coefficient of the intermediate of the reaction to prevent escape into the bulk solution,
and decreasing the overall amount of bioconjugate in the solution to prevent the pathway form
being inhibited by the eventual increase of metabolite concentration in solution.

In comparison, L₅L assemblies of MDH and CS were used to examine the layering effect
of sequential enzymes found in multienzyme complexes such as the PDC. By controlling the
orientation of enzymes in the complex (i.e. how deeply embedded each enzyme is) it was
hypothesized that differences in sequential activity would determine an optimal orientation for a
multienzyme complex. However, it was determined during the course of these experiments that
the PE assembly itself served to slow diffusion of intermediates, leading to a buildup of
oxaloacetate within the PE layers to form a pool of metabolite that equalized the rates of reaction
between the different orientations tested.

Because the same set of enzymes was used for both of the above tasks, a direct
comparison between the two immobilization techniques can be given. While direct adsorption is
known to denature enzymes,⁹ L₅L deposition was thought to have less impact on the structure of
enzymes.¹⁰ However, the specific activity results presented in chapter 2 (direct adsorption) and
chapter 4 (L₅L assemblies) show that while immobilization decreases the activity of enzymes in
both models, MDH and CS activities in L₅L assemblies is far lower than in AuNP bioconjugates.
The difference in rate here may be due more to the effect of PEs on the diffusion of metabolites
than the structure of embedded enzymes, as exemplified by the reduction in specific activity
when more PE layers are present between the enzymes and solution.

To further advance the model design of artificial multienzyme complexes, two
Ni(II)NTA functionalized scaffolds were created. Solid supports created using AuNPs provide a
model of static multienzyme complexes, like the PDC, and a dynamic support created using
LUVs which allow for the lateral diffusion of immobilized enzymes across the surface of the lipid bilayer, similar to membrane bound complexes.

While his-tagged MDH and CS were not available for this study, immobilized LDH and TGase were characterized and compared to free enzymes. The results show that immobilized and free LDH display the same $K_M$ value, which is significantly different from the $K_M$ of LDH:Au, the direct adsorption equivalent. Because the his-tag – Ni(II)NTA interaction is non-destructive, reversible, and can be used with different types of scaffolds, this model is promising for the future study of multienzyme complexes.

**Future Directions**

Recently, the Keating group has begun to isolate his-tagged enzymes from the de novo purine biosynthetic pathway. These enzymes will be used in conjunction with Ni(II)NTA functionalized AuNPs and LUVs to explore the effects of dynamic proximity on the rate of sequential reaction. It has been previously proposed that this pathway is regulated by dynamic colocalization to turn the pathway on and off as needed by the cell. While microscopy has been used to see the association of these enzymes in vivo, the rate of activity for each enzyme and the overall pathway are not easily measured in the cell. The experimental models presented here, in conjunction with computational modeling similar to what is presented in chapter 3 can be used to understand the pathway and control its activity artificially.

Finding new and innovative ways to control pathway activity in metabolism may lead to new methods for pathway inhibition and regulation. This may result in new drugs to aid in the treatment of metabolic diseases, where alterations in an enzyme causes a disease and cancer, where DNA production is unchecked.
References


VITA
Jacqueline D. Keighron

Education

2010 Pennsylvania State University – University Park, PA
Ph.D. in chemistry: Advisor Dr. Christine Keating

2004 Moravian College – Bethlehem, PA
B. S. Biology and Chemistry

Peer Reviewed Publications


Conferences/Presentations

