Using Nanoparticle Scaffolds to Probe and Enhance the Catalytic Activity of Single and Coupled Enzyme Systems

D. Hastman*,***, M. Chiriboga**,*** and I. Medintz***

* Fischell Department of Bioengineering, University of Maryland, College Park, College Park, MD 20742, USA
** Department of Bioengineering, Institute for Advanced Biomedical Research, George Mason University, Manassas, VA 20110, USA
*** Center for Bio/Molecular Science and Engineering, Code 6900, U.S. Naval Research Laboratory, Washington, D.C. 20375, USA, Igor.medintz@nrl.navy.mil

ABSTRACT

The use of nanoparticle (NP) scaffolds for enzymatic systems has proven to be a useful tool for sensing specific enzyme activity as well as enhancing the catalytic rates of these systems. Although, NP-induced enhancement has been shown with a variety of different enzymes, this is not always the case, and the mechanism and magnitude of enhancement can drastically vary between enzymes. Using semiconductor quantum dots (QDs) as a model nanoparticle scaffold, we have examined a variety of single enzymes systems, which has provided a fundamental understanding of many of the processes involved in NP-induced enhancement. As we continue to probe such systems, we have started to implement QD-enzyme conjugates into complex multienzyme cascades, with the goal of preserving QD derived enhancement in the larger system. Here we discuss some of our this using QDs with single enzyme systems as well as our work to integrate QD-enzyme bioconjugates into more complex multienzyme systems.

Keywords: enzyme, nanoparticle, biosensing, catalysis, synthesis

1 INTRODUCTION

The long and arduous process of evolution through natural selection has delicately shaped biological systems to function with an efficiency and precision that cannot be replicated through man made processes. Current recombinant genetic methods require us to hijack entire microbiological systems to efficiently manufacture desired molecular products. The field of cell-free synthetic biology attempts to discard the cell and excess molecular machinery in order to distill chemical and enzymatic pathways to the most essential components of the system. Once the basic components are isolated, they can be used in vitro to preform their innate function. Typically, an enzymes function is specific to a certain type of chemical substrate. First, the enzyme binds the freely diffusing substrate forming the enzyme-substrate complex. Second, the enzyme catalyzes a reaction, and finally the enzyme releases the product to diffuse away in solution, allowing for the binding of another substrate. Each step of this system is described by the Michaelis-Menten enzymatic kinetic model [1]. Enzymes can also be engineered or manipulated to increase their efficiency and productivity. To this end, synthetic nanomaterials such as semiconductor quantum dots (QDs), gold nanoparticles (AuNPs) and de novo synthesized DNA have been used to enhance or augment enzymatic output [1-4]. QDs offer a size determined, monodisperse central scaffold on which to arrange enzymes or substrates. Through a large library of surface ligands and attachment chemistries, it is easy to assemble various proteins to the QD surface. For example, one common method of bioconjugation to QDs is via the integration of a poly-histidine (His) sequence onto the protein amino acid sequence. This (His) sequence is able to attach to the QD surface through metal affinity coordination with the Zn²⁺ on the outer layer of the QDs [5-8]. In addition to ease of conjugation, QDs have broad absorption spectra which allows them to be excited at a variety of wavelengths, and a discreet narrow photoluminescence that is quite useful for Förster resonance energy transfer (FRET) based sensing. In these biosensing systems, the QD acts as a central scaffold to dye decorated peptides and proteins surrounding it that engage in FRET with the QD. These peptides can be designed to be cleaved by a specific enzyme, which separates the dye from the QD surface and “turns off” the FRET of the system allowing for protease sensing. This process can be spectroscopically monitored and used to probe enzyme activity. In our experimental models two overarching regimes exist; substrate (peptide) attached to QD, or enzyme attached to QD. The former lends itself more naturally to sensing applications, while the latter experiments are more of a foray into the nature of NP induced enzymatic enhancement. The eventual goal is to be able to modularly integrate these enzymes and nanomaterials into complex multienzyme cascades. Enhanced enzyme performance lends itself naturally to a range of applications in biosensing, bio-remediation, energy harvesting and industrial syntheses [7, 9-11].
2 SINGLE ENZYME SYSTEMS

2.1 Proteolytic Sensing Using QD Scaffolds

Generally speaking, these types of sensors all follow the same principle. A specific proteolytic enzyme is chosen along with a corresponding peptide substrate. The substrates amino acid sequence is then modified to be book ended at one end with a poly-histidine sequence for QD attachment and the other end is labeled with a dye acceptor fluorophore [8]. Our initial system was designed to monitor the activity of the protease kallikrein. This sensor followed a two-step assay methodology in which substrate enzymatic digestion occurred, then QD was added to the system to sequester the peptide in solution via $(\text{His})_6$ coordination. The resulting FRET-based readout was representative of the amount of peptide that had been cleaved by kallikrein, as only peptide that had not been cleaved would engage in FRET with the QD. The activity of kallikrein could then be fit to the Michaelis–Menten model [5]. In later sensing systems, the substrate was attached to the QD surface before enzymatic digestion occurred, then QD was added to the system to sequester the peptide in solution via (His)$_6$ coordination. The resulting FRET-based readout was representative of the amount of peptide that had been cleaved by kallikrein, as only peptide that had not been cleaved would engage in FRET with the QD. The activity of kallikrein could then be fit to the Michaelis–Menten model [5]. Later sensing systems, the substrate was attached to the QD surface before enzymatic digestion as shown in the schematic in Figure 1A. However, it became clear that not all NP sensors designed in this format could be represented by the Michaelis–Menten model. For example, systems designed to detect elastase and collagenase revealed that while the former could be described by the Michaelis–Menten model the latter deviated as the time course of the reaction proceeded. We speculate this deviation to arise from distinct restructuring of the microenvironment around the QD. This restructuring is a result of the interplay between the chemical and physical properties of the adjoined parties e.g. QD, enzyme, ligand, substrate, solvent, etc. [6]. Perhaps most notably, our work with trypsin displayed a substantial 35-fold enhancement in enzyme activity when the substrate was attached to the QD compared to that of the free substrate in solution. Simulations and empirical observations both point towards peptide substrate availability and enzyme binding site accessibility as being the most important factors that modify enzyme performance when substrate is displayed on a QD [12, 13]. This initial work has validated the use of QD-peptide bioconjugates for biosensing and prompted further investigation of the observed enhancements seen with QD-enzyme systems.

2.2 Enzyme Enhancement on QD Scaffolds

In contrast to QD-substrate systems which focus on sensing proteolytic activity over a range of environmental conditions, QD-enzyme systems are focused on maximizing enzyme catalytic performance. This type of enhancement is applicable to bio-remediation, energy harvesting and pharmaceutical production, to name but a few applications. Figure 1B displays a schematic of a typical QD-enzyme bioconjugate system. Phosphotriesterase (PTE), a crucial enzyme in the remediation of organophosphates, which form a class of potent nerve agents, displayed up to 4-fold enhancement in enzymatic rates and 2-fold enhancement in enzyme efficiency when conjugated to QDs [14, 15]. Other work using PTE with graphene electrochemical chips, AuNPs, and QD-DNA-peptide hybrids has shown to further augment PTE activity [1, 2, 4, 16]. Large, bulky, and even substrate diffusion limited enzymes also can reap the

Figure 1. (A) Schematic of a QD-substrate system. The QD acts as a scaffold to dye-labeled peptide substrates. When intact, the dye labeled peptide engages in FRET with the QD. Once the enzyme has cleaved the substrate, FRET is reduced, allowing for enzyme activity to be monitored in real time. (B) Schematic of a QD-enzyme system. The QD acts as a scaffold to the enzyme as the substrate diffuses freely in solution. Typically, the enzyme kinetics can be monitored by the absorbance of an optically active product or cofactor, barring this, mass spectral and HPLC analyses can be used.
benefits of QD conjugation. For example, the enzyme β-galactosidase which converts lactose into galactose and glucose displayed a 3-fold enhancement in its catalytic rate when attached to QDs. β-galactosidase is a relatively large enzyme that precludes it from being scaffolded by a single QD and as such multiple QDs are attached to the enzyme and effectively surround it. The diffusion limited nature of β-galactosidase and corresponding enhancement suggests QDs exhibit a super-diffusional property providing local accumulation of substrate [3, 17]. Of note should be that greater enhancement was seen utilizing smaller 525 nm QDs versus larger 625 nm QDs, suggesting QD size or curvature has a significant effect on the system. We hypothesize there are generally four mechanisms contributing to enzyme enhancement on QDs: (1) higher enzyme density, (2) super-diffusional rates of substrate accumulation due to a modified microenvironment around the QD, (3) QD surface and ligand chemistries which lead to more stable/active enzymes, (4) favorable enzyme or substrate active site orientation/availability [18]. Table 1 displays some of the catalytic enhancements observed for various representative QD-enzyme systems.

### 3 MULTI-ENZYME SYSTEMS

#### 3.1 Preserving Nanoparticle Enhancement in a Coupled Enzyme System

It is of great interest to synthetic biologists to implement NP-enzyme conjugates into complex multienzyme cascades and preserve the NP-induced enhancement within the larger system. To this end, we investigated a coupled 2-enzyme system consisting of horseradish peroxidase (HRP) attached to a QD scaffold and glucose oxidase (GOX) free in solution [19]. GOX and HRP were chosen as a model enzyme system as they are a very common enzyme pair and are one of the most understood coupled reaction systems [19-23]. Initially, we probed HRP activity in an isolated system to determine the effect of the QD scaffold. The HRP was purchased commercially and functionalized with a maleimide that allowed it to be conjugated to a thiolated-peptide displaying a terminal (His)_6 tag. The high affinity of the His-tag for the QD surface allows for the ratiometric loading of HRP on to the QDs. When HRP was attached to QDs at ratios ranging from 0.05 to 20, the HRP turnover rate increased by a factor of 2. The catalytic enhancement is attributed to the affinity of TMB substrate for the QD surface. The affinity of TMB to the QD could be shown experimentally and accounting for this in our kinetic modeling produced simulations that are in good agreement with the experimentally observed enzymatic enhancement. Next, we investigated the 2-enzyme system of GOX and HRP to determine if the enhancement derived from the HRP-QD conjugate could accelerate coupled system. In order to probe any QD associated enhancement in the dual enzyme cascade, the HRP reaction needs to be the rate limiting step. When operating in this regime (1-10 GOX to HRP), the kinetic enhancement of the HRP-QD was preserved within the 2-enzyme system. The largest QD-associated enhancement (~3 fold) was achieved at a ratio of 10 GOX to HRP, which is very similar to the enhancement for the QD-HRP alone. This work is the first to show that a NP-induced rate enhancement can be maintained in a multienzyme cascade were component enzymes are free in solution. This is an important finding, as not all enzymes are expected to manifest enhancement when displayed on a NP surface and to achieve more complex biosynthetic pathways it may require the coupling of NP displayed enzymes and non-assembled enzymes. Preserving NP-derived enhancement within these multienzyme systems is a promising step towards achieving complex biosynthesis though cell-free enzyme engineering.

#### 3.2 Intermediary Channeling in a Coupled Enzyme System

Substrate or intermediary channeling is the process in which two enzymes in close proximity engage in the “handoff” of an intermediary (product of the first enzyme and substrate for the second). When properly implemented, channeling is capable of augmenting the activity of coupled enzymes...
enzymatic steps by orders of magnitude [24]. We have recently colocalized two coupled enzymes on the same NP scaffold to determine if channeling can occur in such a system [25]. Here, we use the well-characterized enzymes pyruvate kinase (PykA) and lactate dehydrogenase (LDH), which are involved in two sequential steps that take place in downstream glycolysis prior to entry into the Krebs cycle. In this system, PykA will catalyze the first reaction, producing pyruvate, which acts as the substrate in the following reaction with LDH. QDs were chosen as a model NP scaffold and both PykA and LDH were expressed to display a terminal (His)_6 tag for QD assembly. Before exploring the coupled enzyme system, the effect NP-immobilization has on each individual enzyme was determined. For PykA, the QD scaffold inhibits the catalytic activity at low ratios of enzyme to QD but at higher ratios of >5 PykA per QD, enzyme activity is no longer diminished. The inhibition at lower ratios is attributed to the confirmation of the PykA on the NP surface. At higher ratios, more steric hindrance is present due to the loading of other PykA enzymes and this crowding happens to create a more favorable orientation or environment for PykA attachment. When LDH is assembled on to the QDs, a substantial rate enhancement is observed. For low ratios of LDH to QD (<1), LDH turnover increased ~20 to 60 fold. At higher ratios the enhancement decreased but was always present. This enhancement is attributed to an improvement in enzyme stability and a preservation of the LDH tetrameric structure when displayed on the QD surface. Once the effects of NP-immobilization for each individual enzyme were known, the coupled system was investigated. The colocalization of these two enzymes on the same QD lead to >100 fold improvement in the rate of coupled activity, part of which is attributed to the improvements to LDH activity when displayed on the QD, but the bulk of the improvement is attributed to channeling of the pyruvate intermediary between PykA and LDH. Experimental evidence of the channeling effect was shown by physically disturbing the system through vigorous shaking of the samples, which is meant to disassociate the pyruvate from the QD localized environment, preventing channeling. When shaking was present, the kinetics of the system decreased indicating the disruption of channeling, yet shaking had no effect on the individual QD-enzyme systems or the coupled system without QD present. Extensive kinetic modeling of the system also provided further evidence of pyruvate channeling between PykA and LDH. We are currently planning far more detailed experiments to further understand the nature of the pyruvate channeling mechanism, nevertheless this work highlights the potential of colocalizing enzymatic pathways on NP scaffolds for intermediary channeling.

4 CONCLUSIONS/OUTLOOK

To date, a multitude of distinct NP-enzyme conjugates have exhibited enhanced kinetic activity and improved long term stability [3, 14, 19, 25, 26]. As we continue to investigate the underlying mechanisms from which these NP-induced enhancements arise, the recent implementation of NP-enzyme conjugates into multienzyme systems has validated NP scaffolds as a viable tool that can be used in complex enzymatic cascades. The ability to preserve the NP-derived enhancement of a single enzyme within a coupled enzyme system should allow for the optimization of many cell-free synthetic pathways. Furthermore, we have shown that the colocalization of coupled enzyme systems onto a single NP can promote intermediary channeling. Using NP scaffolds for enzyme enhancement holds great long-term promise for industrial and pharmaceutical synthesis, yet such systems are innately very complicated and it is critical that we continue to probe the mechanisms that produce kinetic enhancements within more complex multienzyme systems.

REFERENCES