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RPPR Final Report
as of 21-Nov-2018

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Proposal Number: 68495CHRIP **Agreement Number: W911NF-16-1-0183**
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Title: Acquisition of an advanced circular dichroism spectrometer to understand structure-function relationships in (bio)catalytic nanostructures
Begin Performance Period: 01-May-2016 **End Performance Period:** 30-Apr-2017
Report Term: 0-Other

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Major Goals: The goal of this DURIP award was to procure a circular dichroism spectrometer with advanced stopped-flow and fluorescence capabilities.

Accomplishments: The full amount of the grant ($134,700) was used for the purchase of a Jacso J-1500 circular dichroism spectrometer with fluorescence scanning and stopped-flow capabilities, and associated supplies (e.g., sample cuvettes). The CD system was delivered in September 2016 with Jasco led instrument installation and training occurring on 09/27/2016. The following equipment is now operational in PI Wheeldon's lab at the University of California, Riverside (UCR):

- Model J-1500 Spectropolarimeter with dual prism monochromator; CD, LD and Abs measurement modes
- Model FMO-522 scanning emission monochromator
- Model PTC-517 single position Peltier temperature control system
- Extended wavelength range: 450 - 1250 nm
- Model F250 Compact recirculating cooler
- Water-cooled Peltier thermostatted stopped-flow accessory
- A series of sample cuvettes ranging from 1 mm to 1 cm path lengths
- On site installation and training

As described in the following section, the equipment is currently used and maintained by the Wheeldon research group at UCR and supports on-going projects funded by the ARO (MURI, W911NF-14-1-026), DTRA (HDTRA1-14-1-0045), and AFOSR (FA9550-17-1-0270).

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Training Opportunities: Students in PI Wheeldon's lab were trained on the new equipment during the installation of the equipment, a J-1500 CD Spectrometer from Jasco Inc.

Results Dissemination: The equipment supported by this grant was used in the publication of two works.

1. Lang X, Zhu L, Gao Y, Wheeldon I*. Enhancing enzyme activity and uptake in nanostructured inorganic-protein enzyme aggregates. Langmuir, 2017 (in press).

2. Gao Y, Or S, Toop A, Wheeldon I*. DNA nanostructure sequence-dependent binding of organophosphates. Langmuir 2017;33(8):2033-2040.

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Protocol Activity Status:

Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: PD/PI
Participant: Ian Wheeldon
Person Months Worked: 1.00
Funding Support:
Project Contribution:
International Collaboration:
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Other Collaborators:

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Article Title: DNA Nanostructure Sequence-Dependent Binding of Organophosphates
Authors: Yingning Gao, Samson Or, Aaron Toop, Ian Wheeldon
Keywords: DNA nanostructures, molecular binding interactions
Abstract: In this work, we use a series of simulation-guided binding assays and spectroscopy techniques to investigate the binding of selected organophosphtates, methyl parathion, paraoxon, their common enzyme hydrolysis product p-nitrophenol, and double stranded DNA fragments and DNA DX tiles, a basic building block of DNA-based materials. Docking simulations suggested that the binding strength of each compound was DNA sequence-dependent. Experimental binding assays confirmed sequence dependent binding and that paraoxon bound to DNA with Kd's between ~10 and 300 u?M, while methyl parathion bound with Kd's between ~10 and 100 u?M. Changes in biding affinity were due to changes in binding mode as revealed by circular dichroism spectroscopy.

Acknowledged Federal Support: **Y**

Final Report:

Acquisition of an advanced circular dichroism spectrometer to understand structure-function relationships in (bio)catalytic nanostructures

Ian Wheeldon
Department of Chemical and Environmental Engineering
University of California, Riverside

1.0 Project Status.

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As described in the following section, the equipment is currently used and maintained by the Wheeldon research group at UCR and supports on-going projects funded by the ARO (MURI, W911NF-14-1-026), DTRA (HDTRA1-14-1-0045), and AFOSR (FA9550-17-1-0270).

2.0 Research Summary.

Since the acquisition of the CD spectrometer, we have published two manuscripts with critical measurements made using the requested equipment. The first manuscript was published in the American Chemical Society's journal *Langmuir* (Gao et al. **2017**, *33* (8), pp 2033–2040).[1] The second manuscript was also published in *Langmuir*, accepted for publication on Aug. 16th 2017.[2] These published works are directly related to the proposed research on understanding the effects of molecular scaffolds on the kinetics of spatially organized multienzyme cascades, understanding ligand binding interactions in DNA-nanomaterials, and engineering multienzyme materials. In addition to the published research, ongoing experiments are focused on understanding the pre-steady state kinetics of multimodal reaction cascades and engineering organic/biocatalytic cascades.

2.1 Summary of Results Towards the Proposed Research.

2.1.1 *Understanding the Mechanisms of Kinetic Enhancement in Nanostructure Biocatalysts*.

We have recently discovery a new effect of enhanced biocatalysis in nanostructured enzyme-DNA complexes.[3-5] The concept is shown in Figure 1: the binding of a substrate to DNA in an enzyme-DNA complex can be used to enhance catalytic efficiency (k_{cat}/K_M) by increasing the local concentration of substrate and consequently decreasing the apparent Michalis constant of the enzyme ($K_{M,app}$). Figure 1C shows the measured binding of a reactant, TMB, to different DNA sequences that are attached to the model enzyme horseradish peroxidase (HRP). Figures 1E and 1F demonstrate the change in kinetics due to the binding of TMB to the HRP-DNA complex. Overall, this work shows that we can engineer a 2.6-fold improvement in HRP efficiency. Importantly, this enhancement is to an enzyme that is already fast (k_{cat} > 50 1/s) and efficient, and that the enhancement is due to engineering outside of the enzyme's active site. As such, we expect that this effect can be translated to any arbitrary enzyme with an appropriate biomacromolecule used for reactant binding. The translation and generalization of this enhancement strategy relies on our ability to understand the mechanism(s) of reactant binding. The advanced CD spectrometer acquired here has enabled the critical experiments for us to do so with an enzyme system for the hydrolysis of organophosphate nerve agents.

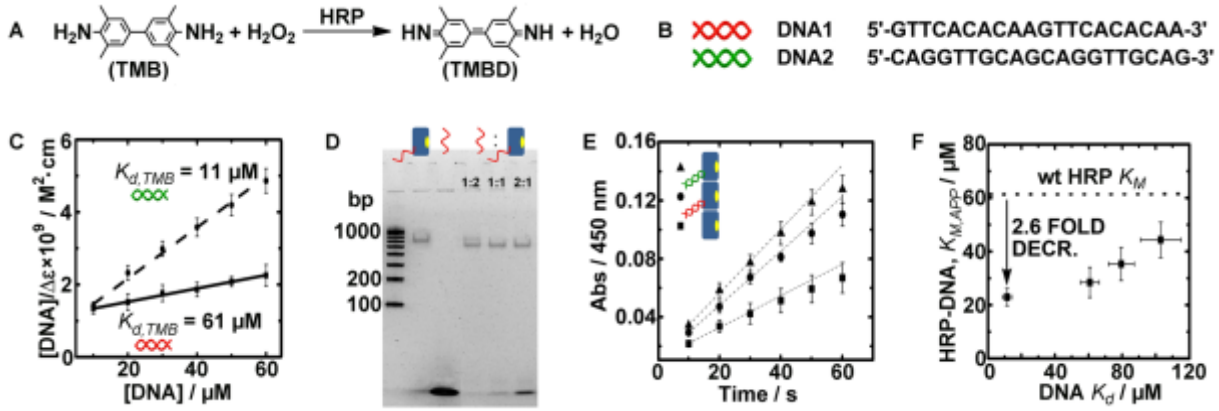

Figure 1. Molecular interactions between substrates and DNA-modified HRP enhance kinetics. (A) HRP reaction with TMB. (B) Two 20 bp DNA sequences used to modify HRP, complementary strands not shown. (C) UV-vis binding assays of TMB to DNA. (D) Electrophoretic mobility shift assay of HRP modified with ssDNA1 and various ratios of a complementary DNA strand. (E) Kinetic of HRP and DNA modified HRP with 50 □M TMB. (F) The observed relationship between $K_{M,APP}$ and K_d of TMB to the DNA conjugated to HRP. The characterization of enzyme-DNA nanostructures shown here are limited to indirect methods of structural characterization (D), cannot identify binding mechanism (C), and are limited to steady-state kinetics. The advanced CD spectrometer requested here will significantly enhance the characterizations shown here to include pre-steady state kinetics, direct evidence of structure, and mechanisms of substrate to DNA binding. Results taken from Gao, Wheeldon et al. [1].

Our recently published manuscript in *Langmuir* describes the DNA-nanostructure sequence-dependent binding of model organophosphate nerve agents.[1] Using a combination of microscale thermophoresis (MST) binding experiments and CD spectroscopy analysis, we discovered that paraoxon and methyl parathion (model organophosphate nerve agents) are major groove binders and intercalators of DNA. MST experiments revealed that these compounds bind

in the micromolar range (~1 mM to ~300 mM) depending on DNA sequence and structure. The CD spectrometer acquired in this award enabled a deeper understanding of the modes of binding. CD spectra of three different 20 base pair DNA sequences (DNA1, -2, and -3) in the presence and absence of methyl parathion, paraoxon, *p*-nitrophenol, and diethyl hydrogen phosphate are shown in Figure 2 (note: *p*-nitrophenol and diethyl hydrogen phosphate are products of the hydrolysis reactions and were therefore studied along with the organophosphate reactants). In the absence of small molecules, the spectra are representative of B-form DNA. The positive CD peak at ~275 nm results from base pair stacking and the negative band at ~245 nm is due to right-handed helicity of the structure.[6] Two minor peaks at ~210 and ~220 nm are also observed. The 210 nm peak arises from β-N-glycosidic linkage between nitrogenous bases and deoxyribose sugars and the 220 nm peak stems from hydrogen bonding between the nitrogenous bases of opposite strands.[7]

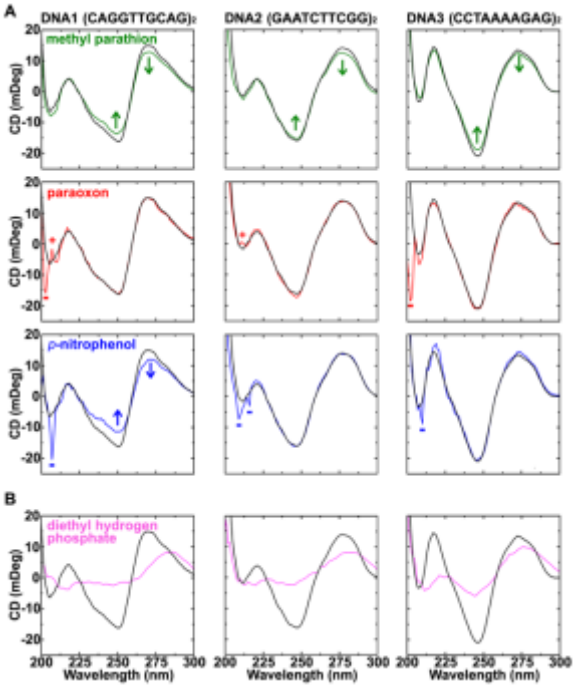

Figure 2. Circular dichroism (CD) analysis of organophosphate-DNA binding. (A) CD spectra of DNA1, -2, and -3 with methyl parathion, paraoxon, and *p*-nitrophenol. (B) CD spectra of DNA1, -2, and -3 with diethyl hydrogen phosphate. Spectra were acquired in 50 mM TRIS-HCl buffered solution, pH 7.4 at room temperature. Spectra in the absence of binding ligand are shown in black, while spectra in the presence of methyl parathion (green), paraoxon (red), p-nitrophenol (blue), and diethyl hydrogen phosphate (pink) are colored. Results published in by Gao, Wheeldon et al. in [1].

Small molecule intercalation in DNA can produce two distinct effects: (i) decreased CD signal at 245 and 275 nm due to weakened base pair π- π stacking and disrupted helical structure;[8-10] and, (ii) induced CD at ~210 nm resulting from specific intercalator-DNA electronic interactions.[11] The first case is observed with methyl parathion, the 245 and 275 nm peaks are reduced when observing DNA1 and -3 in the presence of methyl parathion. With DNA2,

only the 275 nm peak is reduced suggesting weak binding that disrupts base stacking but does not significantly disrupt helicity. In the case of paraoxon, base stacking and helicity are not disturbed, but an induced CD signal appears at ~210 nm. Previous studies have shown that induced CD with intercalation is dependent on the angular orientation of the small molecule relative to the base-pair dyad axis: positive CD is induced when the intercalator is oriented parallel to the dyad axis (see Figure 2a paraoxon with DNA2), while a perpendicular orientation yields a negative induced CD (see Figure 5a paraoxon with DNA1 and -3).[12] Induced CD is also observed with *p*-nitrophenol (DNA1, -2, and-3), and in the case of DNA1 reduced signal at 245 and 275 nm suggests disrupted base pair stacking and helicity.

In the case of the paraoxon PTE hydrolysis product diethyl hydrogen phosphate, MST binding assays and docking predictions suggested very weak binding. CD analysis supports this conclusion, as B-form DNA characteristics are lost in the presence of diethyl hydrogenphosphate (Figure 2b). In all cases the 275 nm peak is red-shifted and CD signal is substantially reduced. In the absence of measurable binding, the observed changes in CD spectra suggest a loss of DNA structure preventing groove binding or intercalation.

The study of organophosphate binding to DNA was critical in translating our novel enzyme engineering strategy to the hydrolysis of organophosphates with the enzyme phosphotriesterase (PTE). As shown in Figure 3, the conjugation of DNA1, -2 and -3 to PTE enhances catalysis in the same manner as with HRP modified with DNA nanostructures (see Figure 1). Paraoxon binds to DNA1 with a K_d of ~60 μM, suppressing the apparent K_M of PTE from 70 μM to 11 μM. Similarly, DNA3 and DNA2 exhibit K_d values of 136 and 287 μM and reduce $K_{M,app}$ to 21 and 38 μM, respectively. These novel results were made possible by the CD analysis of organophosphate binding to DNA and DNA nanostructure.

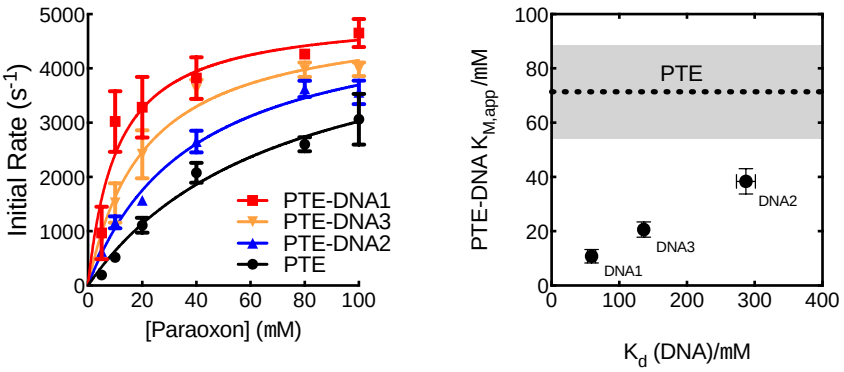

Figure 3. Kinetic analysis of enzyme-DNA nanostructures for enhanced hydrolysis of organophosphate nerve agents. (left) The kinetics of phosphotriesterase (PTE) and PTE-DNA conjugates with paraoxon. The kinetic analysis reveals that the k_{cat} of PTE is unchanged by the conjugation of PTE (~5000 s^{-1} for all constructs), but that the apparent Michaelis constant ($K_{M,app}$) is reduced when double stranded DNA with binding capacity to paraoxon is conjugated to PTE. (right) The relationship between reactant binding strength (K_d) and $K_{M,app}$ for paraoxon and PTE-DNA nanostructures.

2.1.2 *Novel Characterization of Multi-Modal Cascade Catalysts*.

A primary focus of the ARO MURI on Bio-inspired Design of Adaptive Catalysis Cascades (PI Minteer, co-PI Wheeldon) is the development of reaction cascades that control the transport and flux of reaction intermediates along the designed cascade. To this end, we have developed novel nanostructured cascade catalysts comprised of two distinct catalytic modalities. These catalysts combine selective alcohol oxidations by engineered thermostable enzymes and the organic catalyst TEMPO. Designed nanostructures of these two catalysts enable selective alcohol oxidation: TEMPO exhibits high selectivity towards primary alcohols, while the engineered alcohol dehydrogenase from *P. furious* (AdhD) is highly selectively towards secondary alcohols (Figure 4 and refs [13, 14]).

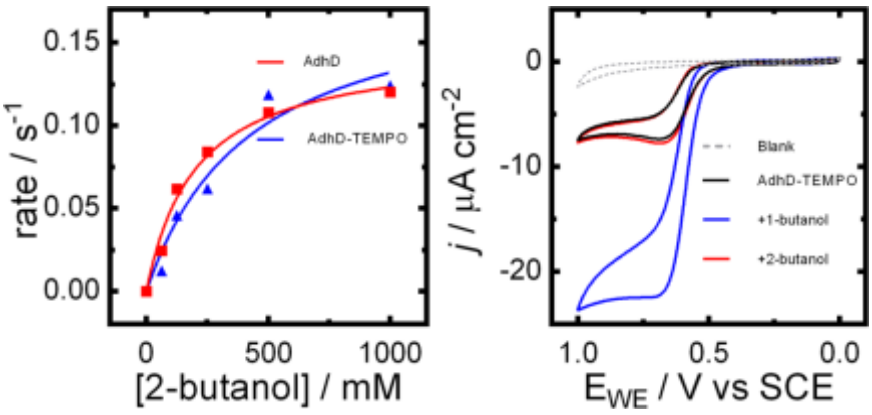

Figure 4. Kinetic analysis of the AdhD-TEMPO conjugate. (left) The kinetics of AhdD in the bifunctional construct towards the secondary alcohol 2-butanol. (right) Electrochemical activity of TEMPO in the bifunctional construct towards 1-butanol. Combined, this data shows that both catalytic modalities (AdhD and TEMPO) are active towards their preferred reactants and that their activities are maintained after the construction of the bifunctional unit.

The chemical conjugation of a glycidal-TEMPO derivative to the AdhD enzyme creates a bifunctional catalyst with the capability of selectively oxidizing both primary and secondary alcohols. We have explored the simultaneous oxidation of 1-butanol and 2-butanol using AdhD-TEMPO conjugates. As shown in Figure 4, the AdhD-TEMPO conjugate is capable of oxidizing primary alcohols (by TEMPO activity) and secondary alcohols (by AdhD activity). Figure 5 compares the kinetics and the AdhD/TEMPO cascade when AdhD and TEMPO are i) mixed together and freely diffusing in solutions, and ii) conjugated into a single bifunctional unit. The kinetic analysis shows that there appears to be no steady-state kinetic advantage when TEMPO is conjugated to AdhD (Figure 5, right).

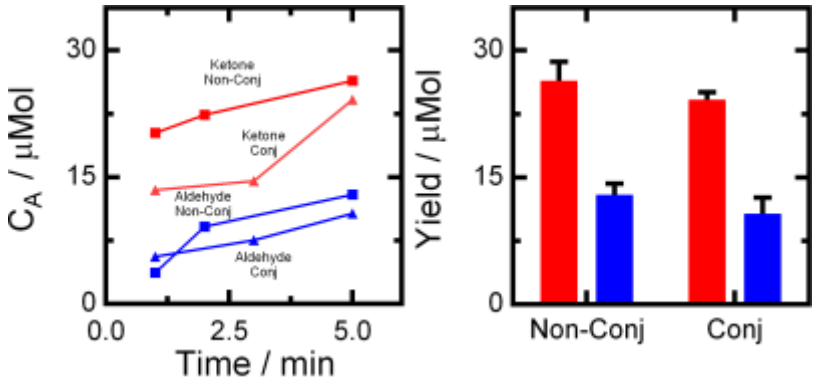

Figure 4. The simultaneous oxidation of 1- and 2- butanol by the bifunctional AdhD-TEMPO and freely diffusing AdhD and TEMPO. (left) Concentrations of butanone (red) and butyraldehyde (blue) produced by the freely diffusing systems (squares) and the bifunctional AdhD-TEMPO (triangles) over a 5-minute reaction. (right) The butanone and butryaldehyde yields after reaction completion. This comparison of freely diffusing and bifunctional systems reveals that there is no difference in steady-state reaction kinetics when TEMPO is conjugated in close proximity to the AdhD active site. Ongoing pre-steady-state kinetics experiments with the acquired stopped-flow system will enable a more detailed understanding of the benefits and limitations of spatially organized multi-catalysts.

In collaboration with Dr. Chia-en Change (Chemistry, UCR), we have developed Brownian Dynamic simulations capable of exploring the molecular effects of enzyme nanostructures on enzyme kinetics.[3] We have continued this collaboration with the study of AdhD-TEMPO catalysis for the successive oxidation of primary and secondary alcohols in diol molecules. Our preliminary simulation results suggest that the conjugation of TEMPO in close proximity to the AdhD active site should create a preference for the reaction cascade of AdhD followed by TEMPO and disfavor the cascade order of TEMPO followed by AdhD. More specifically, when TEMPO is conjugated to His95 (the predominant conjugation site, located ~1.4 nm from the AdhD active site) the probability of a diol intermediate diffusing from the AdhD active site to TEMPO is 40%. Conversely, when a diol first reacts at the TEMPO site, the probability that it will react at the AdhD active site is reduced to ~4%. With the bifunctional AdhD-TEMPO now in hand, we are using the stopped-flow capabilities of the J-1500 CD spectrometer system to understand the pre-steady-state kinetics and experimentally confirm the simulation results. These experiments will enable a more direct comparison to the molecular level simulations and lead to a better understanding of the potential benefits (and limitations) of spatially organized multi-catalytic systems. These ongoing experiments address the primary role of the acquire equipment, that is to enhance the capabilities of the ARO funded MURI on cascade catalysis and better understand the structure, function, and kinetics of multienzyme and multi-catalyst reaction cascades.

2.1.3 *Advanced Characterization of Enzyme-Nanostructures*

A major focus of our research is the development of new technologies for the controlled assembly spatially organized multienzyme systems. To this end, we have explored enzyme-inorganic crystal complexes for the fabrication of multienzyme core-shell particles. The fabrication method and production of particles made from two model proteins are shown in Figure 5. This materials technique allows for the controlled assembly of two-enzyme systems in mixed

and core-shell configurations. Using the model enzyme HRP, we have explored different synthesis conditions to increase enzyme immobilization and activity in the protein-$Cu_3(PO_4)_2$. This work was recently accepted for publication in *Langmuir*.[2]

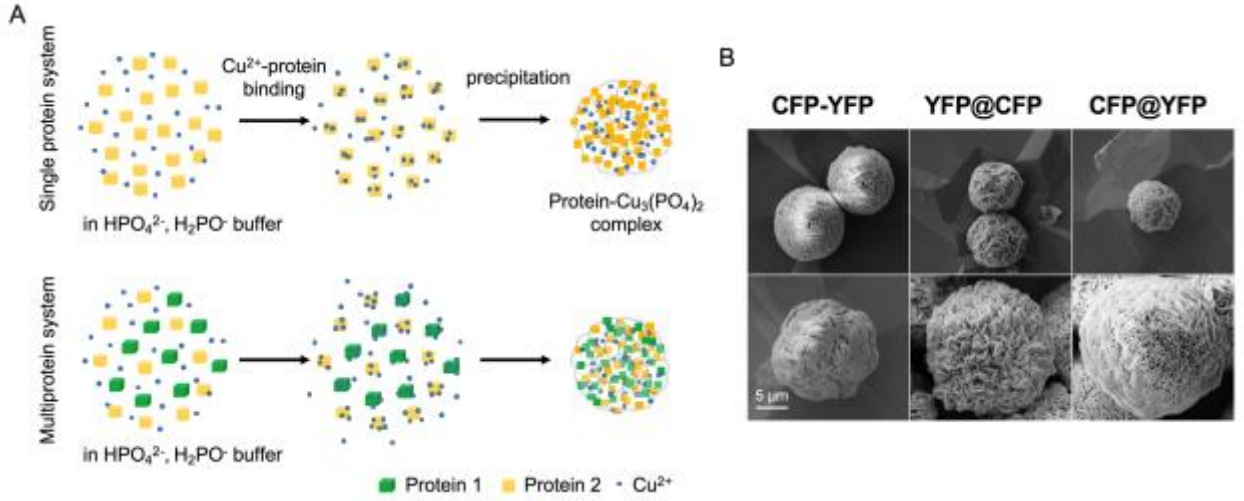

Figure 5. The synthesis of multiprotein-$Cu_3(PO_4)_2$ complexes. (A) Schematic diagrams of the synthesis of single and multiprotein systems. (B) SEM images of CFP/YFP- $Cu_3(PO_4)_2$ complexes, including particles with mixed CFP and YFP, YFP@CFP, and CFP@YFP. The 5 μm scale bar applies to all images.

This work is descried in this report because a critical characterization of the multienzyme material required CD spectroscopy. As shown in Figure 6, enzyme-$Cu_3(PO_4)_2$ particles were characterized by FT-IR and CD spectroscopy. The FT-IR measurements confirm that the enzyme (in this case HRP) is incorporated in the particles, while the CD analysis demonstrates that there is little to no change in enzyme secondary structure. Importantly, the CD analysis provides direct evidence of enzyme incorporation and an indirect method of determining enzyme structure.

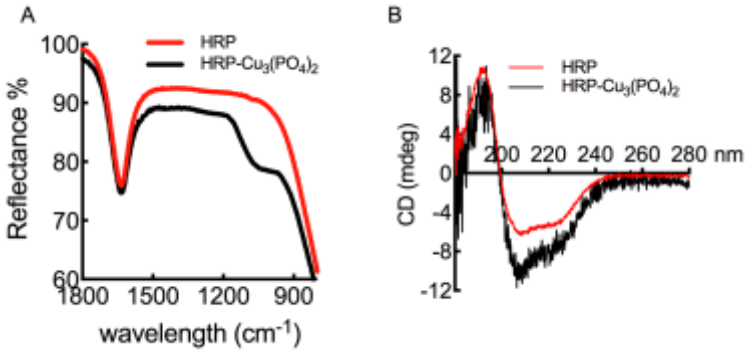

Figure 6. Spectroscopy analysis of HRP-$Cu_3(PO_4)_2$ particles. (A) FT-IR spectra of 37 °C synthesis particles. (B) CD spectra of HRP and HRP-$Cu_3(PO_4)_2$ particles. CD spectral peaks characteristic of a-helical secondary structure (positive CD signal at 190 nm and negative CD signal at 208 and 222 nm) were observed.

As described in our manuscript, the results shown in Figure 6 were further developed to enhanced enzyme loading in enzyme-$Cu_3(PO_4)_2$ particles. These results are not directly related to CD measurements and are therefore not described in this report. We plan to use the enzyme-inorganic particles described here for the assembly of multienzyme systems to explore the effects of spatial organization of cascade reaction efficiency.

2.2 Summary of Other Research of DoD Interest

In addition to the research described in Section 2.1, we are also using the newly acquired equipment to advance other DoD funded projects and research of interest to the DoD. The first example is a collaborative work by Shelley Minteer (Utah), Scott Banta (Columbia University), and the PI of this award Ian Wheeldon (UCR). This work builds on the AdhD-TEMPO system described above in Section 2.1.2, but focuses on using the AdhD protein structure as a molecular scaffold to provide substrate specificity to TEMPO. This work is in the early stages and we plan to use the J-1500 CD spectrometery to characterize potential change in protein structure with the conjugation of TEMPO into the active site of the enzyme. The second example is research that was recently funded by the AFOSR. In this project, PI Wheeldon will use the CD spectrometer to monitor the conformational change of selected transcription factors in response to changes in solution redox conditions and applied electrode potentials. These two projects are ongoing and are expected to generate new data over the next few months.

3.0. Enhancing Graduate and Undergraduate Student Education.

In addition to the research accomplishments described above, the acquisition of the CD spectrometer has also enhanced student education in the Chemical and Environmental Engineering Department at UCR. PI Wheeldon instructs a senior elective course in Biochemical Engineering Principles (CHE 124; 15 – 20 students per year), which teaches the fundamentals of engineering biochemical systems including enzyme reaction kinetics. A laboratory component (CHE 124L) is associated with the lecture course. Beginning in the fall quarter of 2017 (the first offering of the course since the acquisition of the equipment), we will incorporate discussion and experiments related to CD spectroscopy and stopped-flow kinetic measurements.

Of particular interest to the class is pre-steady state kinetics, a non-traditional topic for undergraduates; its inclusion in our lecture discussions will significantly increase student education and training. Traditional steady state kinetics evaluates the steady state turnover of enzyme reactions under defined conditions, including strict limitations on the ratio of enzyme to substrate concentration (i.e., that [Enz] << [Sub]). Stopped flow pumps allow for the evaluation of enzyme kinetics in the millisecond timescale prior to the reaction reaching steady state. In such systems one can monitor the single turnover of enzymes and quantitatively describe the micro-kinetic constants that are not attainable from steady-state analysis. Such experiments and analysis will provide students with a strong connection between classroom theory describing traditional equilibrium constants (typical Michalis-Menten constants) and reaction rate constants, and the hands-on experiments necessary to quantify these values.

With respect to graduate education, PI Wheeldon is developing an MS program focused on Industrial Biotechnology. One course in the program (Upstream Bioprocessing II) is focused on quantitative analysis of bioprocesses, including enzyme and bioreactor kinetics. Data acquired

with the CD spectrometer supported by this award will be used for classroom discussions as well as homework problems focused on pre-steady-state kinetics.

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