



A Self-Assembling Protein Hydrogel Technology for Enzyme Incorporation onto Electrodes in Biofuel Cells (YIP)

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TEXAS ENGINEERING EXPERIMENT STATION COLLEGE STATION

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Final Report

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14. ABSTRACT The proposed research aims to develop a novel protein hydrogel technology for high-density, high-activity enzyme immobilization on electrodes in enzymatic biofuel cells. Enzymatic biofuel cells hold great potential for providing flexible, compact energy to devices such as remote sensors and future miniature unmanned vehicles. However, current enzymatic biofuel cells suffer from low power densities (due to low enzyme concentrations) and short life-spans (due to low enzyme stability in vitro). These limitations derive largely from less-than-ideal techniques for immobilizing enzymes on electrodes. We propose to address these limitations by developing a new, highly stable self-assembling protein hydrogel platform for high-density enzyme immobilization on electrodes. The key features of the proposed protein hydrogel are: (1) conditional assembly of a highly stable protein hydrogel in response to the mixing of two soluble protein block copolymers, each functionalized by one fragment of a split intein, and (2) incorporation of 'docking station peptides' into the self-assembling protein copolymers which serve as anchors for high-affinity immobilization of enzymes end-fused to docking protein tags.					
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A Self-Assembling Protein Hydrogel Technology for Enzyme Incorporation Onto Electrodes in Biofuel Cells

Final Report

Zhilei Chen

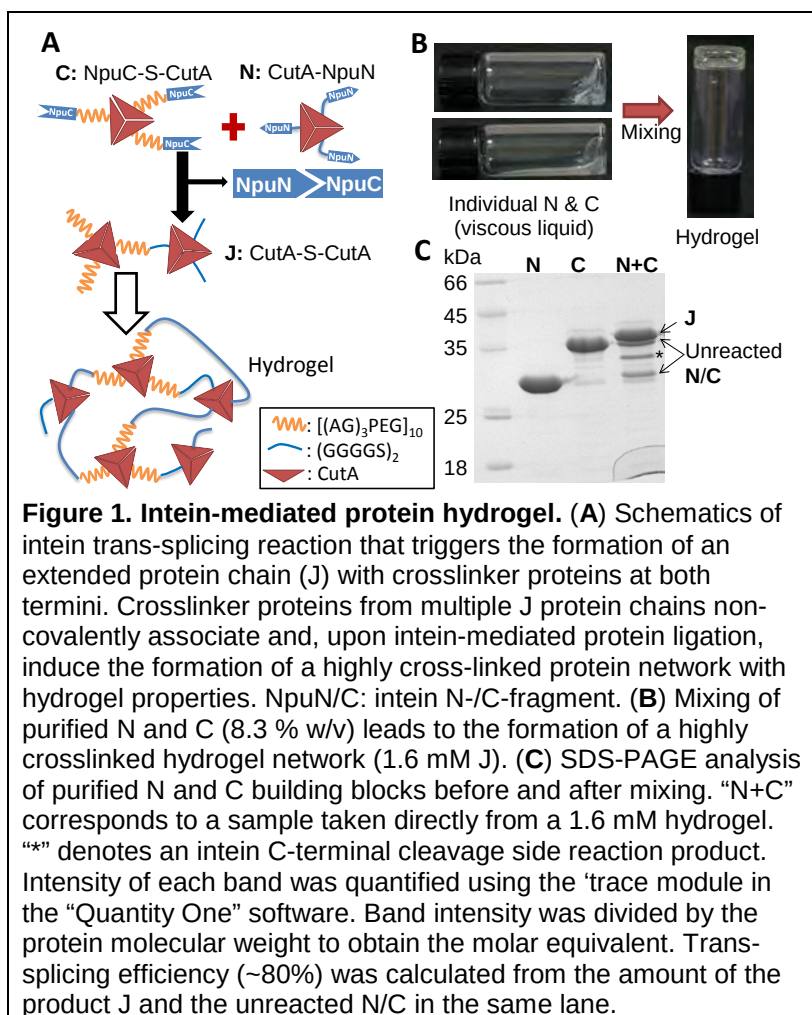
A. Overview: Enzymatic biofuel cells have the potential to provide flexible, compact, and inexpensive energy for low-powered devices such as remote sensors and future miniature unmanned air and land vehicles. However, current enzymatic biofuel cells are severely limited by their low power density (due to a low active enzyme concentration) and short life-span (due to low enzyme stability *in vitro*). These limitations derive largely from less-than-ideal techniques for immobilizing enzymes on electrodes. In this study, we employ an innovative protein hydrogel technology for enzyme immobilization on electrodes. This technology, enables high-density electrode incorporation of multiple enzymes in an ordered 3-dimensional space.

In the first stage, we constructed protein building blocks able to self-assemble into 3D protein hydrogel upon mixing. We first constructed a protein hydrogel triggered by intein-mediated protein *trans*-splicing reaction¹. However, the intein reaction requires reducing conditions which may compromise the activity of certain enzymes. To bypass the use of reducing agents for hydrogel assembly, we next replaced the intein with a PDZ-domain-containing protein-ligand pair whose interaction was reinforced by an engineered disulfide linkage². Both hydrogels exhibit very high solution stability and are stable over a wide range of pHs and temperature. Finally, a “docking station” peptide was incorporated into one of the hydrogel building blocks which allows simple and stable immobilization of “docking protein”-tagged target enzyme into the hydrogel. Physically entrapping conductive carbon nanotube and immobilizing a laccase enzyme into the hydrogel matrix efficiently converted the hydrogel into a biocathode that was able to provide stable current for over one week.

In the second stage, we further enhanced the enzymatic biofuel cell current density by controlled orientation of the small laccase enzyme onto the conductive carbon nanotubes³. The overall current density is relatively low in our engineered enzyme-loaded protein hydrogel, indicating that a vast majority of the immobilized enzymes cannot participate in current generation. The electron harvesting efficiency of a direct electron transfer (DET) electrode is exponentially inversely related to the distance between the enzyme's catalytic center and the electrode surface. Due to the relatively large size, the ability of an enzyme to transfer electrons to the electrode is enzyme orientation dependent and only a small percentage of the immobilized enzymes are electrically wired. To address this problem, we developed a click chemistry approach in which we functionalized the carbon nanotube electrode with cyclooctyne and incorporated the unnatural amino acid 4-azido-L-phenylalanine (AzF) into a selected site in the enzyme. Click chemistry reaction between cyclooctyne and AzF enables the directional enzyme immobilization. The resulting click enzyme electrode exhibited very high enzyme wiring efficiency and current stability.

In the third stage, we aimed to further enhance the current density of our enzymatic biofuel cell electrode. Based on what we learned in the first stage, a protein hydrogel forms upon the formation of an inter-connected protein network. In our previous protein hydrogel, the protein network was formed between two different hydrogel building block proteins. Thus, a maximum of 33% molar amount of the target enzymes can be incorporated into a protein hydrogel, significantly limiting the amount of total enzymes that can be incorporated onto the electrode. Taking advantage of the highly efficient and catalyst-free copper-free click chemistry reaction, we constructed an enzyme-only protein hydrogel electrode. The enzymes were individually functionalized with dibenzylcyclooctyne (DBCO) and azide (N_3) to form enzyme-DBCO and enzyme- N_3 , respectively, and then mixed to form a click protein hydrogel. We showed that DBCO-/ N_3 -functionalization only had minimum effect on the enzyme activity and the successful formation of a click protein hydrogel. Manuscript describing this work is in preparation.

Overall, we developed novel highly stable and flexible protein hydrogels, and applied these protein hydrogels in the synthesis of high efficient enzymatic biofuel cell. We published 3 peer-reviewed journal articles in high impact journals with another one in preparation. Knowledge gained from this study should have a broader impact in the enzymatic biofuel cell field and the electrochemical research community in general. Furthermore, the protein hydrogel developed in this project exhibit high tissue compatibility and modularity, and is currently being explored for tissue engineering application in my lab.



Publications derived from this project:

1. Ramirez, M., Guan, D., Ugaz, V. & Chen, Z. Intein-triggered artificial protein hydrogels that support the immobilization of bioactive proteins. *J Am Chem Soc* **135**, 5290-5293 (2013).
2. Guan, D., Ramirez, M., Shao, L., Jacobsen, D., Barrera, I., Lutkenhaus, J. & Chen, Z. Two-component protein hydrogels assembled using an engineered disulfide-forming protein-ligand pair. *Biomacromolecules* **14**, 2909-2916 (2013).
3. Guan, D., Kurra, Y., Liu, W. & Chen, Z. A click chemistry approach to site-specific immobilization of a small laccase enables efficient direct electron transfer in a biocathode. *Chem Commun (Camb)* **51**, 2522-2525 (2015).

B. Detailed Research Activities

B1. Two-component self-assembling protein hydrogels

Proteins are nature's building block and indispensable in living organism. Protein-based hydrogels have a wide variety of applications in research and in biotechnology thanks to the intrinsic properties of proteins. We first developed an intein-triggered protein hydrogel that utilizes two synthetic soluble

protein block copolymers,

CutA-NpuN and NpuC-S-CutA, each containing a subunit of a trimeric protein (CutA) that serves as a crosslinker and one half of the naturally split DnaE intein from *Nostoc punctiform* (NpuN, NpuC) (**Fig. 1**). CutA is a small trimeric protein (12 kDa) from thermophilic bacteria *pyrococcus horihoshii*^{4, 5} that exhibits ultrahigh stability (able to retain its trimeric quaternary structure in solutions after boiling in SDS buffer). We reasoned that its very strong intersubunit interactions should discourage subunit exchange between different crosslinkers and enhance hydrogel stability⁶. The NpuN and NpuC intein pair was selected for its extraordinarily quick reaction kinetics ($t_{1/2}=63$ s) and very high trans-splicing yield (75-85%)^{7, 8}. The S fragment, $[(AG)_3PEG]_{10}$ ^{9, 10} is a flexible polyanionic linker and was incorporated as the midblock for water retention. Mixing of the two protein block copolymers initiates an intein *trans*-splicing reaction that reconstitutes a self-assembling polypeptide flanked by crosslinkers, triggering protein hydrogel formation. The resulting hydrogels are very stable under both acidic and basic conditions, and at temperatures up to 50 °C.

However, intein reaction requires reducing condition, which is not compatible with many enzymes, especially laccases that harbor metal ions in their active centers. To circumvent this problem, we

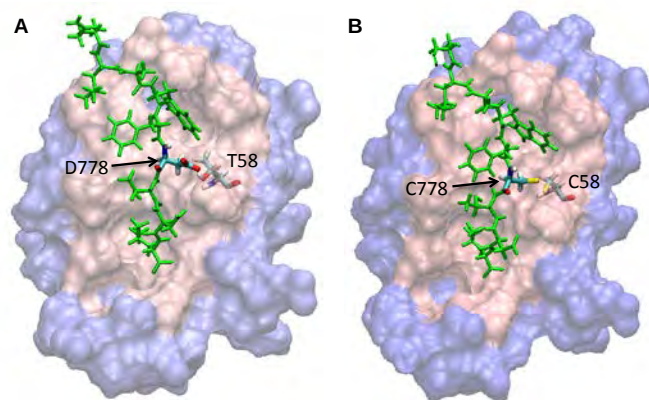
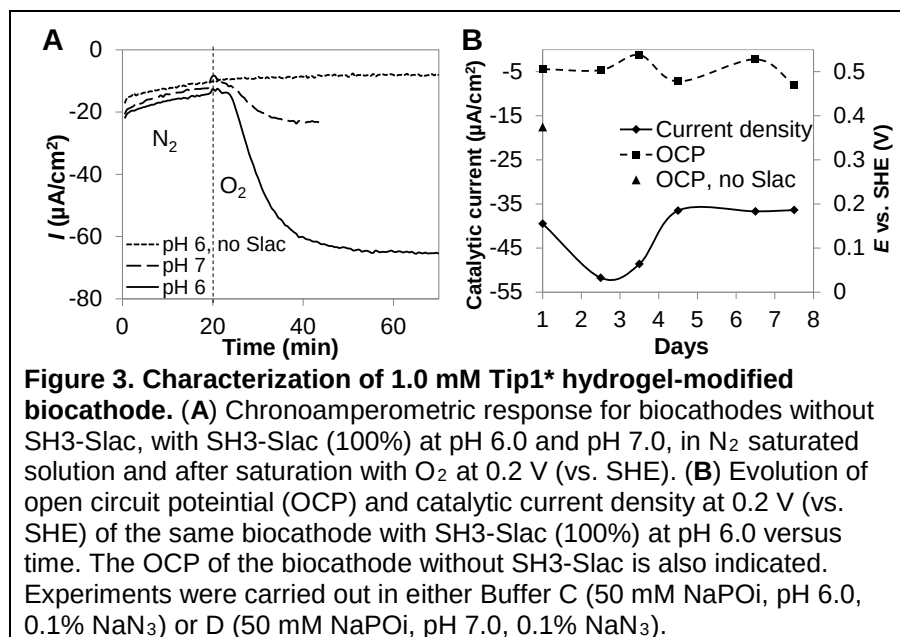


Figure 2. Crystal structure of Tip1-Tip1_{lig} (PDB code: 3IDW). (A) Representation of wild-type Tip1 – Tip1_{lig} interaction. Tip1 is shown in purple surface mode. Residues in Tip1 that are within 5 Å of Tip1_{lig} (green) are shown in pink color. Tip1_{lig} is shown in green licorice mode. Residues T58 and D778 are shown in element colors. (B) Representation of dsTip1 and dsTip1_{lig} interaction containing the substitutions T58C and D778C.

synthesized a new self-assembling protein hydrogel based on the bioaffinity of a pair of engineered proteins, Tip1 and Tip1_{lig}. Tip 1 is the tax-interacting protein-1 that binds to its peptide ligand (Tip1_{lig}, QLAWFDSDL)¹¹. We synthesized two hydrogel building blocks, CutA-Tip1 and CutA-Tip1_{lig}, and mixing of these two building blocks result in the near instantaneous formation of a protein hydrogel. However, this new hydrogel suffered from poor solution stability that was attributed to the weak affinity between the wild-type Tip1 and Tip1_{lig} ($K_d \sim 0.19 \mu\text{M}$)¹¹, making the material prone to intermolecular domain swapping⁶. We subsequently applied rational design and introduced a pair of Cys residues into Tip1 and Tip1_{lig} based on the crystal structure to form dsTip1 and dsTip1_{lig}, which form a disulfide bond with each other (**Fig. 2**). We replaced the wild-type Tip1 and Tip1_{lig} in the hydrogel building block with dsTip1 and dsTip1_{lig} to form CutA-dsTip1 and CutA-dsTip1_{lig}. This new hydrogel exhibited high solution stability, similar to that of the intein hydrogel.



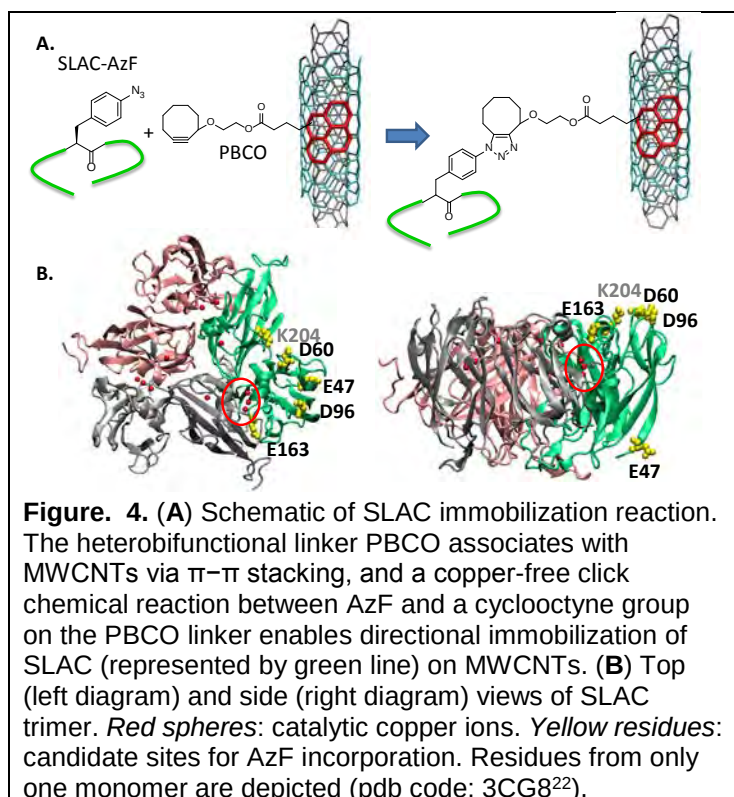
Next, we demonstrated the application of dsTip1 hydrogel in a biocathode. A small laccase from *Streptomyces coelicolor* (Slac)¹² was immobilized in the hydrogel, together with carboxylated multiwall carbon nanotube (cMWNT). Incorporation of Slac and cMWNT did not compromise the integrity of the hydrogel or increase the leaching rate of the immobilized Slac. The Slac-containing hydrogel was loaded onto a pyrolytic graphite electrode and the chronoamperometric response was monitored at 25 °C. At pH 6.0, a cathodic current maximized at -65.5 $\mu\text{A}/\text{cm}^2$ was generated in the presence of O₂ sparging (**Fig. 3**). Hydrogel lacking SH3-Slac did not generate any current. These results demonstrate that enzyme-

functionalized dsTip1 hydrogel has the potential to be used for current generate in biocatalytic biofuel cells.

B2. Site-specific immobilization of a small laccase in a biocathode

In our study, the Slac enzyme directly transfers electrons generated from catalysis to the carbon nanotube to be harvested by the electrode. The electron harvesting efficiency of direct electron transfer (DET) electrodes depends primarily on the distance between the enzyme's catalytic center and the electrode surface^{13, 14}. Due to the large size of the enzyme, only those with active site correctly oriented on the electrode can transfer electrons to the electrode. Numerous approaches have been employed to enhance DET between the enzyme and the electrode¹⁵⁻¹⁸. One commonly pursued strategy is to surround the enzymes in a highly conductive matrix (e.g. a high-surface-area nanomaterial)^{17, 19-21}, similar to that used in our dsTip1-Slac hydrogel electrode.

To further increase the electron-transfer efficiency between the enzyme and the electrode, we sought to develop a facile strategy for the site-specific directional immobilization of enzymes on DET electrodes. An unnatural amino acid 4-azido-L-phenylalanine (AzF) was incorporated into specific



sites of Slac^{2, 12, 23}. A heterobifunctional crosslinker, cyclooctynyloxyethyl 1-pyrenebutyrate (PBCO), was synthesized and used to functionalize MWCNTs on buckypaper via π - π stacking. A copper-free cyclooctyne-azide cycloaddition reaction²⁴⁻²⁶ between AzF on the enzyme and cyclooctyne on the MWNT enabled directional immobilization of SLAC on the buckypaper.

A high electron transfer efficiency of 28.7% was obtained for E47AzF SH3-SLAC on the PBCO-modified electrode. It is noteworthy that SLAC is a trimeric protein with diameter < 8 nm²² and the average distance between different MWNT in buckypaper is ~100 nm. Thus, it is likely that only one of the monomers of the trimeric protein can be correctly oriented onto the electrode via the PBCO linker. Under this single monomer tethering assumption, the effective maximum electron transfer efficiency for SH3-SLAC is only 33.3%. Thus, the 28.7% transfer efficiency achieved by E47AzF SH3-SLAC on the PBCO-modified electrode suggests that a large percentage of the actively immobilized E47AzF SH3-SLAC may accept electrons from the electrode unhindered. The second highest electron transfer efficiency in our study, 7.5%, was obtained for D60AzF SH3-SLAC on the PBCO-modified electrode. In this case, too, the PBCO linker enabled superior (~4-fold enhanced) electron transfer relative to control electrode modified with 1-pyrenebutanoic acid succinimidyl ester (PBSE). The choice of PBCO vs. PBSE linker did not significantly impact the electron transfer capability of the other two mutants or WT SH3-SLAC.

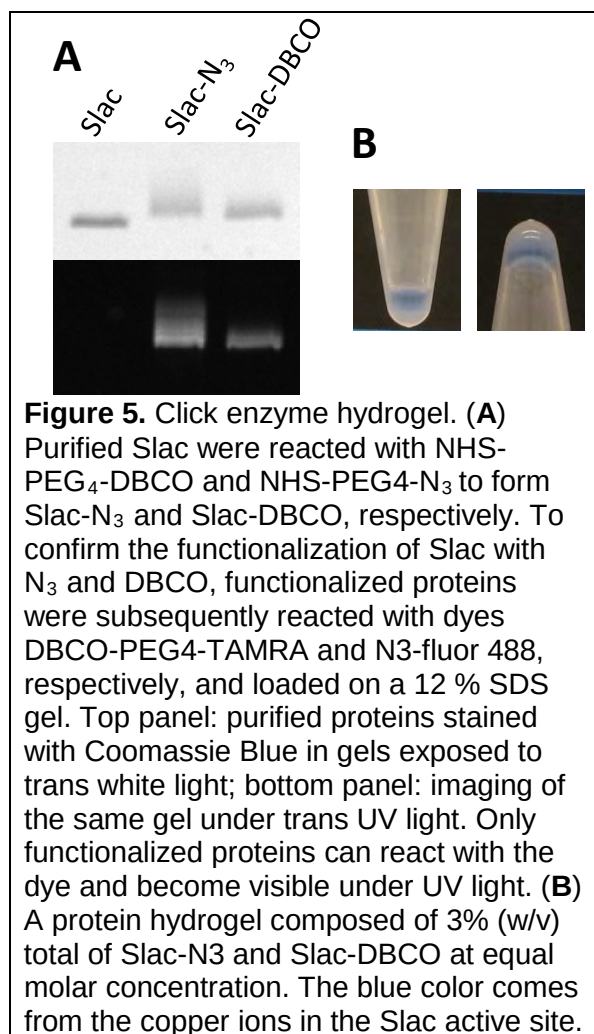


Figure 5. Click enzyme hydrogel. **(A)** Purified Slac were reacted with NHS-PEG₄-DBCO and NHS-PEG₄-N₃ to form Slac-N₃ and Slac-DBCO, respectively. To confirm the functionalization of Slac with N₃ and DBCO, functionalized proteins were subsequently reacted with dyes DBCO-PEG₄-TAMRA and N₃-fluor 488, respectively, and loaded on a 12 % SDS gel. Top panel: purified proteins stained with Coomassie Blue in gels exposed to trans white light; bottom panel: imaging of the same gel under trans UV light. Only functionalized proteins can react with the dye and become visible under UV light. **(B)** A protein hydrogel composed of 3% (w/v) total of Slac-N₃ and Slac-DBCO at equal molar concentration. The blue color comes from the copper ions in the Slac active site.

B3. Two-component click enzyme hydrogel.

To further increase the current density generated by enzymes in a protein hydrogel, we sought to engineer an enzyme-only protein hydrogel, using enzyme itself as the hydrogel crosslinker, thus eliminating the need for additional proteins as the hydrogel building blocks. To achieve this, Slac enzymes were separately functionalized with dibenzylcyclooctyne (DBCO) and azide (N₃) to form Slac-DBCO and Slac-N₃ via reaction with NHS-PEG₄-DBCO and NHS-PEG₄-N₃, respectively. The reaction conditions were optimized so each enzyme is modified with 1-2 functional group, resulting in 3-6 functional groups on each Slac trimer (**Fig. 5A**). DBCO- and N₃-modified Slac retained the same catalytic activity as the unmodified Slac. The resulting DBCO-/N₃-functionalized Slac trimers were mixed at 1:1 molar ratio in PBS and incubated at 37 °C, overnight, resulting in the formation of a blue-Slac hydrogel (**Fig. 5B**). Ongoing experiments are to determine the stability and current density generated by the Slac hydrogel, and compare it to that generated by Slac immobilized in the dsTip1-hydrogel.

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Abstract

Enzymatic biofuel cells have the potential to provide flexible, compact, and inexpensive energy for low-powered devices such as remove sensors and future miniature unmanned air and land vehicles. However, current enzymatic biofuel cells are severely limited by their low power density (due to a low active enzyme concentration) and short life-span (due to low enzyme stability in vitro). These limitations derive largely from less-than-ideal techniques for immobilizing enzymes on electrodes. In this study, we employ an innovative protein hydrogel technology for enzyme immobilization on electrodes. This technology, enables high-density electrode incorporation of multiple enzymes in an ordered 3-dimentional space.

In the first stage, we constructed protein building blocks able to self-assemble into 3D protein hydrogel upon mixing. We first constructed a protein hydrogel triggered by intein-mediated protein trans-splicing reaction. However, the intein reaction requires reducing conditions which may compromise the activity of certain enzymes. To bypass the use of reducing agents for hydrogel assembly, we next replaced the intein with a PDZ-domain-containing protein-ligand pair whose interaction was reinforced by an engineered disulfide linkage. Both hydrogels exhibit very high solution stability and are stable over a wide range of pHs and temperature. Finally, a "docking station" peptide was incorporated into one of the hydrogel building

blocks which allows simple and stable immobilization of “docking protein”-tagged target enzyme into the hydrogel. Physically entrapping conductive carbon nanotube and immobilizing a laccase enzyme into the hydrogel matrix efficiently converted the hydrogel into a biocathode that was able to provide stable current for over one week.

In the second stage, we further enhanced the enzymatic biofuel cell current density by controlled orientation of the small laccase enzyme onto the conductive carbon nanotubes. The overall current density is relatively low in our engineered enzyme-loaded protein hydrogel, indicating that a vast majority of the immobilized enzymes cannot participate in current generation. The electron harvesting efficiency of a direct electron transfer (DET) electrode is exponentially inversely related to the distance between the enzyme's catalytic center and the electrode surface. Due to the relatively large size, the ability of an enzyme to transfer electrons to the electrode is enzyme orientation dependent and only a small percentage of the immobilized enzymes are electrically wired. To address this problem, we developed a click chemistry approach in which we functionalized the carbon nanotube electrode with cyclooctyne and incorporated the unnatural amino acid 4-azido-L-phenylalanine (AzF) into a selected site in the enzyme. Click chemistry reaction between cyclooctyne and AzF enables the directional enzyme immobilization. The resulting click enzyme electrode exhibited very high enzyme wiring efficiency and current stability.

In the third stage, we aimed to further enhance the current density of our enzymatic biofuel cell electrode. Based on what we learned in the first stage, a protein hydrogel forms upon the formation of an interconnected protein network. In our previous protein hydrogel, the protein network was formed between two different hydrogel building block proteins. Thus, a maximum of 33% molar amount of the target enzymes can be incorporated into a protein hydrogel, significantly limiting the amount of total enzymes that can be incorporated onto the electrode. Taking advantage of the highly efficient and catalyst-free copper-free click chemistry reaction, we constructed an enzyme-only protein hydrogel electrode. The enzymes were individually functionalized with dibenzylcyclooctyne (DBCO) and azide (N3) to form enzyme-DBCO and enzyme-N3, respectively, and then mixed to form a click protein hydrogel. We showed that DBCO/N3-functionalization only had minimum effect on the enzyme activity and the successful formation of a click protein hydrogel. Manuscript describing this work is in preparation.

Overall, we developed novel highly stable and flexible protein hydrogels, and applied these protein hydrogels in the synthesis of high efficient enzymatic biofuel cell. We published 3 peer-reviewed journal articles in high impact journals with another one in preparation. Knowledge gained from this study should have a broader impact in the enzymatic biofuel cell field and the electrochemical research community in general. Furthermore, the protein hydrogel developed in this project exhibit high tissue compatibility and modularity, and is currently being explored for tissue engineering application in my lab.

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Archival Publications (published) during reporting period:

1. Ramirez, M., Guan, D., Ugaz, V. & Chen, Z. Intein-triggered artificial protein hydrogels that support the immobilization of bioactive proteins. J Am Chem Soc 135, 5290-5293 (2013).
2. Guan, D., Ramirez, M., Shao, L., Jacobsen, D., Barrera, I., Lutkenhaus, J. & Chen, Z. Two-component protein hydrogels assembled using an engineered disulfide-forming protein-ligand pair. Biomacromolecules 14, 2909-2916 (2013).
3. Guan, D., Kurra, Y., Liu, W. & Chen, Z. A click chemistry approach to site-specific immobilization of a small laccase enables efficient direct electron transfer in a biocathode. Chem Commun (Camb) 51, 2522-2525 (2015).

Changes in research objectives (if any):

Change in AFOSR Program Manager, if any:

Extensions granted or milestones slipped, if any:

AFOSR LRIR Number

LRIR Title

Reporting Period

Laboratory Task Manager

Program Officer

Research Objectives

Technical Summary

Funding Summary by Cost Category (by FY, \$K)

	Starting FY	FY+1	FY+2
Salary			
Equipment/Facilities			
Supplies			
Total			

Report Document

Report Document - Text Analysis

Report Document - Text Analysis

Appendix Documents

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