II.E.1 Development of Water Splitting Catalysts Using a Novel Molecular Evolution Approach

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Objectives

Broad Objectives:

- Develop a novel approach to creating molecular metal-binding peptide catalysts for redox reactions based on high throughput synthesis on an array of electrodes.
- Mimick nature's approach to water splitting.
- Reduce the overpotential by 30%.

Specific Objectives (Fiscal Year 2009):

- Optimize high throughput (>10^4) peptide synthesis of potential metal-binding peptide catalysts of the water splitting reaction directly on CombiMatrix arrays.
- Optimize the multielectrode measurements of water splitting on the CombiMatrix arrays and quantify the baseline catalysis rate of the system.
- Demonstrate several rounds of optimization for catalytic activity.

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Generation By Water Electrolysis section (section 3.1.4.2.2) of the Hydrogen, Fuel Cells and Infrastructure Technologies Program Multi-Year Research, Development and Demonstration Plan:

(G) Capital Costs

(H) System Efficiency

Go/No-Go decision to continue pursuing this approach depends on:

- Ability to measure catalytic signal above noise, and
- Ability to reproducibly synthesize arrays with multiple variable residues.

Technical Targets

Improved Catalytic Performance for Electrolysis:

This project has the goal of generating new, more efficient and more cost-effective catalysts for hydrogen production via electrolysis. Specifically, this work will be applied to reduce the cost of hydrogen to <$3.00/gge (delivered) at the pump by 2017.

Accomplishments for FY 2009

- Multi-step patterned synthesis of peptides in an array.
- Verification of synthesis via direct matrix-assisted laser desorption/ionization (MALDI) spectroscopy on the surface.
- Automation of array synthesis.
- Background current measurements on the arrays.
- Comparing currents from peptides with and without Mn on the arrays.

Introduction

Direct conversion of water to molecular hydrogen and oxygen via electrolysis followed by regeneration of electrical power in a hydrogen fuel cell would be, in principle, an ideal mechanism for the generation and utilization of hydrogen. However, a number of problems still remain to be solved. One of these stems from the fact that the conversion of water to hydrogen via electrolysis using conventional metal electrodes involves a substantial activation energy, necessitating that the reaction be driven by a considerably higher potential than simple thermodynamics would demand. This

overpotential represents a significant energy loss during conversion, impacting the economic practicality of using hydrogen as a fuel in this way.

The biggest part of this overpotential comes from the water splitting reaction at the oxygen evolving electrode (the anode). This is because of the multi-electron nature of the reaction and the high energy, partially-oxidized intermediates that must be formed in order to generate molecular oxygen and protons from water. Fortunately, nature has developed a catalyst, the oxygen evolving complex (OEC) of photosystem II (a complex found in the photosynthetic apparatus of plants), that works with almost no overpotential for this reaction. The OEC contains four manganese atoms that have a structure and chemical environment defined by the surrounding protein. The manganese cluster is directly involved in the redox process and stabilizes the highly reactive intermediates in the oxidation of water. In recent years, a considerable amount has been learned about the characteristics of this complex, including both the redox properties of the manganese atoms at various stages during the four-electron-oxidation of water and the structure of the surrounding protein at moderate resolution.

Approach

Here we propose to use a novel combinatorial biochemical approach to develop manganese binding peptides for modification of the surface of the electrolysis anode used during hydrogen production. The design of these peptides will include features of the OEC and of a model system, developed at ASU, in which bacterial reaction centers lacking the OEC have been modified to bind and oxidize manganese. The approach involves the light-mediated production of large libraries of manganese-binding peptides using a process similar to that employed in the photolithographic generation of deoxyribonucleic acid chips (for example by Affymetrix or CombiMatrix). Each member of the library will be attached to a different microelectrode on a fabricated surface. The current/voltage characteristics of each electrode will be measured in series, looking for the peptide/Mn complexes that result in the lowest overpotential for water splitting. These peptide sequences will then be used as the initial guesses for a subsequent round of molecular evolution, etc. Note that once the peptide-based catalysts are developed in this way, the same types of combinatorial approaches can be used to introduce nonnatural chemical features into the peptides, increasing their resistance to degradation by naturally occurring enzymes and other chemical processes. In principle, similar techniques could also be used to develop catalysts for the hydrogen-evolving cathode (e.g., using hydrogenase as a model) or for the electrodes in hydrogen fuel cells.

Results

Multi-Step Patterned Synthesis of Peptides in an Array

Protection of Terminal Amines – As we seek to synthesize peptides directly on Combimatrix electrode arrays, we have hypothesized that, by protecting α-amines with groups that require milder deprotection conditions (such as dimethoxytrityl and trityl) and protecting side chains with groups that require more stringent removal conditions (like Boc and t-butyl), the side-chain protecting groups will remain stable as we add amino acids to the α-amines during the elongation process. We focused on trityl and dimethoxytrityl protecting groups since they are among the most acid-labile protecting groups available for general organic synthesis. They require approximately 1-3% trifluoromethanesulfonic acid (TFA) for their removal compared to the tert-butoxycarbonyl (Boc) and t-butyl protecting groups, which require 50% and 95% TFA, respectively. We evaluated the stability of trityl and dimethoxytrityl protecting groups in two different electrochemical reagents, 1,2-diphenylhydrazine and hydroquinone. It was found that the trityl groups remained stable in 1,2-diphenylhydrazine solution whereas about 20% were deprotected when exposed to hydroquinone solution. The dimethoxytrityl groups underwent nearly 80% deprotection when exposed to both the 1,2-diphenylhydrazine and hydroquinone solutions without any voltage application.

Based on the stability results of these protecting groups in the two different electrochemical reagents, we decided to move ahead with trityl protection for α-amino groups during manual peptide synthesis and proceeded to deprotect in 1,2-diphenylhydrazine by generation of local acidic conditions due to anodic oxidation. We were able to synthesize peptides involving three electrochemical steps with 40-45% overall yield under these conditions. Four peptides were synthesized in four different regions of a Combimatrix chip. The peptides included control peptides involving no electrochemical steps as well as peptides involving one, two and three electrochemical steps. MALDI spectra showed major peaks corresponding to each of the expected peptides from their respective regions as well as lesser peaks corresponding to peptides synthesized in other regions of the chip (Figure 1). These contaminating peaks may be the result of protons, generated electrochemically from 1,2-diphenylhydrazine, diffusing and deprotecting the trityl groups from amines on silent electrodes. Alternatively, they may be the result of trityl deprotection caused by residual voltage/current in regions where no voltage was applied during each electrochemical step. Even though we obtained unwanted peptides, the ability to synthesize a peptide with three electrochemical steps

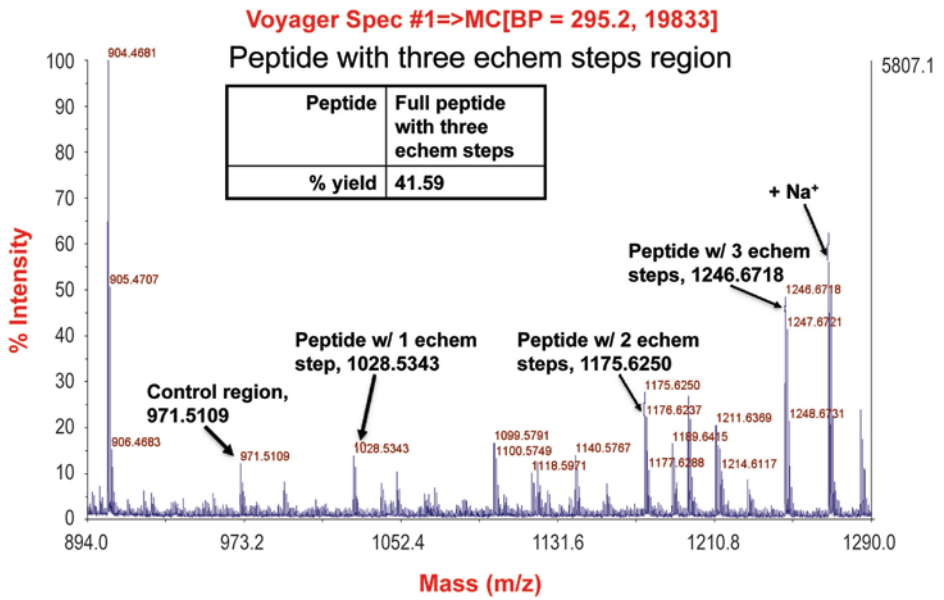

FIGURE 1. Region of the chip where a peptide involving three electrochemical deprotection steps was synthesized. Unlabeled peaks represent peptides containing deletions of one amino acid. This experiment does not involve capping after each coupling step; possibly causing some false high yield. The capping step was incorporated after each coupling step in subsequent experiments.

with an approximate overall yield of 41.5% was a major step forward in peptide library synthesis.

The experiment described above proved very difficult to repeat. This led us to move away from manual synthesis and to focus on automating the process in order to create a more reproducible synthesis system. Automation will be described later.

Verification of Synthesis via Direct MALDI Spectroscopy on the Surface

In 2008, we spent considerable effort developing and modifying an approach for performing matrix-assisted laser desorption ionization mass spectroscopy directly from electrode surfaces so that we could determine exactly what peptides had been synthesized or attached to those surfaces. This has been an essential tool in moving forward. Briefly, when we want to perform chemical product analysis on the surface, peptides are coupled or synthesized directly on top of photolabile linkers, previously coupled to the electrodes. After peptides are synthesized or coupled to this surface, the photolabile linkers are cleaved using ultraviolet (UV) light under dry conditions (to avoid diffusion), an appropriate matrix is added to the region of interest and MALDI mass spectroscopy is performed directly from the surface.

Recently, we have tried a different approach, coating a chip containing peptides synthesized on its surface with the α-cyano matrix for MALDI mass spectrometry before exposing it to UV irradiation to cleave the photolabile linker. The concept was that when cleaved, the peptides should already be co-crystallized with the α-cyano matrix and would be able to be directly analyzed by the MALDI instrument, circumventing the problem of diffusing peptides when matrix is spotted after the UV cleavage of the photolabile linker. Our initial attempts have not resulted in significant desorption of peptide from the surface. We plan to try altering the matrix concentration and composition systematically to see if that will improve the desorption yield.

Automation of Array Synthesis

As mentioned above, there were problems with the reproducibility of manual synthesis. For this reason and for the ability to perform longer syntheses, we automated the hardware/software interface between the Pioneer peptide synthesizer and the Combimatrix Potentiosense electrosynthesis chamber. This has allowed us to automate all of the electrochemical steps and eliminate manual initiation of the Echem software to apply the voltages. Once we completed preliminary experiments to confirm the successful integration of the instruments, we attempted to repeat the synthesis involving three electrochemical steps in fully-automated mode. In initial trials, we synthesized a full peptide with three electrochemical steps, though the yield was low at approximately 10% (Table 1). One of the reasons for the low yield of the full peptide was likely the inefficient coupling of the bulky trityl glycine groups used in the synthesis. The MALDI spectra revealed peaks capped with N-acetyl glycine corresponding to each peptide involving electrochemical steps. Experiments to

TABLE 1. Percent yield summary of a region of the chip where a peptide involving three electrochemical deprotection steps was synthesized in automated mode. MALDI not shown but contains unlabeled peaks corresponding to peptides capped with N-acetyl glycine due to inefficient coupling of incoming trityl glycine.

	% Yield
Control Peptide	36.08
Peptide with 1 Echem Step	35.18
Peptide with 2 Echem Steps	19.30
Peptide with 3 Echem Steps	9.40

optimize the overall yield by introducing the trityl group in situ on the α-amines during the synthesis are currently underway.

Comparing Currents from Peptides With and Without Mn on the Arrays

In prior experiments on gold electrodes, we identified peptides from a designed library with catalytic characteristics that we wished to measure on Combimatrix arrays. They included Peptides 2 and 3, Mn-binding test peptides which exhibited increased current values in the presence of Mn ions in solution, and Peptide 4, a non-manganese binding control peptide which showed no increased current values in the presence of Mn ions in solution. We have attempted array-based analyses by synthesizing, in situ, a checkerboard pattern of either Peptide 2 and Peptide 4 on the same Combimatrix slide, or Peptide 3 and Peptide 4 on the same slide, and then performing water electrolysis measurements to examine the influence of the peptides on the electrodes during electrolysis. In general, our MALDI mass spectroscopy results have demonstrated that we achieved a high percent yield of each peptide when we perform synthesis on the CombiMatrix chip, with relatively low ratios of side products in most cases; which gave us confidence to proceed with water electrolysis measurements. Using this approach, we were successful at measuring current as function of voltage for each peptide. Though there was a large, systematic variation in the current as a function of the position of the array element (apparently an artifact of the reading system), the noise at any particular element was relatively small, allowing us to compare in detail the current/voltage curves for each of the peptides. No significant differences were observed between the putative Mn-binding peptides (Peptides 2 and 3) and the control (Peptide 4) (Figure 2).

Background Current Measurements on the Arrays

Since the redox activity of ferrocene is well known, we performed a control by attaching ferrocene to peptides on the array. The idea was to determine

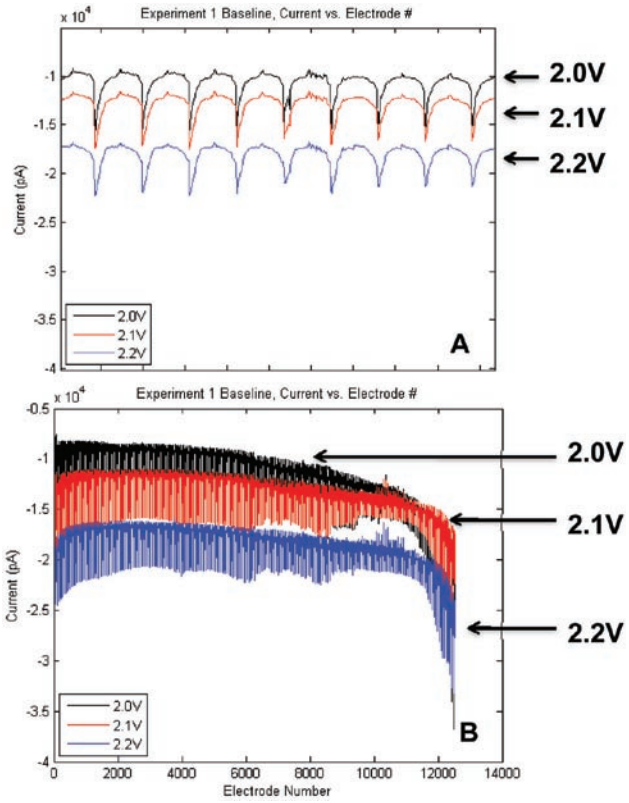

FIGURE 2. A. Selected set of electrodes containing Peptide 2 and Control Peptide 4. B. Plot of current values from all of the electrodes on the chip which contain either Peptide 2 or Control Peptide 4. No significant differences were observed between the putative Mn-binding peptides (Peptides 2 and 3) and the control (Peptide 4). A systematic trend was observed when the curve is blown up, i.e. Figure 3A, is due to the periodic noise from the instrument during measurements.

whether true electrochemical signals associated with the peptides could be distinguished from background. First, to create the ferrocene-containing peptide, ferrocene carboxylic acid was coupled to the alpha amino group of the N-terminus of each peptide by conventional reagents used for amino acid coupling in peptide chemistry. The MALDI spectrometry analysis of the synthesis showed three peaks related to the synthesis, of which one was a full peptide with ferrocene at the N-terminus. Another peak corresponded to the peptide with no ferrocene coupled to it, and the third peak was a peptide containing only one of the cyclopentadienyl rings of the ferrocene coupled at the N-terminus and the rest of the ferrocene molecule getting cleaved under acidic conditions. This synthesis was optimized by increasing the coupling time to increase the intensity of the full peptide with ferrocene.

After optimizing the ferrocene-coupled synthesis to approximately 40% yield as estimated by a MALDI spectrometry analysis (data not shown), we synthesized a range of peptide variants on slides. Some positions on each slide contained ferrocene only. Some contained

peptides that were four amino acids in length with N-terminal ferrocenes, and some contained longer peptides (twelve amino acids) with N-terminal ferrocenes. Some positions on the slide contained neither ferrocene nor peptide. All variants occurred in replicate on regions distributed throughout the slide. We then measured the redox activity on the slides with a voltage sweep. A feature in the oxidative sweep was observed near 0.5 V, somewhat higher than that expected for ferrocene in solution. There was no significant feature in the reductive sweep. The Potensiosense instrument scans the electrodes at fixed voltages and the scan rates are fixed and cannot be altered. Because of this the time between oxidative and reductive sweeps can be lengthy and it is sometimes difficult to obtain reversible oxidation. The oxidiative sweep feature only occurred in the ferrocene containing samples and not in the control samples where no ferrocene was coupled (Figure 3). Apparently, the Potensiosense instrument is able to measure

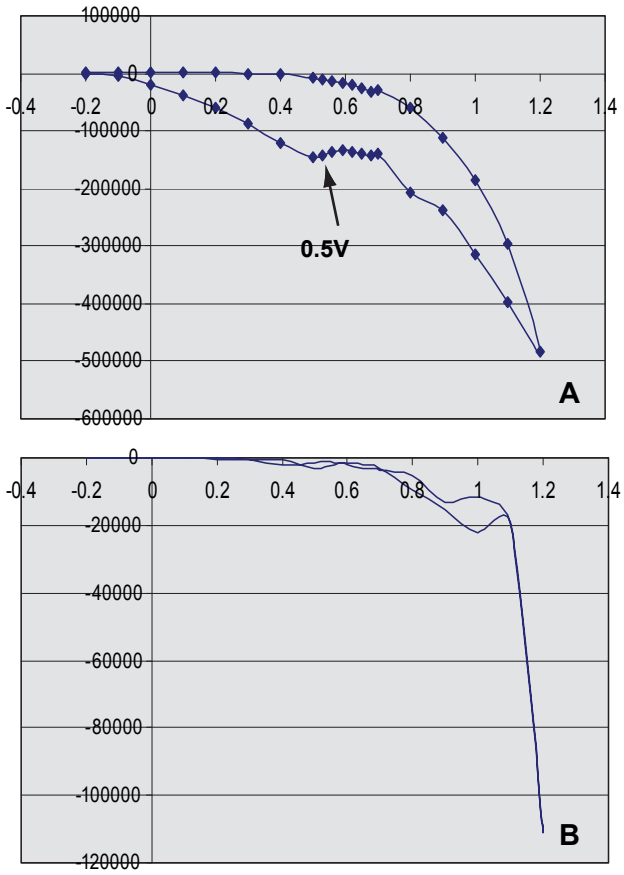

FIGURE 3. A. Region where Ferrocene is directly attached to the surface. B. Control Region with no peptide and Ferrocene. The typical current-voltage curve that we expected to see for ferrocene was not observed. However, a bump in the curve at the right oxidation potential (arrow) for ferrocene was observed, which was lacking in the same region of the control curve where no ferrocene was coupled.

electrochemical signals associated with the peptides if they are large enough. It is likely that for the electrolysis measurements, the platinum is not completely passivated by the combimatrix coating with respect to water oxidation, and at this voltage the background current was too large to detect any small increase due to catalytic activity of the peptide.

Conclusions and Future Directions

During the past year we have:

- Demonstrated multi-step, patterned synthesis of peptides in an array.
- Devised and begun testing an improved method of synthesis verification via direct MALDI spectroscopy on the surface.
- Completed the automation of array synthesis by integrating the fluidics and software our peptide synthesizer with our electrosynthesis instrument.
- Compared currents from peptides, with and without Mn, on Combimatrix arrays.
- Devised and implemented a strategy to evaluate background current measurements on the arrays by incorporating a ferrocene reporter into our syntheses.

Our goals for remainder of the grant period are to:

- Investigate the cause of contaminating peaks on patterned arrays observed during MALDI, possibly resulting from diffusion of protons generated electrochemically from 1,2-diphenylhydrazine that deprotect the trityl groups from amines on silent electrodes, or, trityl deprotection caused by residual voltage/current in regions where no voltage was applied during each electrochemical step.
- Improve the electrochemical deprotection efficiency of trityl protecting groups and enhance the reproducibility of efficient electrochemical deprotection.
- Check the stability of the Boc side-chain-protecting groups during the electrochemical removal of the trityl groups from the α-amino position in order to develop an orthogonal system of protecting groups for peptide synthesis.
- Develop an assay to determine the success of the electrochemical deprotection steps to avoid failure of the synthesis; possibly utilizing fluorescamine for the detection of free amines on the Combimatrix chips during peptide synthesis. Fluorescamine is a dye which possesses a fast amine coupling time (~100 milliseconds) as well as the ability to fluoresce when coupled only to primary or secondary amine functional groups.

- Generate a peptide library using our current capabilities of synthesizing a peptide with two mutant positions.
- Increase the diversity of peptide libraries and measure their catalytic activity.
- Evaluate ability of Potentiosense Instrument to detect catalytic activity as compared to the Cyclic Voltametry Instrument.

References

1. Fodor, 191 Science 251(4995):767.