Final Report

1. **Project Title:** Combinatorial Development of Water Splitting Catalysts Based on the Oxygen Evolving Complex of Photosystem II

Project Period: July 1, 2005 to December 31, 2009

Date of Report: March 31, 2010

Recipient: Arizona State University

Award Number: DE-FG36-05GO15016

Working Partners: The Biodesign Institute at Arizona State University

Cost-Sharing Partners: Arizona State University

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2. **Authorized Distribution Limitation Notices** - None

3. **Executive Summary**

The use of methods to create large arrays of potential catalysts for the reaction $\text{H}_2\text{O} \rightarrow \frac{1}{2} \text{O}_2 + 2\text{H}^+$ on the anode of an electrolysis system were investigated. This reaction is half of the overall reaction involved in the splitting of water into hydrogen and oxygen gas. This method consisted of starting with an array of electrodes and developing patterned electrochemical approaches for creating a different, defined peptide at each position in the array. Methods were also developed for measuring the rate of reaction at each point in the array. In this way, the goal was to create and then tests many thousands of possible catalysts simultaneously. This type of approach should lead to an ability to optimize catalytic activity systematically, by iteratively designing and testing new libraries of catalysts. Optimization is important to decrease energy losses (over-potentials) associated with the water splitting reaction and thus for the generation of hydrogen.

Most of the efforts in this grant period were focused on developing the chemistry and analytical methods required to create pattern peptide formation either using a photolithography approach or an electrochemical approach for dictating the positions of peptide bond formation. This involved testing a large number of different reactions and conditions. We have been able to find conditions that have allowed us to pattern peptide bond formation on both glass slides using photolithographic methods and on electrode arrays made by the company Combimatrix. Part of this effort involved generating novel approaches for performing mass spectroscopy directly from the patterned arrays. We have also been able to demonstrate the ability to measure current at each electrode due to electrolysis of water. This was performed with customized instrumentation created in collaboration with Combimatrix. In addition, several different molecular designs for peptides that bound metals (primarily Mn) were developed and synthesized and metal binding was demonstrated. Finally, we investigated a number of methods.

We have shown that we can create surfaces on glass slides appropriate for patterning peptide formation and have made arrays of peptides as large as 30,000 using photolithographic methods. However, side reactions with certain amino acid additions greatly limited the utility of the photolithographic approach. In addition, we found that transferring this patterned chemistry approach to large arrays was problematic. Thus, we turned to direct electrochemical patterning using the Combimatrix electrode arrays. Here we were also able to demonstrate patterned peptide bond forming chemistry, but yield and consistency of the reaction remains insufficient to create the quality of array required for realistic optimization of catalytic peptide sequences. We are currently exploring both new polymerization

chemistries for generating catalysts on surface as well as adopting methods developed at Intel for creating peptide arrays directly on electronic substrates (silicon wafers).

4. Goals & Objectives vs. Actual Accomplishments

There were seven objectives and associated tasks. Here we look at each of the objectives and discuss how they relate to actual accomplishments. In the subsequent section, we discuss progress for each task in more detail.

- 1) *Optimize light directed peptide synthesis:* The objective was to develop a system for light directed synthesis of peptides and to use this to pattern peptide synthesis, initially on glass slides and later on electrode arrays for catalytic activity assays. We succeeded in creating arrays of peptides on glass slides. In the process, we also developed an approach for performing mass spectroscopy of peptides at specific positions in the array. However, we consistently observed side reactions associated with the removal of the photolabile blocking groups. We decided to discontinue this work and focus on electrochemical synthesis.
- 2) *Develop electrochemically-directed peptide synthesis:* This turned out to be the primary activity of the grant period. We demonstrated the ability to use electrical potential to generate an acid sufficiently strong to remove acid-labile blocking groups from the end of a growing peptide chain. We demonstrated that this could be done while leaving side chain protecting groups largely intact. Many different approaches were explored to optimize this chemistry. We have not yet achieved a level of optimization that allows the synthesis of arrays with sufficient step-wise yields and with the level of reproducibility required for the optimization of catalytic function.
- 3) *Develop surfaces & measure electrochemical baseline:* The objective here was to demonstrate that we could prepare appropriate surfaces to be used in the patterned synthesis of peptides and for electrochemical measurements and then to make baseline measurements. We developed several appropriate surfaces including surfaces that involved attachment through relatively short linkers to peptides as well as three dimensional surfaces that greatly increased the effective surface area. We then developed a particular electrode system that we used as the standard system for comparison and we measured the baseline current voltage curves on that electrode both with and without peptides (non catalytic) present.
- 4) *Fabrication of electrode arrays for light directed synthesis:* We explored the fabrication of electrode arrays with specific surface properties most appropriate for developing the light directed synthesis methods. We experimented with a number of different electrode designs, particularly considering the longevity of the electrodes under conditions of electrolysis and we manufactured some small electrode arrays. There were problems associated with surface stability under the conditions of our electrochemical assays, and with inconsistencies in measurements. When we discontinued the light-directed approach to peptide synthesis for this project, we also discontinued the fabrication of electrode arrays in favor of the commercial arrays produced by Combimatrix.
- 5) *Develop high-throughput electrochemical analysis:* We initiated this task attempting to use the in-house fabricated electrode arrays described above and then transitioned into using the Combimatrix arrays and their ElectroSense platform for making serial measurements of current at thousands of electrodes. This was accomplished and baseline measurements of the current were performed.
- 6) *Optimize catalysis through iterative library design and testing cycles:* We were not able to complete the task of developing a robust enough approach for creating peptide arrays on the Combimatrix electrodes to perform the catalytic optimization. We did design and test an initial set of peptides that bound Mn and decreased the overpotential of the water splitting

reaction to a modest degree (about 15%). Because of the inherent difficulties alluded to above, and described in much more detail below, with the electrochemical approach to peptide array synthesis, we have now adopted a new array fabrication approach that was recently developed by Intel. This involves the synthesis of peptide arrays on silicon wafers (or electronics patterned on silicon wafers) using standard photolithography equipment, masks and photoresist. This is a proven technology but requires the installation of major lithography equipment devoted to the task. We have (now with funds other than DOE moneys) hired the engineer that developed this approach at Intel, have installed lithography equipment donated by Intel and should have the capability to make these arrays reproduced in our laboratories within the next 12 months. At this point we will be able to continue the optimization of electrochemical catalyst development.

5. Summary of Project Activities for Entire Period

The objective of this project was to develop new catalysts for hydrogen production via electrolysis. Specifically, this work aimed to decrease the over-potential for the water splitting reaction forming oxygen and protons from water at the anode of an electrolysis cell. The concept was to optimize catalysts via a molecular evolution approach involving the fabrication of large libraries of metal binding peptides on electrode surfaces. The approach used was to fabricate libraries using a high-throughput, light-directed heteropolymer synthesis approach. As the project unfolded, we also took on board an additional approach for peptide library synthesis that involved direct electrochemical patterning of synthetic reactions on arrays of electrodes. This was performed via a collaboration with CombiMatrix Corporation. The initial guesses for these peptide sequences were made by considering the structure of the oxygen evolving complex of Photosystem II and redox active metal binding sites generated previously in our laboratories involving modifications of the *Rhodobacter sphaeroides* bacterial reaction center structure. The approach then involved iterative modeling, library production and library screening (via electrochemical measurements) to facilitate catalyst discovery and optimization.

As will be described below, the actual integration of the entire high throughput synthesis system with an electrode array and then a measurement system proved to be much more complex than originally conceived. In particular, the actual detailed development of high throughput synthesis on properly modified electrode arrays that could be measured accurately for electrochemical activity ended up being the primary activity of the grant. This covers two tasks primarily (1 and 2). In the first task, we started using a light directed approach to high throughput peptide synthesis which was largely successful, but we latter realized that transferring this to electrode arrays would be problematic. The second task describes an electrochemical approach to high throughput peptide synthesis in which peptides are made directly on electrodes via electrochemical production of acids that remove blocking groups. Here, many problems were encountered in using the peptide synthesis chemistry with the commercially available electrode arrays – in particular, the surfaces of the arrays are sensitive to the acids used during the synthesis and the device structure itself that contains these arrays and associated electronics is sensitive to the solvents being used. We have made a great deal of progress overcoming these issues and have identified new technologies, that mix the acid generating approach of the electrochemical synthesis with the light patterning approach of the light-directed synthesis. We are now moving forward using this new technology (see end of this section).

5.1 Task 1 - Optimize light-directed peptide synthesis

This task built off of an ongoing project in the laboratory to use digital mirror-based virtual masking equipment (basically the same technology used in computer projectors) to pattern the deprotection of photolabile protecting groups from the ends of growing peptide chains on glass surfaces (similar to the synthesis of DNA chips by the company Niblegene-Roche). We set out to

optimize this chemistry and the instrumentation that supported it. This involved both optimization of the surface for synthesis (structure of the surface and associated attachment groups), optimization of the instrumentation (integrated flow cells for synthesis that interfaced with the digital mirror system, optimization of the control software and optimization of the chemistry itself (selection of the best photolabile groups, associated conditions for deprotection, minimization of side reactions and methods for determining the chemical structure of the products produced on the surface). What follows is a time line of the high level project accomplishments. See the quarterly reports for additional detail.

2005: We generated >2,000-feature, 12-mer peptide arrays using light-directed synthesis. We made this array to test our ability to accurately synthesize and mutagenize specific positions within large numbers of peptides. We used normal Fmoc (*N*-9-fluorenylmethoxycarbonyl) solid-phase amino acid synthesis for most of the amino acids (the ones that were not changed in the mutagenesis) and only used the light-directed synthesis on those amino acids whose identities were varied. A major aspect of this work was the development of a methacrylate surface that allowed the production of large amounts of peptide per surface area, the development of scavenging solutions that removed reactive species generated during the photo-deprotection, and screening of a large number of different photolabile groups and derivatives (mostly in the NVOC and NPPOC families).

2006: We synthesized an array of 30,000 peptides on a glass slide (**Fig. 1**).. For this test, we used variants of a peptide known to bind the regulatory protein Gal80. This allowed us to test the concept of library optimization of peptide function on a surface. Binding assays and mass spectroscopy verified that the peptide sequences were synthesized correctly.

Figure 1. Fluorescence image of the surface-synthesized peptide polymer library following binding of Gal80 at 25 pM in 1mg mL⁻¹ E.coli lysate for 1 h. a) Image of one replicate block where the rows and columns describe the layout of the library and provide a substitution index for mutated positions 9 and 12. b) Expanded view of EGE(*)4TEGK(K)9S(*)11(I)12GSC which describes the substitution index for mutated positions 4 and 11 for each white square shown in panel (a). c) Image of the entire peptide library containing three replicate blocks (outlined in white and numbered 1, 2, 3). The total polymer surface area is ~1 cm² with each library feature measuring 45 mm diameter and the template peptide was EGE(W)4TEGK(L)9S(L)11(R)12GSC.

In the course of this work, we discovered that the thickness and homogeneity of the methacrylate surface made a big difference in the times and quality of peptide synthesis. We also fabricated arrays of 100 electrodes that were individually-addressable and designed to fit into our light-directed synthesis chamber. We performed peptide synthesis on the electrode surfaces and

discovered a couple of challenges: the polyindole conducting polymer used as an interface between the electrode and the peptides peeled off of the electrodes due to the many wash steps involved in our synthetic approach and our step-wise yields for the synthesis were low relative to those obtained on our methacrylate test surfaces.

We also developed a means of releasing peptides after synthesis in dry form, making it possible to perform mass spectroscopy directly on the surface at particular features (MALDI – matrix assisted laser desorption ionization). This was extremely powerful as it allowed us to analyze the side products of reactions and optimize to eliminate them.

Towards the end of 2006, two things happened. First, it was determined that interfacing light directed synthesis with fabrication of electrodes and the associated alignment issues were going to greatly complicate reaching our goal of iterative production of peptide arrays that could be assayed electrochemically. There were also problems with specific side reactions occurring, particularly when arginine was present in the amino acid chain, which we were unable to avoid. Second, we had formed a collaboration with CombiMatrix in 2005 that had shown considerable promise to circumvent many of these problems by moving to a system that used electrical potential and electrochemical production of acids to directly create peptide arrays on electrode arrays with no alignment issues (see below). Thus, we discontinued the DOE-funded work on light directed synthesis and focused our attention on developing electrochemically directed peptide synthesis.

This work is covered in the following publications and patents:

Northern, T., Greving, M., Woodbury, N. (2008) "Combinatorial Screening of Biomimetic Protein Affinity Materials" *Advanced Materials*, 2008,20(24), 4691-4697.

Northern, T.R. and Woodbury, N.W. (Arizona State University), "Microarray of Three-Dimensional Heteropolymer Microstructures and Method Therefor," 10/599,770 Nationalized, October 6, 2008.

Northern, T.R.; Woodbury, N.W. and Greving, M.P. (Arizona State University), "Microstructure and Microdomain Microarrays, Methods of Making and Using Thereof," PCT/US2006/023344 Nationalized, June 15, 2006.

Greving, M.P. and Woodbury, N.W. (Arizona State University), "Porous Acrylate Copolymer Films and Uses Therefor," 12/018,398 Non-Provisional, January 23, 2008.

5.2 Task 2 - Develop electrochemically-directed synthesis

Our goal in this task was to design a peptide synthesis scheme that allowed us to selectively deprotect α -amines versus side-chain amino groups that had been protected between successive synthesis steps. Since our electrosynthesis equipment can generate acids at each electrode, we replaced base-labile Fmoc α -amine protection moieties, typically used for peptide synthesis, with acid-labile Boc and Trityl groups which may be deprotected by electrochemically-generated acids before each successive amino acid addition. It was necessary to protect side-chain functional groups during synthesis in order to focus peptide elongation at the α -amines. We searched for side-chain protecting groups that were not susceptible to the chemistry used to deprotect α -amines. After peptide synthesis was complete, we needed to be able to remove the side-chain protecting groups so that the resulting peptides would be functional. We began our syntheses using Boc as the α -amine protection group and t-butyl as the side-chaining protecting groups.

2005: We verified that simple polymerization chemistry could be performed directly on the addressable electrodes of 12,500-element arrays using a system that we purchased from Combimatrix, which employs electrochemical acid formation at specific electrodes. We started out testing standard BOC chemistry (BOC is N-tert-butoxycarbonyl and is removed with moderate acid) deprotection approaches.

We also purchased an instrument, developed by Combimatrix, that can then read the electrochemical currents at each electrode for a given voltage. Initial tests were performed to verify the electrochemical detection capability on an array containing biotin-labeled oligos of varying lengths (purchased from CombiMatrix for the purposes of testing) which was incubated with an HRP-streptavidin fusion protein. HRP emits a continuous electrochemical signal. We looked at linker-length dependence of the electrochemical signals and found that the shorter linkers, which positioned the biotin within a nm of the surface, showed the strongest signals and that the signal decreased rapidly as the linker became longer (**Fig. 2**). The strong linker length dependence was surprising given the size of the HRP-streptavidin fusion.

Figure 2. Electrochemical detection of synthesis by Biotin-Streptavidin (Horseradish peroxidase) assay. Each number represents the length of the Poly T linker on top of which Biotin was chemically coupled.

Using standard Boc chemistry, we then attempted to synthesize peptides, instead of oligonucleotides, on the Combimatrix arrays. We were able to verify that patterned synthesis was possible, by using the biotin/HRP approach described above, but in the initial run we were unable to reproducibly create the desired patterns, a problem that we address later after the automation of the synthesis was in place (see below).

2006: We then integrated the hardware and software between the CombiMatrix electrosynthesis system and a very simple automated peptide synthesizer so that we could completely automate our synthesis processes. Using the biotin-streptavidin fluorescence assay, we verified that yields were comparable between automated and manual syntheses for our 8 to 10-mer peptide range. While this was successful, it became clear that a better synthesizer would ultimately be required. During this time, we attempted to adapt the MALDI mass spectroscopy approaches that we had used for glass slides to the CombiMatrix slides. Initial attempts were unsuccessful because we used a linker that required fairly harsh cleavage conditions resulting in the release of the matrix into solution. This made peptides impossible to see. This problem was later resolved through careful use of trifluoroacetic acid (TFA) vapor treatment and judicious selection of the right type of acid labile linker.

We next performed two short (two coupling) syntheses using the CombiMatrix electrochemical array and again encountered the errors in the software controlling the CombiMatrix electrochemical patterning system described above, resulting in imperfect patterning. The unit was returned to the company for repair and upgrade of the software. Upon return of the instrument, we synthesized a peptide array, made by creating a series of lengths from 2 to 7 amino acids long, of polyglycine. The first two were put on chemically and the last five reactions were performed electrochemically. At the end of each chain, a biotinylated glycine was attached. By comparing the amount of signal (using the HRP assay) for each length of chain, we determined that the yield of each of the electrochemical steps involving plain glycine was about 90%. There was some evidence in this run, however, that the biotinylated glycine showed a lower yield and that as the synthesis went on, the matrix that is on the surface of the commercially produced CombiMatrix slides, was degraded.

To address the automated synthesis issues more completely, a refurbished peptide synthesizer was purchased this then required fairly extensive hardware replacement, electronic integration and software control development. During this time, we continued to work on optimizing the chemistry using manual peptide syntheses, attempted to reduce background noise as much as possible and continued to develop analytical methods (ie – MALDI TOF mass spectrometry) for determining the peptide products formed. An example of the optimization results is shown in **Fig. 3**. Here, 1, 2, and 3 described the number of electrochemical glycine couplings performed. We were able to obtain high coupling yields and low backgrounds in surrounding areas using this approach. Note that in this case we used a fluorescent assay rather than an electrochemical assay.

Figure 3. Different lengths of poly-glycine synthesized on sets of electrodes. Synthesis characterization was done using a Biotin-Streptavidin (Alexa 647)-based fluorescence assay. Each number in the Figure represents the number of glycine amino acids coupled at that location.

We then performed patterned electrochemical synthesis of up to 10-mer peptides on a 12,500-feature array after which we compared the fluorescence from each to determine the stepwise yields. With the longer synthesis, we experienced variable backgrounds and observed signals on some electrodes that were meant to be silent. Otherwise, we found little variability between the lengths indicating efficient yields even for long peptides. We were able to synthesize our 8-mer putative catalytic peptides with an apparent yield of 60% - ample for the activity measurements. We repeated the experiment using 1, 2 and 3-mers after further optimization of backgrounds and the averaged fluorescence between them indicated an average step wise yield of 94%.

2007: Being able to perform couplings is important, but in the end, one must be able to run extended cycles of couplings with multiple amino acids at each point in the peptide (but in different positions on the surface) and then both deprotect the final peptides and show that they are active. It is at this stage of the project that we started to realize one of the major weaknesses with the CombiMatrix approach: the matrix material that they use (proprietary, but apparently a sugar polymer) degrades fairly easily. Much of the rest of the work has been focused on trying to get around this issue. We first really uncovered this as a major problem when we synthesized the hetero-peptides (YGGFL and GGFL) in a checkerboard pattern, and used the commercially available anti-YGGFL antibody to probe and determine if the desired sequence was present. There was only one electrochemical step in this synthesis (Y vs. G at the last step – we synthesize from C to N). Fmoc-Tyr(t-butyl)-OH was used for this synthesis and 95% Trifluoroacetic acid (TFA) for two hours was used for deprotection of the side chain hydroxyl group after synthesis. Fluorescence detection revealed only ring-like patterns from the edges of the electrodes. Further analysis suggested that the chip membrane (the carbohydrate surface) was not stable in the strong acidic conditions required for t-butyl deprotection. We turned to Fmoc-Tyr(o-allyl)-OH for the synthesis since the allyl group did not require acidic conditions for deprotection, but uses instead a soluble palladium reagent. We were unable to achieve high yields of expected product in this way. It was difficult to analyze the products of the reaction completely, but we expect that we simply were unsuccessful at achieving good yields for the deprotection reaction on the surface.

A great deal of iterative effort was then put into optimizing the side-chain deprotection. We were able to develop conditions for t-butyl deprotection using shorter times and different concentrations that left the arrays relatively intact (**Fig. 4**). On the positive side, this reduced the processing time by 75%, from 2hrs to 30 minutes, and allowed for completely deprotecting the t-butyl groups. However, as can be seen in Fig. 4, merely doubling the exposure time completely destroyed the surface of the CombiMatrix chips. This told us that the approach was likely to be very sensitive.

Figure 4. A selected region of the chip was electrochemically deprotected and 5,6-carboxy Fluorescein (fluorophore) was coupled to the free amines generated. Image (a) is a fluorescence scanning image of the selected region after fluorophore coupling to the electrochemically-deprotected region. Image (b) is a fluorescence scanning image of the selected region after 30 min of exposure to 95% TFA and 5% H₂O. Image (c) is a fluorescence scanning image of the chip after an additional 30 min of exposure to 95% TFA solution.

During this same period, we realized that while coupling yield overall was quite reasonable for at least a number of the amino acids, we needed to be able to directly analyze the covalent structure (formula structure at least) of the final molecules to determine if we really created the desired products. Thus we continued to optimize the protocol for performing MALDI spectroscopy directly off the surface of the chips. Because we were using acid to deprotect the N-terminal amines during synthesis, working with acid-labile linker groups as a means of releasing the peptides in situ was problematic. Thus we moved to synthesizing the peptides directly on top of photolabile linkers and then cleaved the linkers using UV light exposure under dry conditions (to avoid diffusion of the peptide from its position on the surface) and subsequently characterized by MALDI directly from the surface. This modified procedure yielded spectra with high signal intensities and allowed direct characterization of the synthesis. In this way we were able to measure precisely what was synthesized at different points on the surface rather than just assaying for addition of fluorescent or electrochemically detected groups.

Our new MALDI assay allowed us to evaluate and optimize our synthesis methods in much greater detail. We developed the MALDI assay using a standard Fmoc peptide synthetic approach (no electrochemical synthesis). This yielded dominant peaks from the desired peptide and its sodium ion adduct (**Fig. 5**). But, when an electrochemical trityl deprotection step was included in the peptide synthesis at the last step, a mixture of products was obtained. It behaved

as though the electrochemical deprotection step was incomplete, which was odd, given the relatively high yields of apparent deprotection we had observed with our end labeling assays. (As we will explain later on, there were other issues, discovered later, associated with instability of the electrochemical synthesis system to the solvents being used that may have led to some of these problems.) To address this problem, we stepped back and assayed a variety of different deprotection conditions (concentrations of electrochemical acid, voltages and time intervals for voltage exposure) and we explored switching from Boc-protected amino acids to trityl-protected amino acids (trityl groups are released at lower acid concentrations). The disadvantage of this is that trityl amino acids are generally not commercially available, but we got around this by using amino acids with the N-terminal protected by Fmoc and then replaced that with trityl as one step in the synthetic procedure on the surface of the chip. As we reexplored the deprotection conditions, surface stability again became a problem under repeated acid treatments. In addition, we had to rethink some of the side-chain protection strategies again. We explored various strategies, particularly benzyl, Fmoc and alloc protecting groups, which are deprotected using hydrogenolysis, base, and Palladium reagents, respectively. This time we were able to find conditions that supported alloc deprotection chemistry on the slides with no apparent damage. However, analysis of the MALDI spectrum obtained when alloc deprotection was used showed that both the desired peptide and a peak 26 Daltons larger was formed. We went back to using acid labile groups (mostly t-butyl) and trying to balance carefully the deprotection conditions against degradation of the surface.

Figure 5. Peptide $\text{NH}_2\text{-KAFGAFGAFG-CONH}_2$ was synthesized on top of a photolabile linker coupled to the chip. Peptide synthesis and Fmoc-photolabile linker coupling was accomplished using a conventional Fmoc synthesis protocol after the chemical deprotection of monomethoxytrityl (MMT)-protected amines present on the chip surface. After synthesis, the linker was cleaved by UV irradiation under dry conditions (no solvents) and then MALDI mass spectrometry analysis of the chip was performed.

At this point, it became clear that there were multiple, interdependent aspects of the array synthesis system that were making it very difficult to optimize one thing (e.g., the stepwise yield) while maintaining another (e.g., surface stability or deprotection yield and purity of product). On top of this, there were both software and hardware problems with the instrument itself that compounded the complexity of the process, though we did not discover these problems until later after we had worked through some of the chemical issues. During 2008 and much of 2009, we focused on systematic optimization across these many variables.

2008: We continued to optimize the electrochemical deprotection efficiency using trityl-protected amino acids. The result of this stage of the optimization was that 10 minutes at 3 V gave roughly 70% deprotection of the trityl group (Fig. 6). The low yields compared to previous experiments may be due to the fact that trityl groups tend to reattach after release and thus sometimes require additional scavengers to remove them efficiently, an approach we tried later. In the experiments of **Fig. 6**, we synthesized peptides with two electrochemical deprotection steps with an overall 50% yield (stepwise yield of 70%). Though this yield was low, it was encouraging as at least it suggested that we might be able to use milder acid conditions than previously.

Figure 6. Peptide NH₂-KLAGAFGAFG-CONH₂, involving two electrochemical deprotection steps before coupling Leu and Ala (1st Ala from the N-terminus of the peptide sequence), was synthesized under fixed voltage (3V) and various time conditions (0min, 2min, 4min & 10 min) on different regions of the chip. Increased time intervals produced increased deprotection efficiencies, as seen by comparing the intensity values of the 4 minute and 10 minute peptide peaks. Unlabeled peaks in the spectrum relate to peptides with deletions of amino acids and some chip membrane-based **chemical moieties**.

We used this approach to synthesize two of the putative catalytic peptides that we had designed, based on biological systems, (Peptides 3 and 2) along with a control peptide (Peptide 4) that was not expected to bind metal or have catalytic activity. We then studied their influence on water electrolysis on the CombiMatrix electrodes. Getting the peptides properly synthesized required considerable optimization. There was a problematic side product that occurred during the synthesis of Peptide 3c (loss of a water molecule and piperidine adduct), which apparently was triggered by synthesis of a Gly-Asp sequence in the peptide. Eventually, we were able to largely suppress this by the use of HOBt in the Fmoc deprotection solution. When we finally were able to synthesize these peptides properly on the surface, we tested them for activity in terms of changing the overpotential for electrolysis, but detected no difference between the potential catalysts and the controls. This was disappointing, as we had seen a small but significant drop in the overpotential when these peptides were compared directly on gold electrodes (see task 6). It seemed likely that the local environment was playing a major role in the activity and we decided that we should try to go through a cycle of optimization on the CombiMatrix surface. Before doing this, however, we wanted to at least verify that the measurement system provided by CombiMatrix was functioning. Thus we designed a ferrocene-based experiment to determine this.

Since the redox activity of ferrocene is well known, to determine how well synthesized peptides were communicating with electrodes, and to differentiate the peptide signals from the instrument noise, we synthesized various ferrocene-containing peptides on Combi slides at 40% yield (**Fig. 7**). Some positions on each slide contained ferrocene only. Some contained peptides that were four amino acids in length (NH₂-Fc-GKFG-CONH₂) with N-terminal ferrocenes, and some peptides were full-length (9 amino acids, NH₂-Fc-KFGKFGKFG-CONH₂) 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. **Fig. 8** is an I-V comparison of the two extremes: the full-length, ferrocene-coupled peptide versus the plain slide containing neither ferrocene nor peptide. In order to get a more complete picture, we attempted to run a piece-wise current-voltage curve for the different electrodes, by cycling through voltages and measuring currents. A bump in the curve at the right oxidation potential for ferrocene was observed, which was lacking in the same region of the control curve where no ferrocene was coupled. It is likely the case that under the conditions we were running the tests, it was not possible to achieve true equilibrium current/voltage relationships. The Combimatrix Potensiosense instrument scans the electrodes at fixed voltages and the scan rates are fixed and cannot be altered. This is very different from the Cyclic Voltammetry instrument where the voltage sweep is continuous and scan rates can be varied. However, ferrocene at least appeared to give a signal, suggesting that there was some communication between the surface and the peptides.

Figure 8. Panel (a) represents current measurements at various fixed voltages corresponding to electrodes possessing full-length, ferrocene-coupled 9-mer peptides and panel (b) represents measurements from the control region.

2009: Because of the low yields and side reactions associated with the α -amine deprotection and coupling reactions, we decided to compare protection of α -amines with trityl (Trt) and dimethoxytrityl (DMT - highly acid labile in 1 and 3% TFA). The hope was that we could generate yet less harsh deprotection conditions for the dimethoxytrityl. We also varied the synthesis in a solution, using one of the solutions more commonly used by CombiMatrix containing hydroquinone. Most of what we had done previously used 1,2-diphenylhydrazine electrochemical solution originally published for amide synthesis by CombiMatrix, but this really was designed with Boc groups in mind. Several trials in the hydroquinone-based solution revealed that the DMT and Trt protecting groups were removed in the control regions of the chip, where no voltage was applied. About 17% of the Trt groups were removed and about 75-80 % of the DMT from the peptides in these regions. As seen previously, Trt remained stable in 1,2-diphenylhydrazine solution. Subsequent measurements using MALDI to look at the products confirmed that approximately 80% of the DMT groups were getting cleaved solely by the exposure of the chip to the solution with no voltage applied. Trityl groups were more stable, as in the previous test, and underwent about 15% deprotection when exposed to the hydroquinone-based electrochemical solution. We repeated these tests in the 1,2-diphenylhydrazine electrochemical solution. Again about 80% of the DMT groups were deprotected from the peptide N-terminus with no voltage applied. In contrast the Trt groups remained completely stable under these conditions. Additionally, the stability of the DMT and Trt groups in the hydroquinone-based electrochemical solution was tested on beads. Surprisingly, the both DMT and Trt groups remained stable in the electrochemical solution on beads. This implied something about the electrode, either the nature of the surface or some error in the hardware/software electronic control was at the root of the problem (see below).

We also attempted a few completely different deprotection strategies. In one case we tried to reduce piperidine hydrochloride electrochemically, at cathodes, and release piperidine *in situ*, assuming that the *in situ*-generated piperidine would then carry out the base cleavage of the Fmoc protecting groups on the amines (**eq. 1**). All of the variables of the experiment such as voltage, time, and concentration of piperidine hydrochloride were tested. At best, we were able to achieve 25% removal of protecting groups at -2V, 20 minutes, and 0.6M piperidine hydrochloride. We did not pursue this strategy further.

In another approach, Carbobenzyloxy (CBZ) protecting groups can be chemically removed from amines by hydrogenolysis on palladium or platinum surfaces. We tested the idea that the hydrogen generated during piperidine hydrochloride reduction at cathodes could do the hydrogenolysis of the CBZ groups utilizing the platinum surface of the electrodes as catalyst for the reaction (**eq. 2**). MALDI demonstrated that this concept was not compatible with our platform

since all of the CBZ groups present on the N-terminus of the peptides remained intact. We did not pursue this strategy further.

Finally, we attempted to electrochemically remove alloc protecting groups from α -amines, which are also widely used in peptide synthesis. For the deprotection of these groups, a Palladium(0) catalyst was required. We generated *in situ* Palladium (0) from Palladium(II) by its reduction at the cathode (**eq. 3**). Again, we were not able to consistently observe deprotection. We did not pursue this strategy further.

Based on these results, we moved forward using trityl protection for α -amino groups during 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 44% overall yield under these conditions (**Fig. 9**). In one set of experiments, four peptides were synthesized in four different regions of a Combimatrix chip. The peptides included: Control peptides involving no electrochemical steps, peptides involving one electrochemical step, peptides involving two electrochemical steps and peptides involving three electrochemical steps. MALDI spectra showed peaks corresponding to each of the expected peptides from their respective regions as well as some contaminating peaks corresponding to peptides synthesized in other regions of the chip (**Fig. 10**). These contaminating peaks may have resulted from protons, generated electrochemically from 1,2-diphenylhydrazine, diffusing and deprotecting the trityl groups from amines on silent electrodes or 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 with an approximate overall yield of 44% was a major step forward in peptide library synthesis.

Figure 9. Region of the chip where a peptide involving three electrochemical deprotection steps was synthesized. Unlabeled peaks represent peptides containing deletions of one amino acid.

We worked on improving the total yield of the peptide involving three automated electrochemical synthesis steps. After changing our trityl coupling strategy, from an amide coupling reaction using trityl glycine to a nucleophilic substitution reaction using trityl chloride followed by capping with N-acetyl glycine, we were able to achieve 9% increase in the total yield of the product.

Experiments were conducted to determine if the addition of peptides on chains in unintended regions of the chip were due to residual current on all of the chip electrodes which led to the generation of acids, resulting in removal of trityl protecting groups on those unintended regions, or whether it was due to the diffusion of protons generated electrochemically on the addressed electrodes to the non-addressed ones, causing deprotection. Electropolymerization of pyrrole on specific electrodes suggests that there is no residual current in the circuits of the chip and that the problem is likely the diffusion of electroacids from one region to another (**Fig. 11**). It may be that the voltage and time conditions that we currently apply to generate acids, 3V for 10 minutes, is a long enough interval for the protons to diffuse from one end of the chip to another. If this is true, even smaller regions spaced further apart may not significantly decrease the levels of contamination.

We reverted to testing the electro-deprotection of the monomethoxy trityl group (MMT) (combi chips when purchased have the amines present on the surface protected with MMT groups. MMT and DMT groups are derivatives of Trityl groups and are very similar in acid lability. Therefore it is assumed that electrochemical conditions tested for efficient deprotection of MMT groups will be very close to the conditions needed for Trityl deprotection) from the primary amines on the electrode surface and checked diffusion of the electro-generated acids by exploiting the N-terminal endorphin sequence's (YGGFL, $K_d = 7.1\text{nM}$) high affinity interaction with anti-YGGFL antibodies. Twenty different current and time conditions were tested for electro-deprotection and each condition had seven replicates (seven sets of 32 electrodes) on the chip surface. After the electro-deprotection step, the N-terminal endorphin sequence was synthesized with three glycines coupled at the C-terminus of the peptide ($\text{NH}_2\text{-YGGFLGGG-CONH}_2$), to act as a linker by regular Fmoc synthesis. The intent was that peptide synthesis would only take place on the electrodes whose primary amines were electrochemically deprotected. After the synthesis, the chip was incubated with antibodies for the fluorescence-based characterization. Fluorescence scanning of the chip revealed that several of the current/time conditions successfully deprotected the MMT groups from the primary amines (**Fig. 12**). Of all conditions tested, the one with $0.75\mu\text{A}$ per electrode and a reaction time of 60s showed the highest fluorescence from the selected electrodes which corresponded to high electro-deprotection efficiency. All seven replicates on different parts of the chip showed similar fluorescence and the neighboring electrodes did not show any signal higher than the average background fluorescence which would mean that there was no diffusion of electro-acids generated from the selected electrodes to the neighboring silent electrodes.

Figure 12. This fluorescence image displays the relative efficiencies of twenty different current and time conditions tested for the electrodeprotection of trityl groups on a Combimatrix chip. Seven replicates of sets of electrodes on different parts of the chip underwent electrodeprotection by each condition onto which N-terminal endorphin sequence, NH₂-YGGFLGGGG-COOH was synthesized. Fluorescence detection was done based on the sandwich assay involving binding with anti-endorphin antibody, biotin conjugated 2^o antibody and Alexa-647 conjugated streptavidin protein.

Once a current/voltage condition was determined for the electro-deprotection of MMT groups, we decided to further evaluate the deprotection efficiency under the selected condition. Since fluorescence-based detection is an indirect characterization technique and does not give an estimate of percent efficiency of a chemical step, MALDI-based characterization of the synthesis was carried out. The efficiency was calculated using this approach by quantifying the relative intensities of the m/z peaks observed in the spectrum. Also, the side products of the synthesis were determined. In this experiment, a peptide NH₂-AKAFGAFGAFG-CONH₂, was synthesized on the chip using Fmoc synthesis methodologies. The second lysine from the N-terminus of the peptide had a trityl group protecting the ϵ -amino group on its side chain. The chip was divided into four sets of electrodes. In two sets of electrodes, electrochemistry was performed (electrochemical region) and the remaining two sets of electrodes did not undergo any electrochemical step (control region). Once the peptide was synthesized, the electrochemical deprotection step was carried out in the two electrochemical regions of the chip using 110mM solution of 1,2-diphenylhydrazine in acetonitrile as the electroacid-generating reagent, at 0.75 μ A and 60s. After the electrochemical step, proline was coupled to the free ϵ -amines. The chip was then treated with 1% trifluoroacetic acid solution to chemically remove all the trityl protecting groups present on the electrode array surface. If the electro-deprotection step worked, the peptide m/z peaks from the two electrochemical regions should include proline; whereas the two control regions of the chip would show peptide peaks with no proline. What we found was that the two electrochemical regions showed peptide including the proline m/z peaks in the MALDI spectrum as expected, however the control regions of the chip showed no peptide peaks at all. The same experiment was carried out on a different chip with one variation: instead of trityl protection, methyltrityl (mtt) was used. The result was essentially identical. We expect that the lack of the expected peptide in the control region has something to do with the MALDI detection efficiency, which can vary dramatically for peptides with different amino acid sequences.

We repeated the experiment performing all the chemical steps manually and no automated synthesis using the peptide synthesizer was performed. This was done so as to exclude the possibility that the inconsistent result could be due to problems with the synthesizer. In this experiment, a leucine was coupled, instead of proline, after the electrochemical deprotection step. Also, after chemical deprotection of the trityl groups, an N-acetyl glycine amino acid was coupled to the ϵ -amines of the lysine in the control regions as well as the lysines whose ϵ -amines did not get deprotect by the electrochemical step in the electrochemical regions. The MALDI spectrum of the experiment showed peptides m/z peaks from both the electrochemical and control regions in this experiment, though leucine coupling had apparently occurred with low yield as had the chemical deprotection step of the trityl group. However, it was clear that the electro-deprotection efficiency of trityl protecting groups was efficient and there was diffusion of electroacid to the control regions. We intend to repeat the experiment to understand the poor yields of the coupling and chemical deprotection steps.

We are slowly working through the technical hurdles associated with building peptide arrays on electrodes. However, technical hurdles remain: 1) the chemical and mechanical integrity of the polymer surface on the Combimatrix slide is not yet adequate for repeated exposure to organic solvents and acids, and 2) the integration of the Combimatrix e-chem synthesizer and the peptide synthesizer need to be optimized and validated. As will be described under conclusions, we are also

exploring now a new technology (using different funding) for creating peptide arrays on electrode surfaces.

5.3 Task 3 – Develop Surfaces & Measure Electrochemical Baseline

2005: We initially performed IR spectroscopy and Current/Voltage measurements to characterize in-house fabricated electrodes leading to the fabrication of electrode arrays (see task 4). The idea was to use these arrays and perform light-directed peptide synthesis on them. IR spectra for the bare gold electrode, the electropolymerized indole and for the peptide bound to the electropolymerized indole showed the expected peaks, verifying that the peptide was attached (in particular, upon peptide binding the peaks for the carboxylic acid appeared). Addition of polyindole predictably decreased the current at voltages high enough to split water due to its partially insulating properties. When peptide was added, the rate of electrolysis improved somewhat.

2006: We developed appropriate electrolysis conditions to consistently measure complete current vs. voltage relationships and use these to calculate a specific value related to catalysis. We saw relative currents (ratio of the currents at 0.3 V overpotential with and without Mn or using a designed Mn-binding peptide vs. a control peptide) of about 1.05. In other words, our designed Mn-binding peptides gave a slightly higher electrolysis current in the presence of Mn than in its absence or compared to peptides that did not bind Mn (**Fig. 13**). This relative measurement represents a way of setting our baseline and the modest Mn-dependent increase in current represented a reasonable point to optimize from.

Figure 13. Electrolysis curves for two electrodes” Red: the Mn-binding peptide without Mn, Blue: The Mn-binding peptide with Mn. The X-axis is voltage in volts. The Y-axis is current in mAmps.

We spent considerable time early on, working to design better gold electrode arrays and make more robust fabrication methods (that was before turning to the CombiMatrix platform). In this approach, we first electropolymerized aminated indole on the surface and then either synthesized

the peptide on that or attached a previously synthesized peptide. To alleviate the problem of polyindole conductive polymer layers cracking and separating from the gold electrodes after prolonged use, we mixed 2% of thiol-modified indole monomer into the indole monomer mixture used for the electropolymerization of the indole film, which created a very robust attachment to the gold surface, making it possible to run a much larger range of electrochemical tests and synthetic reactions on the gold/indole surface without damage. However, the thiol-modified indole monomer is time consuming to prepare and, at higher voltages (above 2.0 volts), the gold layer itself tended to detach in our original configuration. We, therefore, altered the electrode fabrication, passivating everything except the window where the electrode itself is exposed with a polymer. Since gold surfaces were giving us trouble, we tested platinum electrodes. The polyindole binds well to the platinum without having to use any kind of specific attachment function, saving considerable time, though increasing the expense of generating large arrays. We decided that gold would be sufficient for the range of voltages that we needed to use. (Neither gold nor platinum were envisioned for the final electrodes, just as test surfaces for developing catalytic function.)

We performed a series of electrochemical measurements as a function of indole layer thickness on the surface and discovered that our measurements were drifting with time and the overall current as a function of voltage was decreasing steadily. We initiated a series of studies of the surface and its behavior to prolonged measurement and determined that the edges of the electrode were clearly breaking off due to micro air bubbles that were likely forming under the edges, presumably because water was leaking under them. As the electrodes degraded, the current due to electrolysis would decrease. We, therefore, initiated a redesign of the electrode fabrication, employing a layer of the photoresist, SU-8, everywhere on the gold except over the actual region of electrode activity. No degradation of the electrode was visible in the new design. We proceeded to perform careful IV measurements and noticed that there was a significant decrease in voltage for the first three IV curves and then the measurements stabilized. The issue with the initial current drop has to do with a thin layer of material that forms over the gold during that time and apparently comes from the SU-8. This did not pose a significant problem in the measurements.

2007: Task 3 complete.

5.4 Task 4 - Fabricate electrode arrays for light directed synthesis

2007: Once the measurements on individual gold electrodes with indole and bound peptide were repeatable to within a few percent, we proceeded to develop arrays. We fabricated an array of gold electrodes, but about that time elected to put our emphasis on the commercial CombiMatrix arrays, as described in Tasks 2 and 5.

5.5 Task 5 - Develop high-throughput electrochemical analysis

2006: We did a series of tests using the initial Electrosense instrument that was delivered to us by Combimatrix and found that it was too limited for electrolysis measurements. We then worked with CombiMatrix to modify a newer version that they had since developed, ensuring that it could be used for the kinds of measurements we needed. This delayed our ability to make the necessary measurements until that instrument was delivered in late June, 2007

Figure 14. Currents from all electrodes, while stepping down the voltage 200mV each time. Tris Buffer was used for measurements

2007: **Figure 14** shows a baseline measurement using the newer CombiMatrix instrument. The y-axis is the current and the x-axis is the electrode number (proceeding from electrode 1 to 12,500 on the right). This allows us to perform either comparative current measurements at each electrode or to perform a series of current measurements at different voltages. There is a read-drift in the current as the values from each electrode are being read over time (it takes a second or so to read them all). This is easily removed by simply using every hundredth electrode as a control and adjusting the baseline according to that.

2008: We successfully attached complete peptides to electrode arrays including one of our designed Mn peptides (Peptide 2, NH_2 -EGHPGDSSS-COOH which gave positive results on gold electrodes as described in Task 6) and a control peptide (Peptide 4, NH_2 -NGHPGQGSS-COOH which does not bind any manganese and therefore has no catalytic activity), as confirmed by MALDI mass spectroscopy. However, current/voltage measurements of the type in Fig 13 above could not distinguish differences in the catalytic activity of the Mn-binding and control peptides on the CombiMatrix electrodes for water electrolysis on the functionalized chip. This gave rise to some of the ferrocene measurements discussed in Task 2 that demonstrated that the Electrosense system could measure electrochemical signals. We suspect that we will first need to optimize the activity of the designed catalysts on the CombiMatrix arrays before we will see any signal due to catalysis. This necessarily awaits the ability to generate relatively large peptide arrays needed to compare many peptides.

5.6 Task 6 - 15% increase in catalysis

2006: We have designed and tested seven potential peptide catalysts which were attached to electrodes via our conductive indole surface and current voltage curves were performed on each electrode. Like the OEC of Photosystem II, the peptides coordinate Mn^{2+} . Control peptides were also designed that lacked the residues required for Mn binding (**Fig. 15**).

1. $\text{NH}_2\text{-EGHPGEGYS-COOH}$

2. $\text{NH}_2\text{-EGHPGDSSS-COOH}$

3. $\text{NH}_2\text{-EGHPGDGSS-COOH}$

4. $\text{NH}_2\text{-EGHPGQGSS-COOH}$

Figure 15. Sequences and chemical structures of catalytic peptides 1, 2, 3 and control peptide 4.

Early measurements on a simple gold electrode showed that the voltage (and thus the power consumption) required to achieve 1.3mA of current dropped by 10% for Peptide 2, which corresponds to a 15% drop in the overpotential required to achieve this current (**Fig. 16**)

Figure 16. I/V curve comparing catalytic efficiencies of peptides 1, 2, 3 & polyHis, bound with Mn and control peptide (which does not bind Mn) on gold electrodes, for anodic oxidation of water during water electrolysis. Full-length peptides were coupled to polyindole surfaces on gold electrodes and incubated with sub-mM MnCl_2 in PBS buffer. Electrolysis measurements were done in TRIS buffer.

We have found, however, that this is very sensitive to conditions, the nature of the surface and the nature of the attachment, making it hard to reproduce.

5.7 Task 7 - 30% increase in catalysis

Not initiated

5.8 Task 8 – 45% increase in catalysis

Not initiated

6. Products Developed under the Award and Technology Transfer Activities

a. Publications

Matthew P. Greving, Pallav Kumar, Zhan-Gong Zhao, Neal W. Woodbury. 'Feature-level MALDI-MS characterization of in situ-synthesized peptide microarrays,' *Langmuir*. 2010 Feb 2;26(3):1456-9.

Pallav Kumar, Arman Ghodousi, and Neal W. Woodbury. 'Investigation of the 2-(2-nitrophenyl) propyloxy carbonyl protecting group substituent-effect on the rate of photolysis and yield of amine.' Manuscript in preparation. (Will submit to *Tetrahedron Letters*.)

Pallav Kumar, Zhan-Gong Zhao, Karl Maurer, JoAnn Williams, James P. Allen, and Neal W. Woodbury. 'The removal of the trityl group by electrochemically generated acid and use of an addressable electrode array for peptide synthesis.' Manuscript in preparation. (Will submit to *Journal of Combinatorial Chemistry*)

Northen, T., Greving, M., Woodbury, N. (2008) "Combinatorial Screening of Biomimetic Protein Affinity Materials" *Advanced Materials*, 2008,20(24), 4691-4697.

b. Patents

Northen, T.R. and Woodbury, N.W. (Arizona State University), "Microarray of Three-Dimensional Heteropolymer Microstructures and Method Therefor," 10/599,770 Nationalized, October 6, 2008.

Northen, T.R.; Woodbury, N.W. and Greving, M.P. (Arizona State University), "Microstructure and Microdomain Microarrays, Methods of Making and Using Thereof," PCT/US2006/023344 Nationalized, June 15, 2006.

Greving, M.P. and Woodbury, N.W. (Arizona State University), "Porous Acrylate Copolymer Films and Uses Therefor," 12/018,398 Non-Provisional, January 23, 2008.

7. Modeling - Not applicable