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Report

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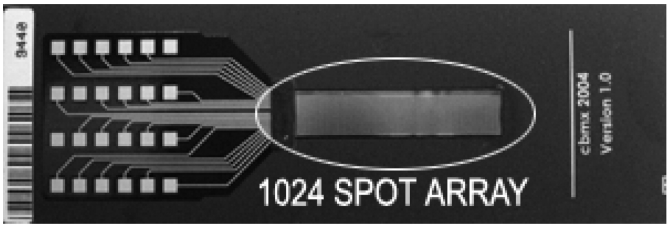

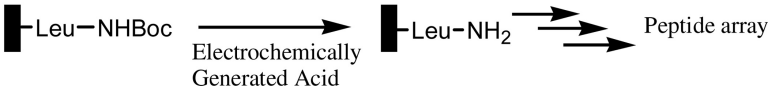

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Reports

The Removal of the *t*-BOC Group by Electrochemically Generated Acid and Use of an Addressable Electrode Array for Peptide Synthesis

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Rapid developments in the field of DNA arrays have led to a number of methods for their in situ synthetic preparation, including photolithography using both fixed and programmable masks, ink jet printing of reagents, and electrochemical deprotection techniques.[1] These techniques have led to a number of commercially available DNA microarray products.[2] The in situ synthetic preparation of a peptide array, however, has proven significantly more challenging. An in situ synthetic peptide array using photomasking techniques was originally reported by Fodor in 1991.[3] This methodology was extended in 2000 to an addressable masking technique.[4] For lower density arrays, synthesis using spotted reagents has also proven useful.[5,6] The immobilization of biosynthesized proteins directed by DNA hybridization has been shown for arrays of up to 12 proteins.[7] Given the synthetic challenges of constructing a peptide array, most recent work involving peptide arrays has utilized arrays produced by spotting presynthesized peptides or isolated proteins.[8] However, the development of a reliable synthetic procedure for massively parallel in situ synthesis of peptides on an array would allow access to a much more extensive array than can now be achieved. Herein we report the use of an electrochemically generated acid (EGA) for the removal of the *t*-BOC protecting group at selected positions on an electronically addressable array. Use of this deprotection technique allows the construction of different peptide chains at different places on the array using commercially available *t*-BOC reagents.

The substrate used for this work was a CombiMatrix CustomArray.[9] This unit consists of a semiconductor silicon chip with an array of 1024 individually addressable platinum electrodes and a porous overlaying layer on which is synthesized a DNA oligomer. The electrodes are 92 μm in diameter, reside in a 16 $\times$ 64 grid and can be set to a specified voltage via connection to a PC with appropriate software, allowing different patterns of on and off electrodes to be used at each step in the construction of the array.

The production of acid from an electrochemical reaction has been known for some time.[10] The development of a system for the electrochemical generation of acid using a hydroquinone/benzoquinone system for the removal of the DMT group during DNA synthesis has been reported by Southern.[11] Montgomery[9] reports the use of an aqueous system for a similar purpose. In this work, we chose to use a system based on 1,2-diphenylhydrazine, which is able to act as either the cathodic or anodic reagent in our electrochemical system. Although we have not undertaken a thorough mechanistic investigation of the reaction in our system, phenyl hydrazine derivatives may be oxidized initially to the azo derivative, producing two protons[12] (see Scheme 1). In our previous work with this microarray based platform, it has generally been found to be necessary to add neutralizing or scavenging species to the deprotection mixture to prevent the diffusion of the electrogenerated reagent to unwanted positions of the array. For example, in the generation of a palladium (0) species, a reoxidant was added to ensure that the desired species was present only at the active electrode.[13] When a palladium (II) species was generated, a scavenging reductant was added.[14] However, in the present case, we found that deprotection of the *t*-BOC group was well-contained, and no additional scavenging reagent was required. This remarkable containment of the

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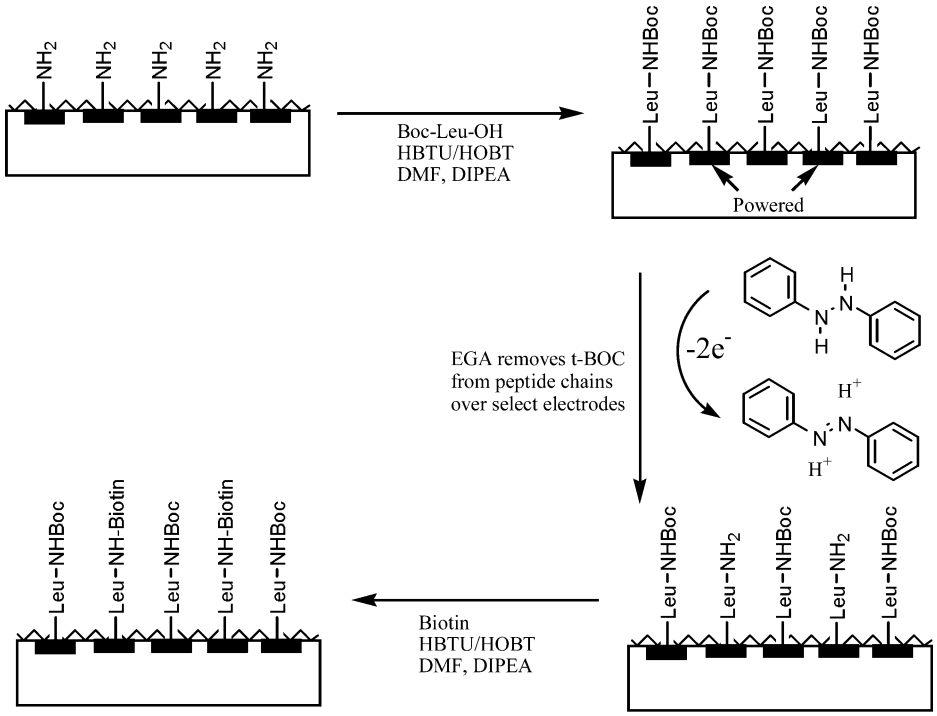
Scheme 1



reaction can be explained by the binding of the acid generated by the diphenylhydrazine itself. In the area immediately around the electrode, the acid is active because little or no diphenylhydrazine is present to protonate. When a proton diffuses away from this area, it encounters a higher concentration of diphenylhydrazine, which it protonates instead of engaging in the removal of the *t*-BOC group (Figure 1).

A CombiMatrix CustomArray DNA chip with a 15-thymidine strand of DNA terminated with a 5′ aminoethoxyethyl modifier[15] at all noncontrol electrodes[16] was exposed to a mixture of boc-leucine (120 mg, 0.52 mmol), HBTU (*O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, 190 mg, 0.50 mmol), HOBT (*N*-hydroxybenztriazole, 67 mg, 0.50 mmol), and diisopropylethylamine (261 μL, 1.50 mmol) in DMF (*N*,*N*-dimethylformamide, 1 mL) to couple leucine to all locations of the array. The chip was washed with DMF (1 mL) and methylene chloride (1 mL), then reexposed to the coupling mixture. After repeating the washing procedure (with an additional ethanol rinse to remove any residual DMF or methylene chloride), the chip was allowed to dry. The boc-leucine-covered chip was then placed in a reaction chamber with a platinum counter electrode and a solution of 1,2-diphenylhydrazine (200 mg, 1.1 mmol) and tetrabutylammonium hexafluorophosphate (400 mg, 1.0 mmol) in methylene chloride (10 mL). The array was addressed using a computer, and select electrodes were held at +3.0 V verses the platinum counter electrode for 60 s. After deblocking, the chip was removed, rinsed with ethanol to remove remaining electrochemical deblocking solution, and allowed to dry. The coupling reaction was repeated using biotin in place of boc-leucine (at 50% concentration due to solubility; see Scheme 1) followed by

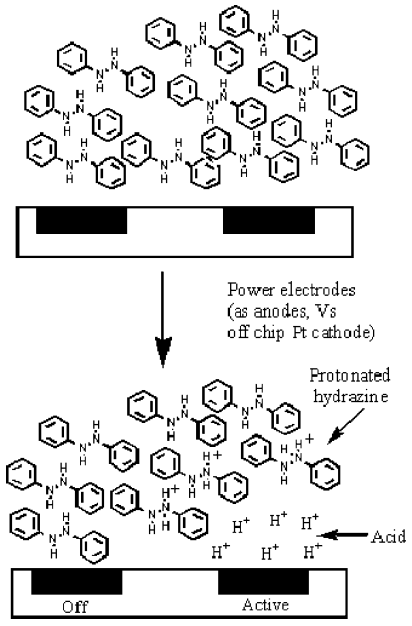


Figure 1.

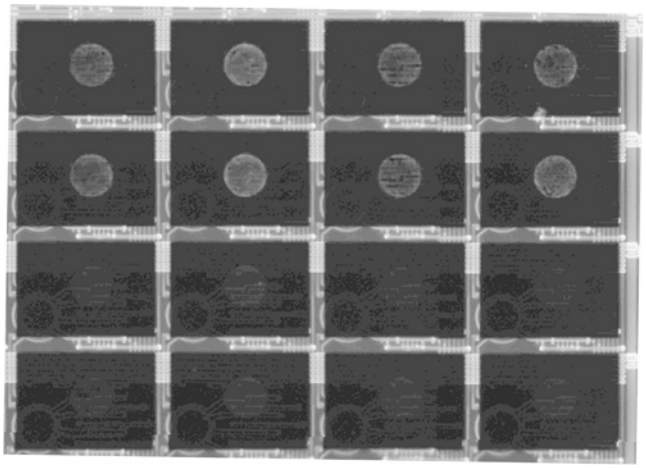
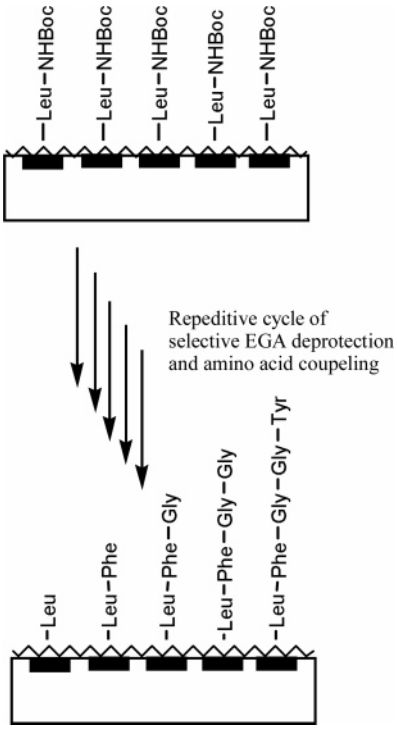


Figure 2. Image of array after Texas Red-labeled streptavidin incubation with biotin-capped peptides on the array.

Scheme 2



incubating in a solution of Texas Red-labeled streptavidin and imaging to visualize the labeled vs unlabeled areas of the array. As expected, the electrochemically deprotected portions of the array that had a free amine group able to bind to biotin show significantly more fluorescence (relative intensity 626 units) than those areas which remained *t*-BOC-protected (relative intensity 67 units; see Figure 2).[17]

Having shown that removal of the *t*-BOC group with EGA is possible, we next decided to use this procedure in the production of a peptide array (see Scheme 2). The N-terminal endorphin sequence YGGFL was chosen for this purpose because it has a known high affinity antibody, and the binding of this antibody is highly affected by the presence of the terminal tyrosine in the sequence. Following the procedures given above, a CustomArray chip was derivatized with boc-leucine and the *t*-BOC selectively removed electrochemically at the desired locations. The coupling process was repeated with boc-Phe-OH, followed again by electrochemical deblocking using a different electrode pattern, and the process iterated through two rounds of boc-Gly-OH, and boc-Tyr (*tert*-butyl)-OH coupling to construct an array of five amino acid chains of different lengths. The entire array

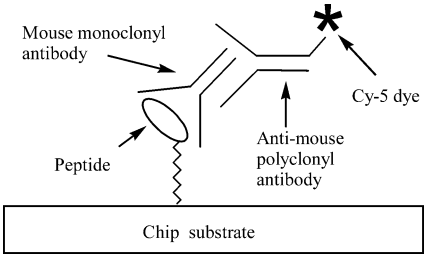


Figure 3. Antibody binding technique used to visualize the endorphin array. The antigen is initially recognized by a mouse monoclonal antibody which is, in turn, recognized by a fluorescently labeled polyclonal anti-mouse antibody.

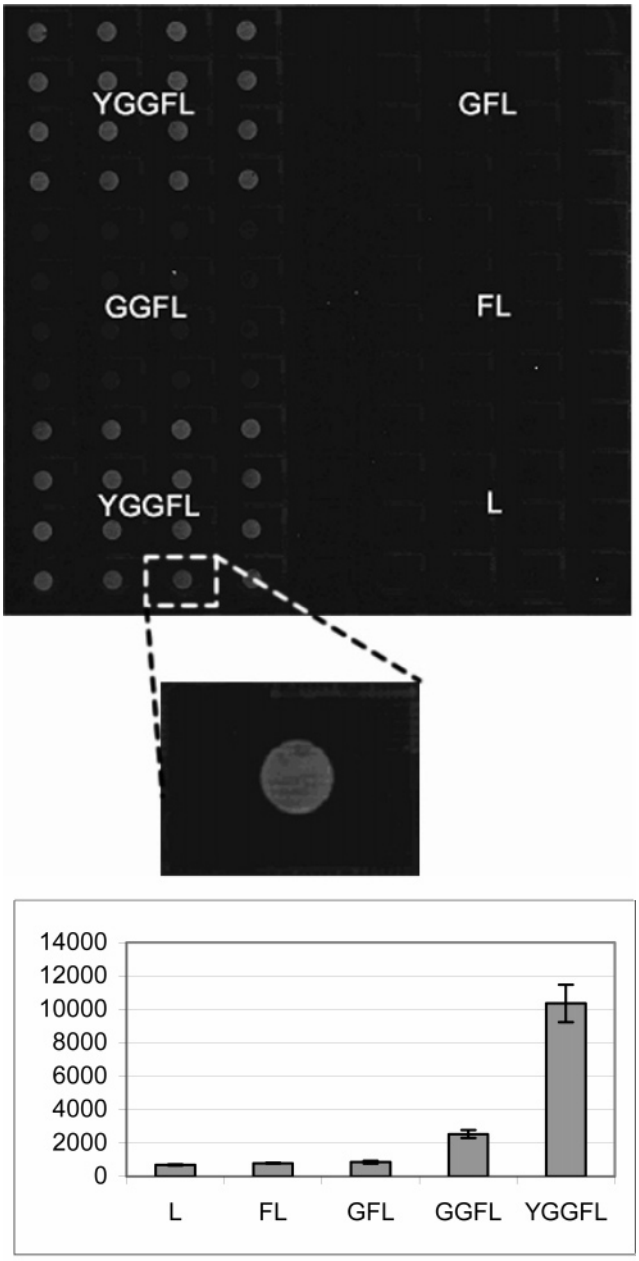


Figure 4. Image of array after antibody staining procedure. Note that the positions with full length endorphin sequence show signal (sequence YGGFL), whereas the tyrosine truncated positions (GGFL) show considerable attenuation, and further truncated positions show essentially no signal.

was then subject to standard chemical deprotection using 40% TFA in methylene chloride (30 min), followed by 90% aq TFA (30 min) to yield an array containing Leu, Leu-Phe, Leu-Phe-Gly, Leu-Phe-Gly-Gly, and Leu-Phe-Gly-Gly-Tyr (see Scheme 2). The array was rinsed with ethanol, blocked with acylated BSA (2 mg/mL in 2XPBS + 0.05% Tween 20, 30 min), incubated with primary anti-β-endorphin antibody[18] (mouse), followed by Cy-5-labeled donkey anti-mouse antibody (see Figure 3) and imaged on an Array Works imager. The label predominantly bound at the positions where the full length endorphin sequence was synthesized (YGGFL, relative intensity 10 354 units), with traces of binding at the des-Tyr positions (GGFL, relative intensity of 2536 units) and negligible binding to the

remaining peptides (GFL 855 units, FL 791 units, F 692 units) (see Figure 4). The fluorescence signal from a single electrode is shown in the expansion. The slight left-to-right striations are due to topology caused by underlying circuitry on the silicon chip substrate.

In conclusion, we have developed a method for electrochemically generating acid in sufficient concentration to remove the *t*-BOC protecting group and have used this method in conjunction with an addressable electrode array to synthesize an array of peptides containing the N-terminus of the endorphin sequence (YGGFL). Although this sequence contains generally easy to work with amino acids lacking the need for side chain protection, we are presently investigating the incorporation of the more challenging amino acids for which discrimination between the acid labile *t*-BOC group and acid labile side chain protecting groups will be required. This array represents only a 5-mer peptide; however, we have applied the technique to the synthesis of up to 10-mer peptides with encouraging results. Beyond this length, further modifications will have to be made to preserve the integrity of the polymer−DNA surface overlaying the electrodes, because damage begins to result from repeated cycling of the electrodes. One of our largest difficulties in performing this work lies in getting a good analysis of the products produced on the chips' surfaces. In this work, the use of antibody interactions allowed us to visualize the desired sequence; however, most other antibody interactions are not as strong as this one, and further development of analytical techniques will be required before coupling or deprotection efficiency can be measured directly. In time, we hope to extend this work to cover all the natural amino acids to make a fully diverse array possible.

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