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Building Addressable Libraries: Site Selective Coumarin Synthesis and the "Real-Time" Signaling of Antibody–Coumarin Binding

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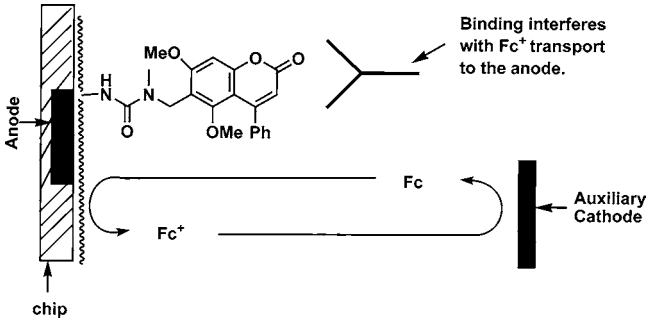
ABSTRACT



The feasibility of using active semiconductor chips containing addressable arrays of microelectrodes for the "real-time" monitoring of biologically relevant binding events has been demonstrated by detecting the binding of a coumarin substrate by an anticoumarin antibody. The coumarin substrate was synthesized proximal to predetermined electrodes on the chip with the use of a Pd(II) reagent that was itself generated by using the selected electrodes. Once the coumarin was synthesized, its binding to the anticoumarin antibody was detected by monitoring the current associated with a ferrocene–ferrocinium ion redox cycle that was established between the electrodes on the chip and a remote auxiliary electrode.

Chip-based molecular libraries can provide a valuable tool for screening molecular interactions involving a variety of biomolecules.[1] Of particular interest are libraries having the molecules located proximal to individually addressable electrodes on an active-semiconductor microarray.[2,3] In principle, the molecules in such a library can be monitored by using the electrodes thereby providing a "real-time" measure of how the molecules interact with biological receptors. With this in mind, we have been working to develop the synthetic methodology needed for synthesizing organic molecules at preselected sites on electrochemically addressable microarrays.[4,5] Taking our lead from initial efforts to use electrochemically generated acid and base on

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(1) For reviews see: (a) Lipshutz, R. J.; Fodor, S. P. A.; Gingeras, T. R.; Lockhart, D. J. *Nature Genetics* **1999**, *21*, 20. (b) Pirrung, M. C. *Chem. Rev.* **1997**, *97*, 473. For more recent examples see: (c) Webb, S. M.; Miller, A. L.; Johnson, B. H.; Fofanov, Y.; Li, T.; Wood, T. G.; Thompson, E. B. *J. Steroid Biochem. Mol. Biol.* **2003**, *85*, 183. (d) Shih, S.-R.; Wang, Y.-W.; Chen, G.-W.; Chang, L.-Y.; Lin, T.-Y.; Tseng, M.-C.; Chiang, C.; Tsao, K.-C.; Huang, C. G.; Shio, M.-R.; Tai, J.-H.; Wang, S.-H.; Kuo, T.-L.; Liu, W.-T. *J. Virol. Methods* **2003**, *111*, 55.

(2) For a description of the chips used here see: Dill, K.; Montgomery, D. D.; Wang, W.; Tsai, J. C. *Anal. Chim. Acta* **2001**, *444*, 69.

(3) For alternative approaches see: (a) Sullivan, M. G.; Utomo, H.; Fagan, P. J.; Ward, M. D. *Anal. Chem.* **1999**, *71*, 4369. (b) Zhang, S.; Zhao, H.; John, R. *Anal. Chim. Acta* **2000**, *421*, 175. (c) Hintsche, R.; Albers, J.; Bernt, H.; Eder, A. *Electroanalysis* **2000**, *12*, 660.

(4) For the use of Pd(0) catalysts see: Tian, J.; Maurer, K.; Tesfu, E.; Moeller, K. D. *J. Am. Chem. Soc.* **2005**, *127*, 1392.

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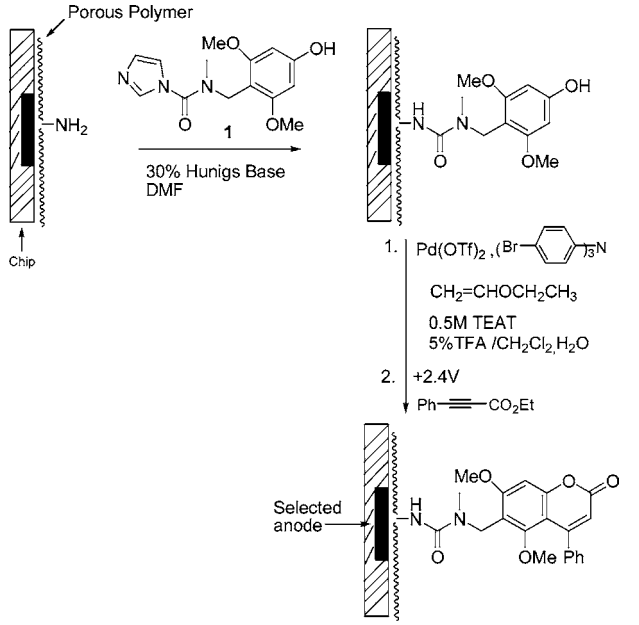
the arrays to build DNA and peptide libraries,[6,7] this work has utilized selected electrodes in the arrays to synthesize both Pd(0)[4] and Pd(II)[5] reagents for effecting chemical transformations on molecules tied to a polymer coating above the electrodes. By utilizing a chemical reagent in the solution above the array that destroys the reagent generated, the desired reactions can be confined to the region of the array immediately surrounding a selected electrode.

With a strategy for exploring new reactions in place and a number of new microarray-based synthetic methods in development, attention has turned toward determning how the microelectrodes in the array could be used to monitor the "real-time" binding of small molecules attached to the array's surface. To date, most efforts to monitor the behavior of molecules on electrode microarrays have focused on either fluorescence based approaches[8] or electrochemical amplification techniques.[9] Both techniques require washing the array following incubation of the library with a desired biological receptor and then capitalizing on an appropriate antibody bioconjugate to effect signaling. While these approaches can be very effective, the washing step is potentially problematic. For example, if one is using a library containing conformationally constrained ligands to probe the three-dimensional requirements of a receptor, then weak binding interactions can provide important information suggesting that a particular conformation is close to being correct. A separate washing step could "wash away" this information thereby changing a positive binding event into a false negative and affording inaccurate information.

For this reason, we sought a method that would allow the electrodes to directly signal a binding event on the array's surface. The overall strategy taken for the development of electrochemical sensors appeared ideal.[10] In this approach, a biological receptor is placed on the surface of a gold electrode. A redox couple is then cycled between the electrode and a remote auxiliary electrode creating a current that can be monitored. The heterogeneous electron-transfer rate is a function of the surface coverage of electroinactive species[11] and therefore as molecules bind to surface bound receptors, the observed current decreases. Several aspects of this approach were of concern with respect to its application to chip-based microarrays. First, rather than attach a large biological receptor to the electrode and then screen a series of solution phase ligands one at a time for their interactions with the receptor, the microelectrode array experiment seeks to accelerate the rate at which ligands can be screened by placing the ligands proximal to the electrodes and then probing their interactions with a solution phase biomolecule in a parallel fashion. Second and more worrisome, the current microarray based chemistry does not attach the molecules to the electrode surface, but rather binds the molecule to a porous polymer covering the entire surface of the chip. Will extra distance between the electrode and ligand/receptor interaction result in a situation where the binding event no longer interferes with the current associated with a secondary redox couple? If the answer is yes, then how do we change the overall approach so that the molecules can be directly bound to the electrodes? If the answer is no, then what distance between the electrode and the molecule being monitored can be tolerated?

To begin addressing these questions and demonstrate the feasibility of the approach, we decided to locate coumarins proximal to the electrodes of an addressable microarray. The ability of the array to signal a binding event would then be determined by treating the array with commericially available, coumarin specific antibodies. With this in mind, our attention turned toward a site-selective synthesis of coumarins on the microarrays. To this end, the synthetic protocol outlined in Scheme 1 was followed.

Scheme 1



The synthesis began by coupling a phenol substrate to the surface of the array with use of an amine based porous polymer.[12] The phenol was placed on the entire surface of

(5) For the use of Pd(II) see: (a) Tesfu, E.; Maurer, K.; Ragsdale, S. R.; Moeller, K. D. *J. Am. Chem. Soc.* **2004**, *126*, 6212. (b) Tesfu, E.; Maurer, K.; Moeller, K. D. *J. Am. Chem. Soc.* **2006**, *128*, 70.

(6) For the lead patent on DNA related work see: Montgomery, D. D. PCT Int. Appl. 1998, 91 pp, CODEN: PIXXD2 WO 9801221 A1 19980115.

(7) For peptide based libraries see: Rossi, F. M.; Montgomery, D. D. PCT Int. Appl. 2000, 52 pp, CODEN: PIXXD2 WO 0053625 A2 20000914. For recent work see: Oleinikov, A. V.; Gray, M. D.; Zhao, J.; Montgomery, D. D.; Ghindilis, A. L.; Dill, K. *J. Proteome Res.* **2003**, *2*, 313 as well as ref 2 above.

(8) For examples, see refs 2 and 4−7 above.

(9) (a) Dill, K.; Montgomery, D. D.; Ghindilis, A. L.; Schwarzkopf, K. R.; Ragsdale, S. R.; Oleinikov, A. V. *Biosens. Bioelectron.* **2004**, *20*, 736. (b) Dill, K.; Montgomery, D. D.; Ghindilis, A. L.; Schwarzkopf, K. R. *J. Biochem. Biophys. Methods* **2004**, *59*, 181.

(10) Murata, M.; Gonda, H.; Yano, K.; Kuroki, S.; Suzutani, T.; Katayama, Y. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 137.

(11) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods, Fundamentals, and Applications*, 2nd ed.; John Wiley and Sons: New York, 2001.

(12) (a) For a previous use of this polymer see: Maurer, K.; McShea, A.; Strathmann, M.; Dill, K. *J. Comb. Chem.* **2005**, *7*, 637. (b) Chips coated with this polymer either as a "blank chip" or a synthesized DNA array are commercially available and can be purchased from CombiMatrix Corporation, 6500 Harbour Heights Pkwy., Suite 310, Muilteo, WA 98275; http://www.combimatrix.com.

the chip supporting the array. The coumarin synthesis then capitalized on a Pd(II)-catalyzed cycloaddition between the phenol substrate and an acetylene in the solution above the array.[13] This chemistry used the methodology developed previously for doing site-selective Pd(II)-mediated oxidations on the arrays.[5] In this way, Pd(0) was generated in the solution above the array by a Wacker oxidation involving $Pd(OTf)_2$ and ethyl vinyl ether. The Pd(0) was then converted to the desired Pd(II) reagent at selected electrodes by using them as anodes to oxidize an amine and generate a radical cation, which in turn oxidized the Pd(0). The Pd(II) catalyst generated in this fashion was confined to the region of the chip surrounding the electrode with the use of excess ethyl vinyl ether. The success of this strategy was analyzed by imaging the array with the use of a biotinylated anticoumarin antibody[14] followed by incubation with a Texas red conjugated steptoavidin.[15] Figure 1 shows a fluorescent microscope

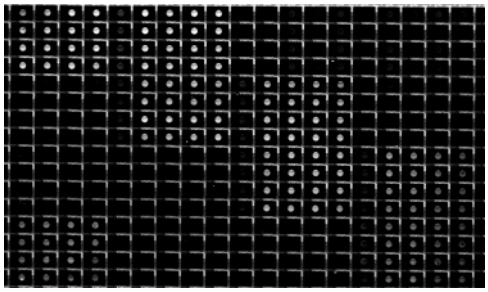
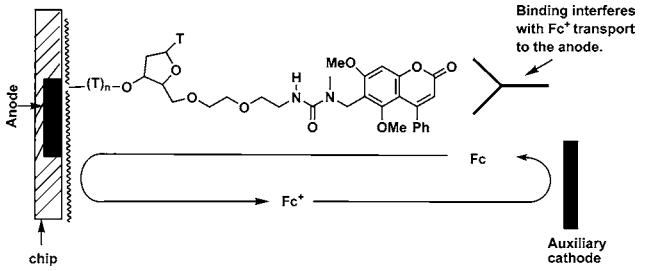


Figure 1. "Zigzag" pattern of electrodes used for site-selective coumarin synthesis.

image of an array where a "zigzag" pattern of electrodes (blocks of 32) were used as anodes. The effectiveness of the confinement strategy is readily apparent. In direct analogy to the use of site-selective catalytic Pd(0) reactions on the arrays,[3] it was clear that the strategy for effecting site-selective Pd(II) reactions was not just effective for oxidation reactions requiring a stoichiometric Pd(II) reagent, but also for transformations utilizing catalytic Pd(II).

Having effectively located coumarins proximal to electrodes on the surface of a chip, attention was turned to the signaling experiments. For this work, an electrode microarray having ca. 12 000 electrodes/cm^2 was utilized.[16] Of the ca. 12 000 electrodes, three blocks of 121 electrodes were employed as anodes for the coumarin synthesis. On the first block, the coumarin was linked to the polymer through a single thymidine unit having an aminoethoxyethyl terminating group (Scheme 2, $n = 0$). On the second block, the coumarin was linked to the polymer by using 5 thymidines

Scheme 2



($n = 4$) having an aminoethoxyethyl terminating group. On the third block, the coumarin was linked to the polymer by using 15 thymidine units ($n = 14$) having an aminoethoxyethyl terminating group. The entire electrode array schematically illustrated in Scheme 2 was then submerged in a solution of an iron redox couple (ferrocene acetic acid/ferrocinium acetic acid) and electrolyte. A platinum counter electrode having an area of 0.75 cm^2 was then placed over the chip. The distance between the chip and the counter electrode was kept at approximately 650 to 800 μm with the use of an O-ring.[17] Cyclic voltammetery was performed from −0.4 to 1.2 V (this potential represents the cell potential and is reported as the potential of the working electrode relative to the Pt counter electrode) at a sweep rate of 100 mV s^{-1} by using the electrodes on the chip as anodes and the remote auxiliary electrode as the cathode. The addition of an anticoumarin antibody was expected to interfere with Fc^+ (ferrocinium acetic acid) transport to the anode on the chip reducing the total current flow.

From the start, only a small signal could be observed for the blocks of electrodes having the coumarins linked to the surface with 5 thymidine units while no signal could be observed for the blocks of electrodes having 15 thymidine units. However, a clear signal could be observed for the block of electrodes having the coumarin linked to the surface with a single thymidine. The results of this experiment are illustrated in Figure 2. The initial current measurement was made in the absence of any antibody and is represented by the black curve. The array was then treated with an anti-2,4-DNP antibody and the current measured giving rise to the red curve illustrated in Figure 2. No significant drop in current was measured indicating that there was minimal nonspecific interaction between the antibody and the array's surface. Following this control experiment, the chip was treated with the anticoumarin antibody used in the imaging experiment illustrated in Figure 1. The current was measured and gave rise to the green curve illustrated in Figure 2. Clearly, the overall strategy employed is capable of providing a "real time" signal in response to a small molecule−antibody binding event. Even with the current level of sensitivity, the use of only 121 electrodes on a 12K chip suggests that libraries of approximately 100 molecules can be rapidly screened.

(13) Oyamada, J.; Jia, C.; Fujiwara, Y.; Kitamura, T. *Chem. Lett.* **2002**, 380.
(14) Available from Vector Laboratories, catalogue no. BA-0606.
(15) Available from Molecular Probes, catalogue no. S872.
(16) For these experiments, a CombiMatrix 12K CustomArray DNA chip with a variable length polythymidine strand of DNA terminated with a 5′-aminoethoxyethyl modifier (Glen Research) was used. For ordering information see ref 11.
(17) The distance between the electrodes was dependent upon the nature of the O-ring material and the pressure used to hold the plates together.

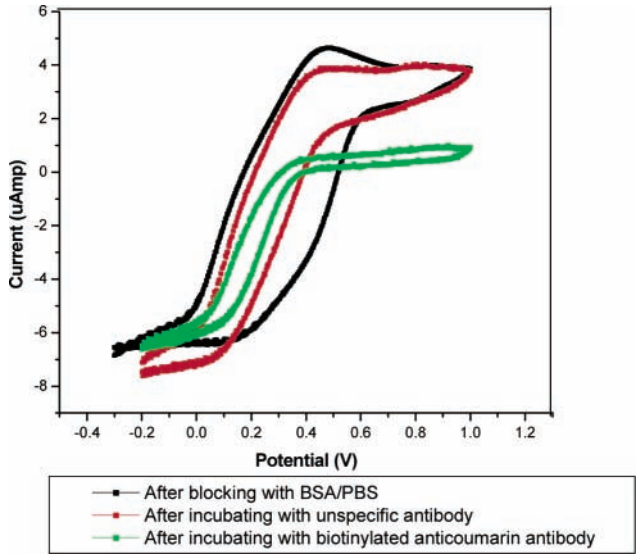


Figure 2. Cyclic voltammetry of ferrocene acetic acid on Pt electrodes with coumarin linked to a polymer through a single thymidine having an amino ethoxy ethyl terminating group. All measurements were performed in PBS buffer and the concentration of the ferrocene acetic acid was 8 mM.

In conclusion, we have demonstrated that the same confinement strategy for isolating Pd(II)-mediated oxidations to site-selective locations on a chip can also be employed for conducting site-selective cycloaddition reactions that utilize a Pd(II) catalyst. This reaction scheme was utilized to synthesize coumarins on the surface of an addressable electrode array. The coumarins were used to probe an electrochemical method for monitoring ligand/receptor binding events on the arrays. The method for detecting binding on the array surface was designed in analogy to existing electrochemical sensors. However, in the case of the array the coumarins were attached to a polymer covering the surface of the chip and not directly attached to the electrodes themselves. The presence of this polymer did not prevent detection of the binding event; however, the stregnth of the signal is dependent on the distance between the coumarin and the electrode used for monitoring its behavior. At the present time, the level of sensititivity obtained would allow for the rapid monitoring of small libraries of molecules. Current work is focusing on optimizing the sensitivity of the system, probing the size of the library that can be monitored, and examining the use of the overall strategy for differentiating between the binding of an antibody in solution with different substrates on the surface of the chip.

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Note Added after ASAP Publication. There were errors in the Abstract graphic and Scheme 2 in the versions published ASAP January 28 and 31, 2006; the corrected version was published ASAP February 1, 2006.

Supporting Information Available: Experimental details for the synthesis of **1**, sample procedures for the chip-based experiments, and CV data with 5 and 15 thymidine linkers on the polymer. This material is available free of charge via the Internet at http://pubs.acs.org.

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