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Building Addressable Libraries: Amino Acid Derived Fluorescent Linkers

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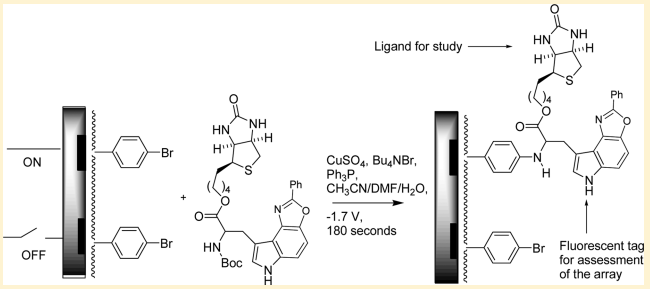
Supporting Information

ABSTRACT: A new amino acid derived fluorescent linker for attaching molecules to the surface of a microelectrode array has been developed. Molecules to be monitored on an array are attached to the C-terminus of the linker, the N-terminus is then used to attach the linker to the array, and the side chain is used to synthesize a fluorescent tag. The fluorescent group is made with a one-step oxidative cycloaddition reaction starting from a hydroxyindole group. The linker is compatible with site-selective Cu(I)-chemistry on the array, it allows for quality control assessment of the array itself, and it is compatible with the electrochemical impedance experiments used to monitor binding events on the surface of the array.



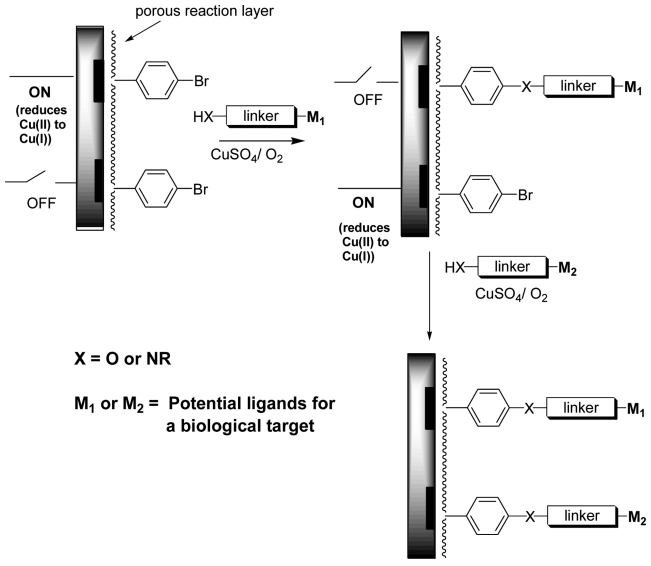
INTRODUCTION

Microarray-based molecular libraries can be powerful tools for probing the binding preferences of biological targets.[1,2] However, current efforts do have several limitations. Efforts to identify the molecules in the library that bind a receptor typically involve multiple washing steps, and it is difficult to fully characterize the molecules in the library. This leads to a loss of information about weak-binding interactions between library members and the receptor, and a lack of quality control with respect to the library itself. One method for potentially avoiding these difficulties would be to probe the interactions with the use of a microelectrode array.[3−5] In such efforts, each unique member of the library is placed or synthesized next to a unique, addressable electrode (or set of electrodes) in the array.[6−9] The electrodes can then be used to both conduct impedance experiments that detect binding-events between the molecules in the library and the receptor in "real-time"[10] and reclaim molecules from the library so that they can be characterized.[11]

The synthetic strategy used to place or synthesize molecules on the surface of the microelectrode array is illustrated in Scheme 1.

In this example, a Cu(I)-catalyzed coupling reaction is used to add a molecule with a heteroatomic nucleophile to an arylbromide[12] on the surface of the array.[9b] The reaction places the molecule next to selected electrodes in the array by using the electrodes to generate the Cu(I)-catalyst. Oxygen is then used to reoxidize the Cu(I)-catalyst in the solution above the array before the catalyst can migrate to electrodes not selected for the reaction. For each new molecule to be added to the array, a new set of electrodes is used to generate the catalyst.

Scheme 1



Central to this effort is the linker used to connect the molecules to the polymer coating on the array. The linker needs to be readily accessible, compatible with both the synthetic and analytical electrochemistry used to build and monitor molecules on the arrays, and fluorescent. To date, no linker has been able to satisfy all three requirements, a fact that

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has been limiting development of the arrays. We report herein a solution to this problem that capitalizes on amino acid derived linkers.

It is important for the linkers to be fluorescent. Microelectrode arrays typically fail when a short-circuit in their wiring permanently turns on all of the electrodes in a region of the array. When this occurs, a reaction on the array cannot be confined. It occurs at both the electrodes selected for generating the reagent of choice and every electrode accidently activated by the short-circuit. Since the software used to assess whether an array is working properly simply checks to see if all of the electrodes are active, it does not detect this problem. In addition, all of the electrodes being active does not mean that they are all working equally well. Does using two different electrodes to run a reaction on the array really place roughly equal amounts of material by both of the electrodes? The use of a fluorescent linker allows one to answer these questions and assess the quality of the array before investing the time, money, and effort needed to build and analyze a molecular library.

Our first attempt at building a fluorescent linker took advantage of a disubstituted pyrene **1** (Figure 1).[13,14] The effort

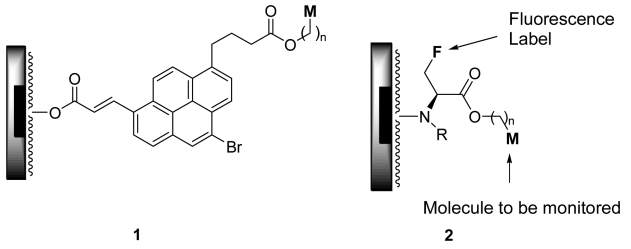


Figure 1. Past (**1**) and newly designed (**2**) fluorescent linkers.

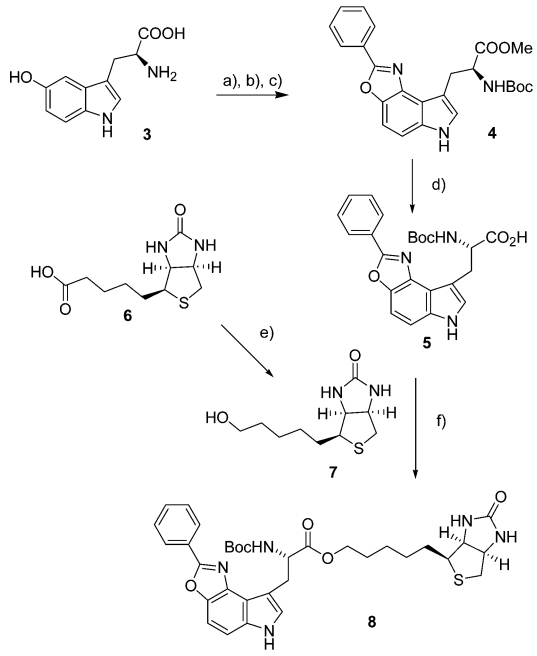
was partially successful in that it allowed us to assess the quality of the array. However, the linker was very difficult to synthesize. Construction of the disubsituted pyrene core of the linker required the bromination of a pyrenyl butanoic acid derivative, a reaction that led to a complex mixture of products, generated only low yields of those products, and could not be scaled up. Because of these synthetic problems, the compatibility of the linker with electrochemical signaling experiments on the arrays could not be determined. A better approach was needed, and it appeared that an amino acid derived linker like **2** might provide this opportunity. The plan called for using the C-terminus of the amino acid to attach molecules for study on the arrays, the N-terminus of the amino acid to connect the linker to the array, and the side chain functionality of the amino acid to build the fluorescent tag. The approach was intriguing because it would allow for variations in the fluorescent group to be made without requiring a change in the overall strategy. It would also capitalize on the very gentle Cu(I)-chemistry outlined in Scheme 1 to attach the molecules to the array.

RESULTS AND DISCUSSION

Two questions about the plan immediately arose. First, what should the fluorescent group be, and second, would the amino acid linker and the fluorescent group selected be compatible with the electrochemical impedance experiments employed for monitoring binding events on the arrays?

The choice of a fluorescent tag was based on the availability of the amino acid starting material and the simplicity of the synthetic route needed for its construction. With this in mind, we elected to take advantage of an oxidative strategy for synthesizing fluorescent groups from hydroxylated indole rings.[15] The synthesis started with 5-hydroxytryptophan (Scheme 2).

Scheme 2. [a]



[a]Reagents and Conditions: (a) $SOCl_2$, MeOH, RT. (b) Boc_2O, Na_2CO_3, Dioxane/H_2O, RT. (c) MnO_2, Benzyl amine, dry THF, 0 °C–RT, 31% in three steps. (d) LiOH, THF/MeOH/H_2O, RT, 92%. (e) $LiAlH_4$, THF, 45 °C, 84%. (f) DCC, DMAP, DMF, RT, 63%.

Following protection of the C-terminus and N-terminus of the amino acid, the fluorescent label was constructed from the hydroxyindol with the use of an oxidative cycloaddition. While the yield of the cycloaddition was low, it provided a readily scalable, one-step synthesis of the fluorescent group that could be varied by simply changing the arylamine used. Next, biotin was attached to the C-terminus of the amino acid derivative so that biotin/streptavidin interactions could be used to determine the compatibility of the linker with electrochemical impedance experiments on the arrays. This was accomplished by saponification of **4** followed by esterification of the resulting acid with alcohol **7**.[16]

Compound **8** was then placed onto an array having 12,544 microelectrodes·cm^{-1} (Scheme 3). The array was coated with a diblock copolymer containing a cinnamoyl functionalized methacrylate block and a poly(*p*-bromostyrene) block.[12] The cinammoyl groups were photochemically cross-linked to provide the necessary stability for the surface. At this point, the *t*-Boc-protected amine in compound **8** was directly coupled to the poly(*p*-bromostyrene) block of the polymer proximal to selected electrodes by using the chemistry highlighted in Scheme 1.[12,17] To this end, the array was treated with a $CH_3CN/DMF/H_2O$ solution containing the substrate, copper sulfate, triphenylphosphine, and tetrabutylammonium bromide. The electrodes selected for the reaction were set at a potential of −1.7 V vs the Pt-counter electrode for 180 s in order to reduce the copper sulfate to Cu(I) where the catalyst was needed. Oxygen was used as a solution-phase oxidant to prevent migration of the Cu(I)-catalyst to remote sites on the array. Initially, the electrolysis reaction was conducted at 10

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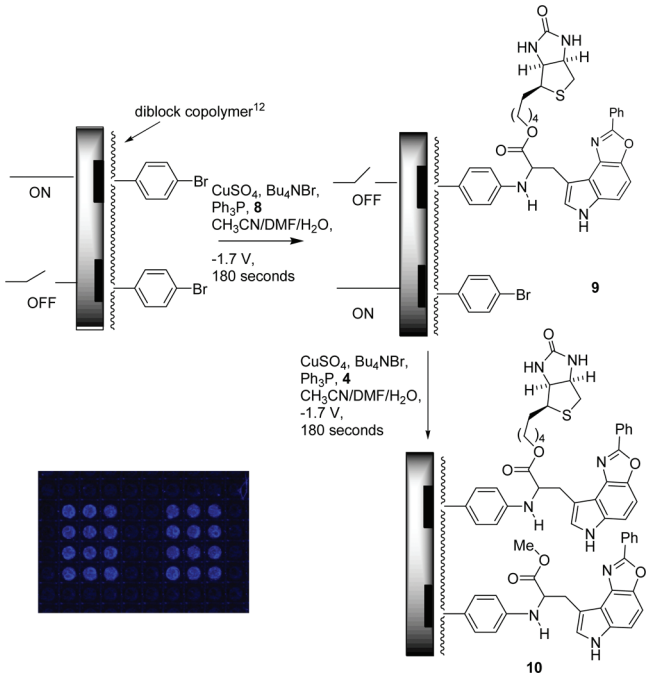
Scheme 3



separate sites on the array each containing 12 microelectrodes. When the reaction was complete, the array was washed with water and ethanol. The reaction was then repeated, but in this second case, compound **4** was used in place of **8** as the substrate and a new set of electrodes was employed. Again, the reaction was run at 10 separate sites on the array each containing 12 microelectrodes. The result was an array that contained both blocks of electrodes that were functionalized with the linker and biotin (compound **8**) and blocks of electrodes that were functionalized with only the linker (compound **4**). When the reactions were complete, the array was washed with water and ethanol and then imaged with a fluorescence microscope. The picture provided in Scheme 3 shows two of the electrode blocks functionalized with the linker and biotin. The image clearly shows that the array worked perfectly. The reaction was nicely confined to the selected electrodes, and roughly equal amounts of material were placed at each of the electrodes.

Interestingly, solution-phase experiments using these same reaction conditions showed that Cu(I) did not catalyze the addition of either a t-Boc protected nitrogen or an indole ring to the arylbromide. Instead, the reaction conditions led to removal of the t-Boc group and then direct coupling of the resulting amine to the arylbromide. The deprotection is not unexpected, since the reaction on the array is an undivided cell electrolysis that generates acid at the anode.

With the functionalized array in hand, signaling experiments were conducted by randomly selecting three of the ten electrode blocks functionalized with the linker plus biotin, three of the ten electrode blocks functionalized with only the linker, and three unfunctionalized blocks of 12 electrodes each from the remainder of the array. The array was then submerged in a series of solutions containing streptavidin and 8 mM $K_4[Fe(CN)_4]_2/K_3[Fe(CN)_4]_3$ in 1× phosphate buffer saline (PBS). The solutions ranged in concentration of streptavidin from 10^{-6} to 10^{-20} M. The first solution used was the 10^{-20} M streptavidin solution. After submerging the array in this solution, a cyclic voltammogram was recorded for the iron in solution at each of the nine selected blocks of electrodes by sweeping the potential at the electrodes from −700 to 700 mV relative to the counter electrode.[18] A sweep rate of 400 mV/s was used. Following this experiment, the array was washed three times with a 10^{-19} M streptavidin solution and inserted into the next solution having 10^{-19} M streptavidin. This was done to ensure a consistent concentration of 10^{-19} M streptavidin across the surface of the array. The cyclic voltammetry experiment was then repeated at all nine blocks of electrodes. The array was then washed three times with a 10^{-18} M streptavidin solution and inserted into the solution having 10^{-18} M streptavidin. The cyclic voltammetry experiment was again repeated at all nine blocks of electrodes. This process was continued until a cyclic voltammogram for the iron in solution had been recorded at all nine blocks of electrodes for every concentration of streptavidin. At this point, the data recorded at one of the blocks of electrodes having biotin on the surface (Figure 2) was examined for the potential (25 mV) at

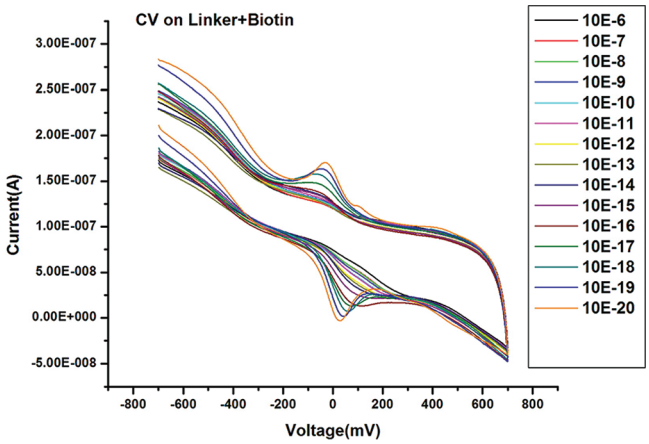


Figure 2. Sample voltammograms for one block of electrodes.

which the greatest drop in current had occurred with increasing streptavidin concentration. The presence of streptavidin causes a drop in current because it binds the biotin on the surface of the array and blocks iron from reaching the electrode below.

The current measured at this potential was then used to compare all of the cyclic voltammograms measured at each concentration of streptavidin used. This data is summarized in Figure 3. Each data point in the figure is the average of the current measured for the three blocks of electrodes used at 25 mV. For example, the data point on the red line at a concentration of 10^{-14} M streptavidin represents the average current measured at 25 mV for this concentration of receptor at the three blocks of electrodes with linker plus biotin on the surface. The point on the green line at this concentration represents the average current measured at 25 mV for the three blocks of electrodes with only the linker on the surface. The data has been normalized and the largest change in current (greatest impedance) observed scaled from zero to one. The largest current measured for each set of data was given a value of zero. In this way, the data directly reflects the binding of streptavidin to the surface of the array. The larger number on the vertical axis, the greater the binding of the receptor to the surface.

The black line in Figure 3 summarizes the data for the three blocks of unfunctionalized electrodes taken at random places

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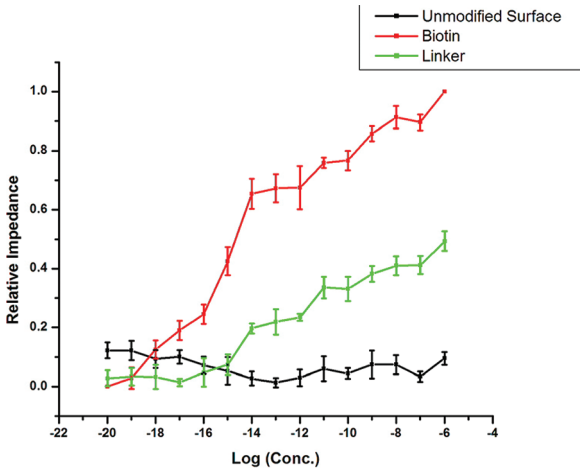

Figure 3. Summary of the CV data for all nine blocks of electrodes. Normalized curves are shown for the impedance measured electrodes functionalized with the fluorescent linker and biotin (red), electrodes functionalized with the fluorescent linker and no biotin (green), and unfunctionalized electrodes (black).

on the array. This data shows that the streptavidin shows no binding to the unfunctionalized polymer coating the array. The green line in the figure shows the data for the three blocks of electrodes functionalized with the linker (**4**) and no biotin. It indicates that the streptavidin does bind the surface once it has been functionalized. The red line shows the binding of streptavidin to the surface of the electrodes functionalized with the linker and biotin. The difference between the green and red lines shows that the presence of the biotin causes a significant increase in the binding of the streptavidin to the surface of the electrodes.[19] The nature of this difference can be seen clearly by taking the difference between the lines (Figure

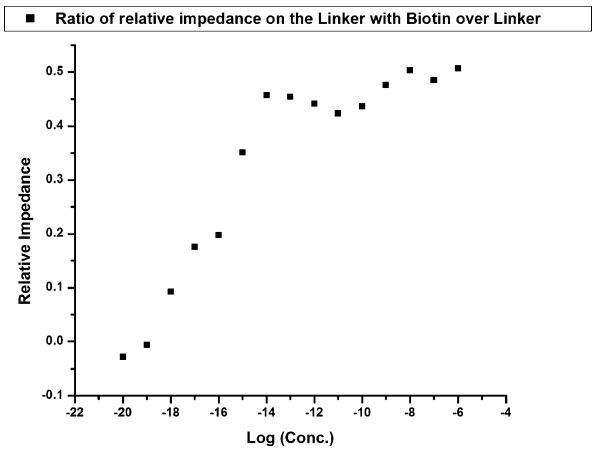

Figure 4. Difference data for the current drops associated with the electrodes functionalized with the linker plus biotin (red line in Figure 3) and the electrodes functionalized with only the linker (green line in Figure 3).

4). Initially, the binding of streptavidin to the surface of the electrodes functionalized with biotin increases much more rapidly with increasing protein concentration than it does to the surface of the electrodes that are functionalized with only the linker. At higher concentrations of streptavidin, this difference falls off, and the rate of change at the two electrodes with increasing streptavidin concentration becomes almost equal (parallel slopes in Figure 3).

Since there is only about 20−50 fmol of material by each electrode in the array, at high concentrations of streptavidin the biotin becomes completely bound. Hence, any further binding of streptavidin at these sites results from nonspecific binding of the streptavidin to the surface. The fact that at these concentrations the rate of change in the impedance at sites with biotin is the same as the rate of change for sites without the biotin indicates that the nonspecific binding of streptavidin to the surface is the same in both places. Hence, the difference in impedance between electrodes functionalized with biotin and electrodes functionalized with only the linker at low concentrations shows the binding of streptavidin to the biotin. Clearly, both the polymer surface coating the electrodes and the fluorescent linker are compatible with the electrochemical impedance experiments needed to monitor binding events on the arrays.

In conclusion, the use of an amino acid derived fluorescent linker provides a convenient handle for site-selectively placing molecules on the surface of a microelectrode array. The linker is easy to synthesize and can be rapidly functionalized with different biological probes to be studied or substrates to be development on the array's surface. The linker enables quality control assessment of the array itself, is compatible with the analytical experiments used to probe binding events on the surface of the array, and is stable enough to allow for the multiple analytical experiments needed to evaluate binding events in a quantitative manner. The linker design reported here will be incorporated into all future efforts to capitalize on the arrays.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details for the synthesis of the fluorescent linker, for coating the arrays with the diblock copolymer, for site-selectively functionalizing the polymer, and for conducting the signaling studies are provided along with the complete ref 15. This material is available free of charge via the Internet at http://pubs.acs.org.

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