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Site-Selective Chemistry and the Attachment of Peptides to the Surface of a Microelectrode Array

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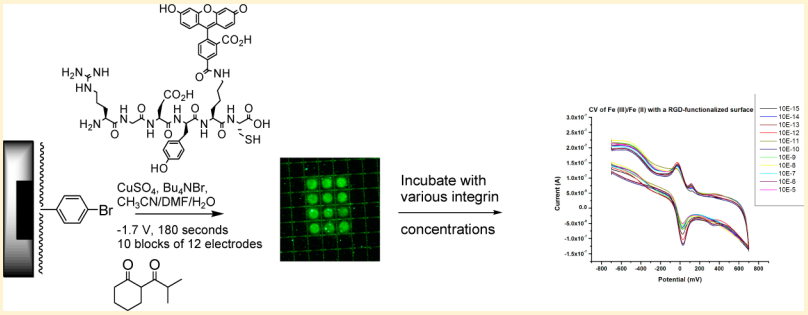
Supporting Information



ABSTRACT: Peptides have been site-selectively placed on microelectrode arrays with the use of both thiol-based conjugate additions and Cu(I)-coupling reactions between thiols and aryl halides. The conjugate addition reactions used both acrylate and maleimide Michael acceptors. Of the two methods, the Cu(I)-coupling reactions proved far superior because of their irreversibility. Surfaces constructed with the conjugate addition chemistry were not stable at neutral pHs, especially the surface using the maleimide acceptor. Once a peptide was placed onto the array, it could be monitored in "real-time" for its interactions with a biological receptor.

INTRODUCTION

Microelectrode arrays have great potential as tools for monitoring the interactions of small molecules and biological receptors in "real-time".[1−3] Typically, when microelectrode arrays are used as bioanalytical sensors, the surface of the array is functionalized with a receptor or antibody, and then the array is used to identify ligands for the receptor in solution.[4−6] We have been working to reverse this approach so that the electrochemical impedance experiments can be used to probe the binding of small-molecule libraries to receptors as those events occur. To accomplish this task, the molecules in the molecular library must be built or placed onto the microelectrode array so that each unique member of the library is located next to a unique, individually addressable microelectrode in the array.[7] The microelectrodes are then used to monitor interactions that involve molecules in the library and a receptor as illustrated in Figure 1.[8]

In this experiment, a current is established at each electrode in the array by the addition of an iron redox couple to the solution above the array. The iron(II) species in the couple is oxidized at the electrodes in the array (the anode), and the iron(III) species is reduced to iron(II) at a remote cathode. When a receptor in solution (green ball) binds to one of the molecules in the library on the array, it impedes the iron(II) from reaching the array. The result is a drop in the current measured at the associated electrode that both signals the binding event and identifies the molecule involved.

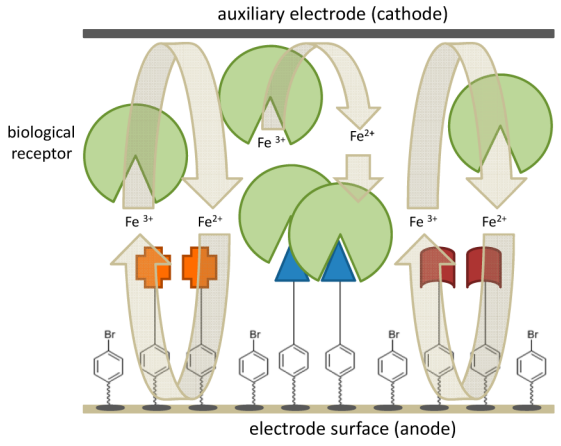


Figure 1. Planned impedance experiment.

One of the most attractive features of this experiment is that it can be used to rapidly provide biological data for a newly synthesized molecule without the need for a labeled receptor or immunological assay. For example, if one has a library of molecules that target a particular receptor already on an array, then a new molecule can be added to the existing library, the impedance experiment conducted with the receptor in question, and the binding-data obtained for the new molecule

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immediately compared with data gathered for the existing library. These data can then be used to guide subsequent synthetic efforts. Of course, key to this experiment is the ability to place a newly synthesized molecule on the array so that it is located exclusively on the electrodes used to monitor its behavior.

With this in mind, we have been developing methods for site-selectively adding peptides and peptidomimetics to the surface of a microelectrode array.[8b] The focus on small peptides and peptidomimetics is motivated by the overall utility of the molecules as probes for interrogating a variety of different biological targets.[9]

AN INITIAL APPROACH

The initial plan called for the use of a thiol-based conjugate addition strategy to add the peptides to a polymer surface-coating on the arrays (Scheme 1).[8b] Our hope was to convert a well-known approach for functionalizing polymers[10] into a site-selective reaction on the array. Initially, two strategies were followed. One placed a maleimide group onto the array, and the second placed an acrylate moiety (as illustrated [Scheme 1]) onto the array. In either case, the surface of the array needed to be coated with a porous reaction layer. This reaction layer provides attachment sites for fixing substrates to the surface of the electrodes. For the exploration of new array-based reactions, agarose is an ideal surface. It is both stable to a host of chemical reagents and easily removed from the surface of the array following the completion of a reaction. This last point is important because it allows for the arrays to be recycled. In this way, a single microelectrode array can be used to probe a variety of different reaction conditions.

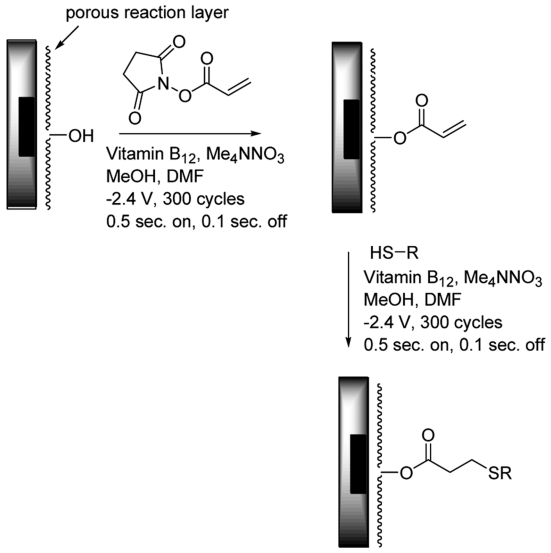
To conduct the sequence outlined in Scheme 1 in a site-selective fashion, the initial placement of the "Michael acceptor" on the surface of the array needs to be confined. This is required because thiol-derived conjugate addition reactions are base-initiated chain reactions in that the product from the conjugate addition reaction is a strong enough base to deprotonate the thiol substrate and regenerate the nucleophile. Hence, the thiolate is not consumed and can migrate to other sites on the array. For example, consider the reaction illustrated in Scheme 2. In this case, the base-catalyzed esterification reaction between the activated acrylate ester and an agarose coating was used to place the "Michael acceptor" by every microelectrode in a 1K-array (an array that has 1028 microelectrodes/cm^2).[11] The conjugate addition with the thiol nucleophile was then conducted by the generation of base at every other electrode in the array. The nucleophile was labeled with a pyrene moiety so that its placement on the array could be monitored with a fluorescence microscope. Following each reaction, the array was washed with an excess of ethanol, water, and then ethanol again. After the washing steps, the array was allowed to dry. As shown in the image provided, the conjugate addition reaction happened to a nearly equal extent at every microelectrode in the array. There was no difference between electrodes selected for the conjugate addition and those that were not.

Fortunately, the base-catalyzed first step in the sequence can be confined.[12] The reaction works by reducing vitamin B_{12} to a radical anion that in turn deprotonates alcohols on the agarose surface. The alkoxides then react with the solution-phase-activated ester, a reaction that consumes the alkoxide and generates a solution that is not basic enough to catalyze additional esterification reactions. Of course, the base being generated at the selected electrodes can migrate to other regions on the array if either the reduced vitamin B_{12} or the alkoxides on the surface of the array deprotonate methanol. Hence, to confine the esterification to selected electrodes on the array requires a confining agent in solution that can consume methoxide. To this end, the use of excess activated ester is ideal (Scheme 3). In the experiment shown, a single electrode in a 12K-array (an array that has 12,544 microelectrodes/cm^2) was used as a cathode to conduct the esterification reaction with the activated ester. The entire array was then used to generate base and trigger the thiol-derived conjugate addition reaction. Clearly, the addition occurred at only the microelectrode used for the esterification.

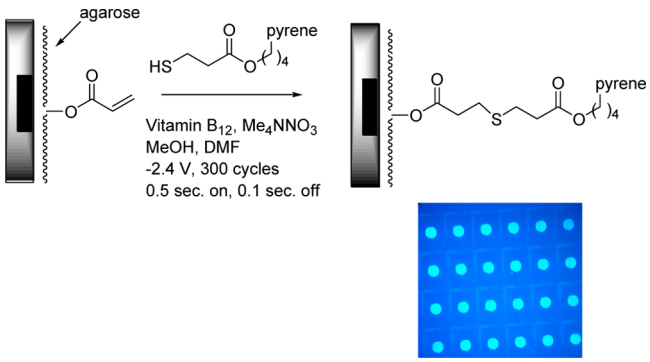
The conjugate addition strategy can also be used to place peptides on the arrays (Scheme 4).

In the reaction shown, a fluorescently labeled RGD-peptide (**1**) was placed by 10-electrodes in a checkerboard pattern on a 1K-array. The success of the reaction was monitored with a fluorescence microscope. The RGD-peptide-functionalized array allowed us to demonstrate the utility of the electrochemical impedance experiment suggested in Figure 1.[8b]

Scheme 1



Scheme 2



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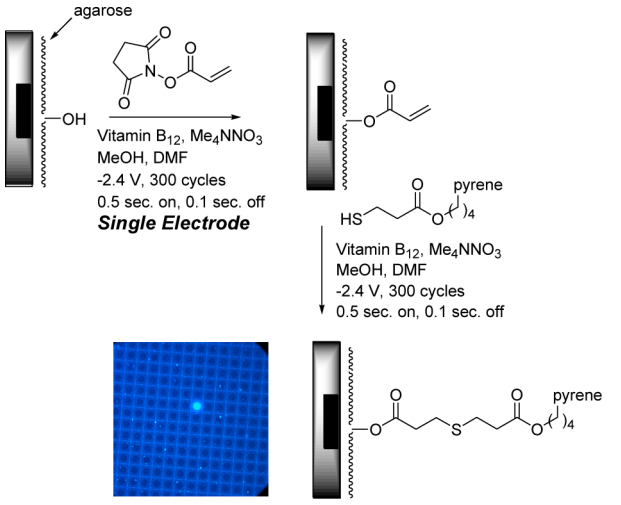
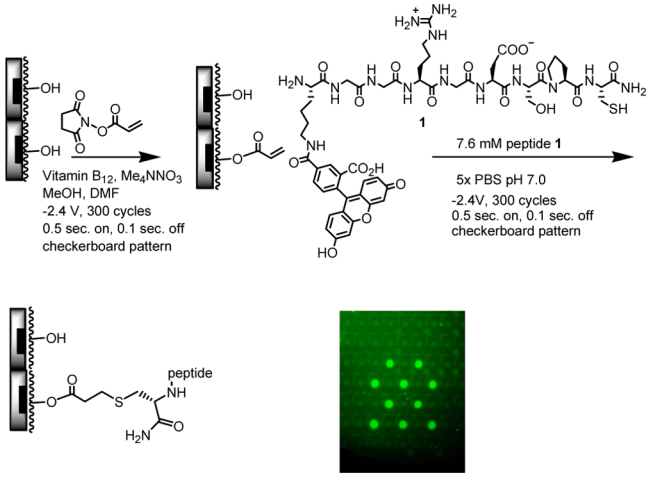
Scheme 3



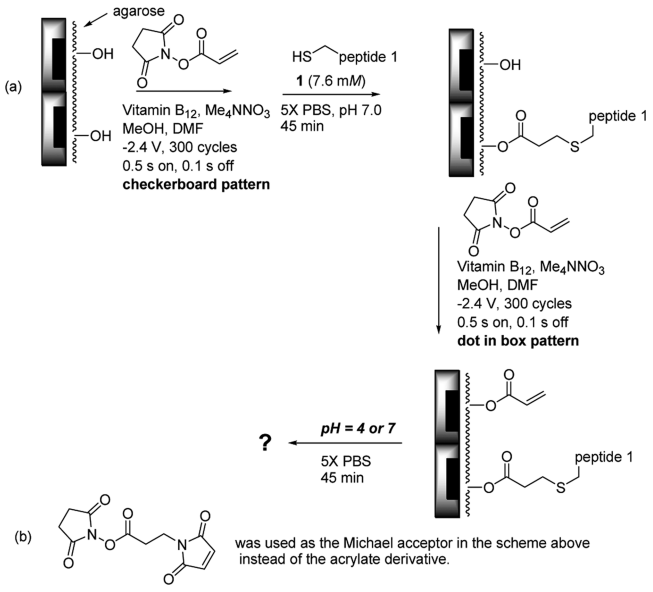
Scheme 4



PROBLEMS WITH REVERSIBILITY AND CONVERSION

Yet, while the thiol-Michael reaction worked and allowed us to conduct an initial signaling study on the array, it was far from ideal. Thiol-based conjugate addition reactions are reversible, and on the array the reverse reaction proved difficult to stop. This compromised the stability of the functionalized surface. The two experiments shown in Scheme 5 highlight this problem. In the first, an acrylate group (**2**) was used as the electron-poor olefin for the conjugate addition. In the second, a maleimide group was used. In both cases, an initial base-catalyzed esterification was used to place the olefin onto a 12K-array in a checkerboard pattern. A conjugate addition reaction was then used to add the RGD-peptide derivative (**1**) to those sites on the array. For the conjugate addition reaction, we found that it was not necessary to pass any current through the cell. Simply incubating the array with the peptide in a 5×PBS, pH = 7 buffer, for 45 min was enough to accomplish the thio-Michael reaction. A fluorescence image of the array was taken to ensure that the reaction proceeded at the selected electrodes. Next, a second set of electrodes was functionalized with the electron-poor olefin. This time a "dot in a box pattern" of electrodes was used. The arrays were then incubated for 45 min in a 5×PBS buffer solution at either pH 4 or pH 7. No

Scheme 5



nucleophile was added to the solution during this second incubation period. A fluorescence microscope was then used to take a second image of the arrays (Figure 2).

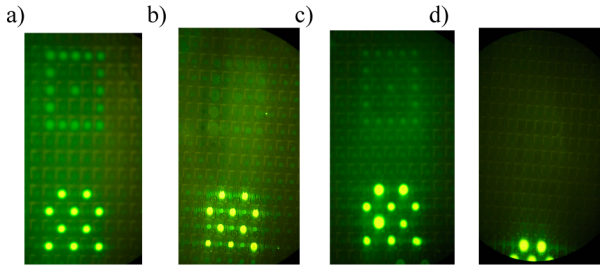


Figure 2. (a) Maleimide as the Michael acceptor, pH = 7; (b) maleimide, pH = 4; (c) acrylate as the Michael acceptor, pH = 7; (d) acrylate, pH = 4.

As can be seen in a and b of Figure 2, when maleimide was used as the electron-poor olefin, the conjugate addition was reversible at pH = 7. This conclusion was reached because the only source of fluorescence during the second 45-min incubation period of the experiment was the peptide originally attached to the checkerboard pattern of electrodes at the bottom of the image. Hence, the fluorescence observed at the "dot in a box pattern" was derived from the originally functionalized electrodes. Incubation at a pH = 4 reduced the amount of the migration but did not stop it. The use of an acrylate group as the "Michael acceptor" led to less of a migration at a pH = 7, but it clearly still occurred (Figure 2c). Fortunately, with the acrylate group the migration could be minimized nicely with the use of pH = 4 conditions (Figure 2d). For this reason, an acrylate group was used as the "Michael acceptor" on the array for all subsequent studies. With that said, *the instability of the maleimide-based system under all of the conditions examined is worrisome in light of its popularity as an attachment strategy for making bioconjugates.*

Since more basic reaction conditions did seem to favor the migration reaction, we worried that some migration might occur during the based-catalyzed esterification reaction used to place the "Michael acceptor" by a second set of electrodes on

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the array. Such a migration would occur if any of the base generated at the selected electrodes for the second placement reaction migrated to electrodes already functionalized with peptide **1**. While the experiment leading to Figure 2d suggested that this was not a problem, a more careful experiment that examined just the second acrylate placement reaction was conducted. In this experiment, the chemistry shown in Scheme 5 was repeated on a 12K-array, and then the array was examined after the second acrylate placement reaction but before the incubation step. The first acrylate and hence peptide **1** was placed on the array in a "checkerboard within a box" pattern. The fluorescence image presented in Figure 3a was

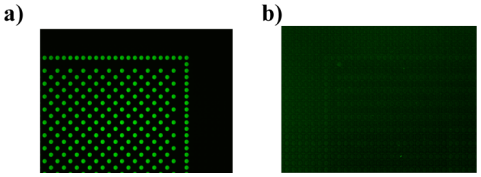


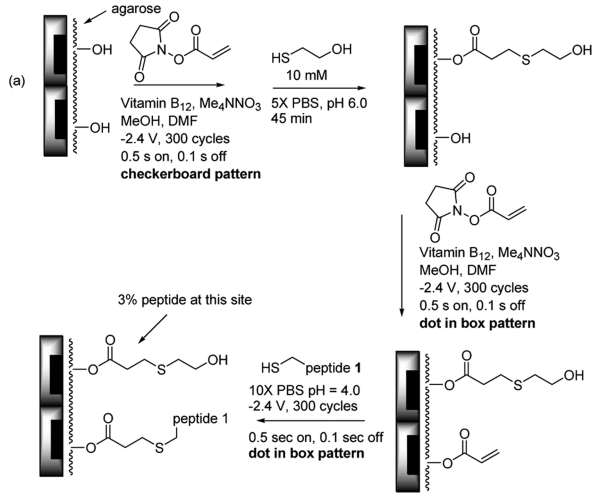
Figure 3. (a) Initial placement of acrylate onto the array followed by a Michael reaction with peptide **1**. (b) Site of the second acrylate placement on the array.

then acquired in order to assess the quality of this placement reaction. The first step of the procedure was then repeated at a second location on the array. This time, the pattern used was a set of "parallel lines in a box". The pattern was selected so that there would be no confusion as to which reaction gave rise to which pattern. An image of the array was then taken with a fluorescence microscope to see if any of the fluorescently labeled peptide migrated from the checkerboard pattern to the parallel lines of acrylate placed on the array. Very faint fluorescence did appear at the second site (Figure 3b). A quantitative comparison of the fluorescence intensity at the two locations on the array indicated that the line pattern had about 0.3 ± 0.3% of the intensity of the checkerboard pattern. Clearly, very little migration of the peptide occurred during the second acrylate placement.

A third issue with the conjugate addition approach was the "completeness" of the initial reaction. As illustrated in Scheme 2, thiol-based conjugate addition reactions are base-initiated chain reactions. Once initiated at any electrode in an array, they occur at every site on the array that has been previously functionalized with a "Michael acceptor". This can be problematic with respect to the placement of multiple molecules on an array. If a conjugate addition to place one molecule onto an array does not go to completion, then unreacted "Michael acceptor" will remain proximal to the electrodes. A thiol-based conjugate addition conducted to place a second molecule at a different site on the array will then place some of the second molecule by the original electrodes as well. The result would be a mixture of molecules by the original electrode.

In order to determine if this was a problem, one site on an array was functionalized with a checkerboard pattern of the acrylate moiety (Scheme 6). The array was then incubated with a 10 mM solution of 2-mercaptoethanol in 5×PBS at a pH = 6 for 45 min. A second area of the array was then functionalized with the acrylate in a "dot in a box" pattern. The electrodes used in this second pattern were then employed to initiate a conjugate addition of peptide **1** to the acrylate moiety in a fashion identical to that illustrated in Scheme 4. This second

Scheme 6



conjugate addition was conducted at a pH of 4 so that migration of thiol groups that were attached to the array would not occur. Hence, any fluorescence that occurred at the first site would be the result of unreacted acrylate at that site undergoing a conjugate addition reaction during the second procedure. The results of the experiment are shown in Figure 4. Figure 4a

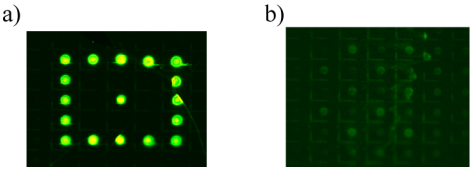


Figure 4. (a) Reaction of peptide **1** with acrylate at the second site on an array.(b) Reaction of peptide **1** with unreacted acrylate at the first site on an array following incubation with 2-mercaptoethanol.

shows the "dot in a box" pattern generated by the second conjugated addition reaction with peptide **1**. The reaction proceeded nicely and was well confined with no migration to the surrounding electrodes. However, a small amount of fluorescence was observed at the initial checkerboard pattern of electrodes (Figure 4b). The initial conjugate addition had not gone to completion. A quantitative measure of the fluorescence image from the two sites indicated that the reaction at the initial site (checkerboard) proceeded to an extent of 3 ± 2% of the reaction at the second site. While the amount of fluorescence at the initial site was small, its presence did indicate that a capping-step would be needed at the site of the initial conjugate addition reaction in order to consume any unreacted Michael acceptor at that location.

The reversibility of the thiol-based conjugate addition reaction was an even greater problem with the use of an amine nucleophile (Scheme 7). In this case, the nucleophile placed on the surface of the array was sensitive to the base conditions used to add the electron-poor olefin to the array. The experiment illustrated began with the placement of peptide **2** onto a 12K-array with the use of a lysine side chain and an acrylate "Michael acceptor" on the array. The acrylate on the array was placed down in a "checkerboard in a box" pattern in the manner outlined above. The conjugate addition reaction was then conducted with the pH = 4 conditions employed for the previous thiol-based addition. The success of the reaction

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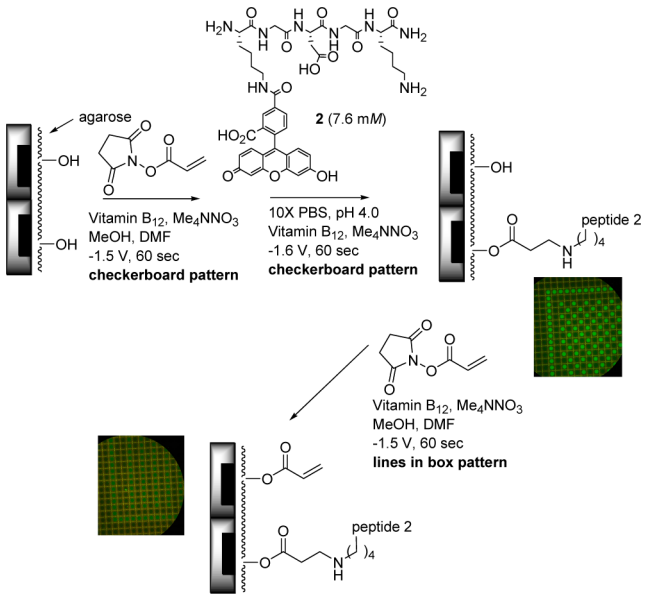
Scheme 7

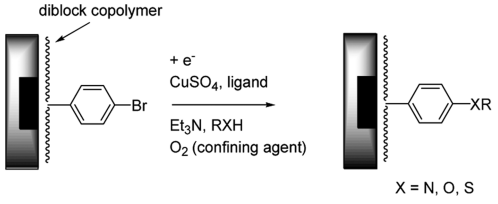


was verified with a fluorescence microscope. The acrylate placement reaction was then repeated with a second set of electrodes in a "parallel lines in a box" pattern. This reaction led to faint fluorescence at this second site, fluorescence that could only arise from migration of the peptide previously placed by the electrodes used in the checkerboard pattern.

A SOLUTION USING SITE-SELECTIVE Cu(I) CATALYSIS

While the reversibility of the conjugate addition of a thiol nucleophile can be stopped by using a pH = 4, this requirement was not compatible with the more biologically relevant conditions needed for signaling studies using intact proteins. At neutral pHs, a library placed on an array would scramble. What was needed was a nonreversible method for placing peptides and peptidomimetics onto an array. To this end, a transition metal-based option appeared ideal. Both Pd(0)- and Cu(I)-coupling reactions have proven to be very useful synthetic tools for site-selectively adding new molecules to microelectrode arrays.[13,14] Of these two approaches, the Cu(I)-based method illustrated in Scheme 8 is particularly attractive.

Scheme 8

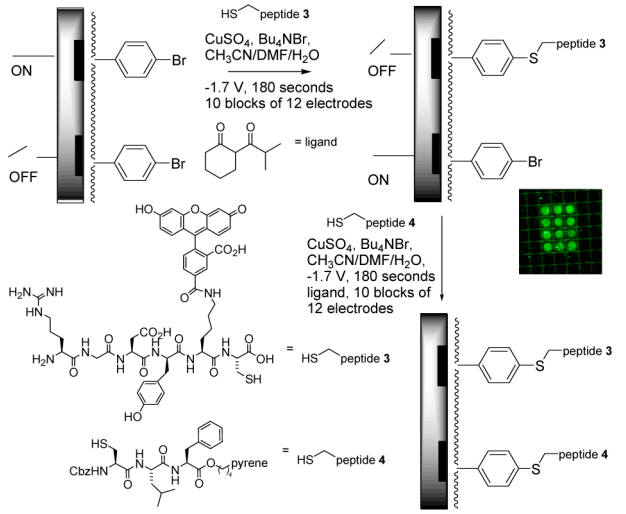


While both Pd(0) Heck and Suzuki strategies work beautifully on the arrays, they require modification of both the peptide and the surface prior to the placement reaction. For a Heck reaction, the peptide must be functionalized with an olefin, and the surface, with an aryl bromide. Frequently, Heck reactions employ activated olefins. However, the inclusion of an activated olefin in the peptide can lead to its polymerization, especially if the peptide contains either a nucleophilic side chain or an unprotected N-terminus. For this reason, the reaction needs to be conducted with either a peptide containing an unactivated olefin,[15] or performed by modifying the surface of the array so that it contains the olefin and then modifying the peptide with the necessary aryl halide. In a similar fashion, the use of a Suzuki reaction would require either the addition of a phenylboronic acid to the peptide or modification of the surface to contain the phenylboronic acid and incorporation of an aryl halide into either the polymer or the peptide.

On the other hand, Cu(I)-catalyzed coupling reactions require no modification of the peptide. The site-selective additions of alcohol-, amine-, and thiol-based nucleophiles to aryl bromide-functionalized diblock copolymers[16] have all been shown to work on both 1K- and 12K-arrays.[14] Hence, with the use of Cu(I) the same peptide substrates synthesized for the conjugate addition chemistry can potentially be used to place molecules on the array in an irreversible fashion. The method offers additional advantages in that Cu(I) reactions show ligand-dependent chemoselectivity of heteroatom-/aryl halide-coupling reactions.[17] In this way, the method has the potential to selectively place peptides onto arrays with alcohol- and thiol-containing side chains even if the N-terminus is not protected. Preliminary results indicate that such selectivity is possible with 1,3-dicarbonyl ligands for Cu(I) leading to much faster reactions between thiol nucleophiles and arylbromides on the surface of an array relative to reactions using alcohol and amine nucleophiles.[14b]

However, is the site-selective generation of Cu(I) on the array compatible with more complex peptide substrates that can serve to chelate the metal? In order to test this idea, peptide **3** was placed on a 12K-microelectrode array that had been precoated with a diblock copolymer (Scheme 9). The diblock

Scheme 9



copolymer was composed of a cinnamoyl-functionalized methylmethacrylate block used to coat the array and then provide stability to the surface after photochemical-cross-linking, and a 4-bromostyrene was used to provide a coupling partner for the Cu(I)-coupling reaction. The functionalized array was submerged in a 7:2:1 acetonitrile/dimethylformade/water solution that contained the peptide, copper sulfate, a 1,3-dicarbonyl ligand for the copper, and tetrabutylammonium bromide as an electrolyte. Selected electrodes in the array were then used as cathodes by setting them to a potential of −1.7 V

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relative to a remote Pt anode. The electrodes were turned on for two periods of 90 s each in order to give rise to the image shown. Oxygen was used in the solution as a 'confining-agent" (oxidant for Cu(I)) in order to prevent migration of the Cu(I)-catalyst to electrodes not selected for the reaction. Upon completion of the reaction, the array was washed with an excess of ethanol and DMF and then allowed to dry. Because the coupling reaction is catalyzed by Cu(I) and not a "chain-reaction" as for the conjugate addition, there was no reaction at any site on the array that was not used for the generation of Cu(I). For the experiment described, 10 blocks of 12 electrodes each were used for the reduction. The fluorescence image shown in Scheme 9 shows one of these blocks of electrodes along with the surrounding electrodes. The very high level of selectivity for the electrodes used is clearly evident.

SIGNALING

With the placement of the peptide on the array complete, two questions immediately arose. Was the peptide available for signaling studies, and was the new surface stable enough for us to conduct the analytic experiment multiple times to establish a relationship between binding on the surface of the array to the concentration of receptor in solution? Answers to these questions were probed with the use of an integrin receptor. To this end, the array made in Scheme 9 was functionalized with a second, non-RGD-peptide, **4**. This second peptide was also placed on 10 blocks of 12 electrodes each. The array was then incubated with various concentrations (10^{-15} to 10^{-5} M) of the integrin receptor $\alpha_{IIb}\beta_{III}$ in a phosphate buffer solution (1×PBS, pH ≈ 7.4) that also contained a 1:1 mixture of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$. For each concentration of the receptor, a cyclic voltammogram was recorded for the iron redox couple. On an array, there is no reference electrode. Hence, the potentials measured represent the potential at the electrode on the array relative to the Pt-counterelectrode located 0.9 mm away.[18] The data obtained for one of the blocks of 12 electrodes functionalized with the RGD-peptide **3** are shown in Figure 5. Note how the peak current at 30 mV for the cyclic voltammogram decreases as the concentration of integrin receptor in solution increases. This decrease in current reflects the binding of integrin to the peptide on the polymer coating the electrodes. The data for three blocks of the electrodes functionalized with peptide **3** are summarized in Figure 6

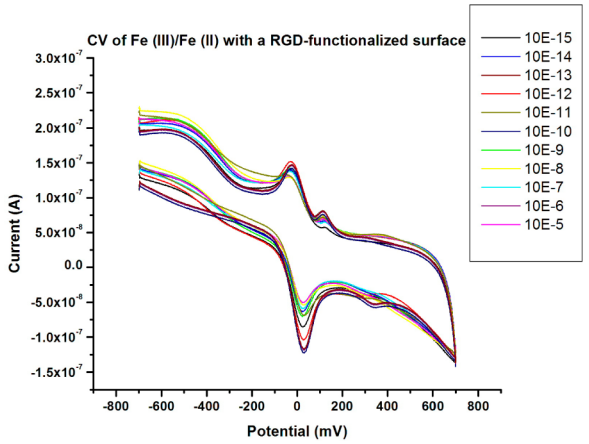

Figure 5. CV data for one block of electrodes functionalized with RGD-peptide **3**.

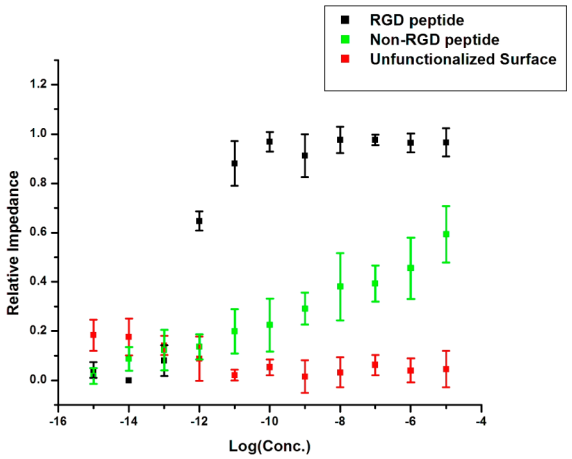

Figure 6. Summary of cyclic voltammetry data.

(black line). Each data point on the line represents the average current recorded for three of the blocks of electrodes at 30 mV. The error bars indicate the spread in the data for the three points.

Figure 6 also includes the summary for data obtained at the electrodes functionalized with non-RGD-peptide **4** (green line) and electrodes that were not functionalized at all (red line). Little impedance was observed at the electrodes functionalized with **4**, indicating a low-level nonspecific binding to the functionalized surface of the array. The steady climb in impedance at the sites functionalized with **4** without any leveling off of the curve suggested that the nonspecific binding that was observed was a result of the functionalized surface and not peptide **4**. There is only 10−50 fmole of peptide bound to the surface of the electrode, and thus binding to the peptide itself would lead to a leveling off of the impedance with increasing concentration of the receptor. This leveling off of the impedance was observed with the binding of the receptor to peptide **3**. At the unfunctionalized electrodes, no binding was observed, indicating that functionalization of the surface did increase the level of nonspecific binding of the integrin receptor to the polymer surface. The level of impedance observed for the electrodes functionalized with **3** relative to those functionalized with **4** clearly demonstrated that the integrin receptor recognized the RGD-peptide on the surface.

Binding of the integrin receptor to the RGD-peptide on the surface of the array was reversible. Incubating the chip used to obtain the data shown in Figure 6 with a buffer solution led to recovery of the current at the electrodes functionalized with the RGD-peptide. Reintroducing the integrin receptor then caused the current to drop again.

At this time, it is not known why the impedance measurement made on the array is more sensitive than the typical nanomolar binding constant associated with RGD−integrin binding. Now that we have a method for placing peptides irreversibly on an array, efforts to understand this effect, to develop the use of the arrays for quantitative measurements, and to explore the chemistry of libraries with the arrays can commence.

CONCLUSIONS

We have found that peptides can be placed site-selectively on microelectrode arrays with the use of either thiol-based Michael chemistry or Cu(I)-catalyzed coupling reactions. Both reactions take advantage of a cysteine in the peptide to provide the

nucleophile for the reaction. The conjugate-addition strategy was confined by controlling the placement of the "Michael acceptor" on the array. These reactions were problematic because of their reversibility. When an acrylate acceptor was used on the array, the retro-conjugate addition could be stopped when the reactions were run at a pH = 4. For a maleimide acceptor, the reversibility of the conjugate addition could not be stopped. The use of a Cu(I)-catalyzed addition of the thiol nucleophile to a arylbromide surface on the array did not suffer from these issues. The reactions were confined with the use of air as an oxidant for the Cu(I)-catalyst generated on the array. With this chemistry, an RGD-peptide was site-selectively placed on a 12K-array along with a second non-RGD-peptide. Once there, binding of the peptide to its integrin receptor was monitored in "real-time" with the use of an electrochemical impedance experiment. The work demonstrates the potential for microelectrode arrays as a platform for analyzing peptide-based molecular libraries.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental and characterization data for all substrates and products; copies of proton and carbon NMR spectra are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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