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New Methods for the Site-Selective Placement of Peptides on a Microelectrode Array: Probing VEGF−v107 Binding as Proof of Concept

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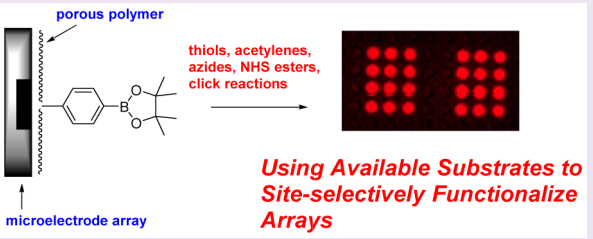
Supporting Information

ABSTRACT: Cu(I)-catalyzed "click" reactions cannot be performed on a borate ester derived polymer coating on a microelectrode array because the Cu(II) precursor for the catalyst triggers background reactions between both acetylene and azide groups with the polymer surface. Fortunately, the Cu(II)-background reaction can itself be used to site-selectively add the acetylene and azide nucleophiles to the surface of the array. In this way, molecules previously functionalized for use in "click" reactions can be added directly to the array. In a similar fashion, activated esters can be added site-selectively to a borate ester coated array. The new chemistry can be used to explore new biological interactions on the arrays. Specifically, the binding of a v107 derived peptide with both human and murine VEGF was probed using a functionalized microelectrode array.



Microelectrode arrays offer a novel platform for analyzing the biological activity of a wide variety of molecules.[1−12] They are particularly intriguing because binding events between molecules fixed on the surface of an array and a biological target can be monitored with the use of a benign redox mediator (usually and Fe(II)/Fe(III) couple).[13,14] The couple is oxidized at the Pt electrodes in the array and reduced again at a remote Pt counter-electrode, and the resulting current monitored at each electrode in the array. When a binding event happens on the surface of the array, the conductivity of the surface is altered, leading to a change in current at the associated electrode. This change in current is detected and its magnitude used to judge the extent of the binding interaction. In this way, the interaction is monitored in "real-time" without any need to label the receptor, label the peptide, or develop an immunological assay with labeled antibodies.

However, what makes the use of microelectrode arrays truly unique relative to alternative approaches is the synthetic capability of the arrays. A wide variety of site-selective reactions can be conducted at individual electrodes in an array, even when the array has thousands of electrodes per square centimeter.[13,15−17] Electrodes can be used to synthesize oxidants, reductants, acids, bases, Lewis acids, nucleophiles, electrophiles, and transition metal reagents and catalysts. In this way, much of modern organic synthesis can be used to place, construct, or manipulate molecules on the surface of an array. These reactions can also be used to site-selectively recover molecules built on the arrays so that they can be characterized following a biological analysis.[13] In this way, the synthetic capabilities of the arrays allow for quality control analysis of an array based library. Because of these combined capabilities, the arrays offer an opportunity to simplify the analysis of medium to larger molecular libraries by enabling their synthesis directly on the device used for their analysis, to then analyze the libraries over extended periods of time while new members are added or built on the surface, and then characterize the molecules made in order to make sure their structures are correct both immediately after their synthesis and over time.

Three key components underlie every site-selective reaction run on an array. The first is a porous polymer surface that coats the array and provides attachment points for fixing molecules to the surface of the electrodes.[18] This polymer must be stable over time, inert to a wide variety of chemical reagents, and compatible with the electrochemical signaling studies. It was this need for chemical inertness and long-term stability that led us to choose polymer platforms for coating the surface and in so doing avoid the less-stable self-assembled monolayer platform used so successfully with alternative methods.[19] The second is a redox stable chemical reagent that can exist in an inert form over the arrays and then be activated by the electrodes in the array. Because of the use of the more stable polymer platform for building a surface on the arrays, direct electrochemical methods for modifying the array are not possible.[20] While this initially appears problematic, the use of indirect methods in which the electrode generates a chemical reagent that in turn conducts reactions on the surface in practice offers tremendous advantages. As mentioned earlier,

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electrodes can be used to make a wide variety of chemical reagents and catalysts. To date, Pd(0), Pd(II), Cu(I), Cu(II), Ce(IV), DDQ, Os(VIII), Ru(VII), triarylamine radical cations, H^+, methoxide, H_2, and Sc(III) have all been used to conduct syntheses on an array. Of course for these reactions to be conducted at selected electrodes in the array, a third key component is needed for each reaction. That component is a confining agent that consumes the reagent generated at the electrodes and returns it to its inactive solution phase state so that the reaction cannot happen on the array at sites remote from the selected electrodes.[13]

With the capability of running almost any chemical reaction site-selectively on an array now in place, we turned our attention toward the development of array-based variants of transformations that are commonly employed in the construction of bioconjugates. The goal of the work is to ensure that the arrays can be used to complement other analytical methods without any need to resynthesize the molecules being evaluated. For example, peptides already functionalized with acetylenes for use in Huisgen-type "click reactions"[21,22] can potentially be site-selectively placed onto a microelectrode array that is coated with the type of stable, chemically inert surface that would allow it to be monitored over extended periods of time. Peptides functionalized for use in Diels–Alder reaction based routes to forming bioconjugates can be site-selectively placed on an array with the same Diels–Alder strategy.[23]

Of these methods, the use of a "click reaction" is particularly attractive because of the reaction broad applicability in chemical biology. For this reason, we developed a site-selective variant of the reaction that coupled molecules with an acetylene to an azide on an agarose coated array.[24,25] In this case, the agarose surface served as a "practice" coating on the arrays for developing the confinement strategy needed for the site-selective reactions. Agarose is not stable and hence can be removed very easily from an array. This is wonderful for reusing the arrays, but impractical if one wants to build a stable surface that enables signaling studies. For this reason, a more stable and very versatile diblock copolymer surface has been developed for use on the arrays (Figure 1).[18]

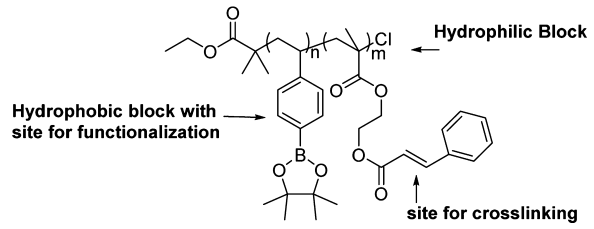
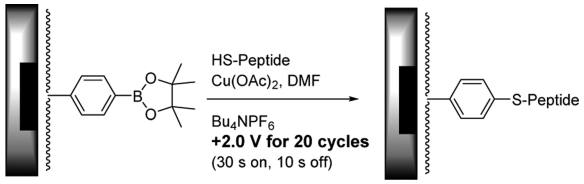


Figure 1. Diblock copolymer used to coat the microelectrode arrays.

This surface is ideal because it is stable for extended periods of time, compatible with a wide variety of both synthetic and analytical experiments, and tunable so that nonspecific binding interactions with biological molecules can be minimized.

Our first strategy for placing peptides onto the borate ester surface took advantage of a thiol nucleophile and the well-known Cu(II)-mediated Chan-Lam reaction (Scheme 1).[26,27] The Cu(II) needed for the reaction was generated by the oxidation of a Cu(I) precursor at the electrodes selected for the experiment. Excess thiol in solution was used as the confining agent for the reaction by reducing any Cu(II) that migrated away from the selected electrodes in the array. Hence, the

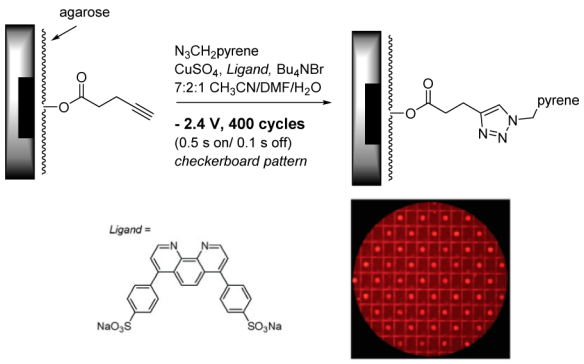
Scheme 1. Placement of Thiol Nucleophiles on a Borate Ester Surface Using a Chan-Lam Coupling Procedure



Chan-Lam reaction only occurred at the desired electrodes. The chemistry was very successful and allowed for peptides functionalized for use in thiol-Michael approaches to be utilized directly on the arrays.

With our first complementary method in place, we turned our attention back to the development of an array-based variant of the Huisgen-type "click reaction." Unfortunately, initial efforts to place molecules functionalized for use in the [3 + 2]-cycloaddition onto arrays coated with the new, more desirable diblock copolymer surface were not successful. The initial plan was to simply move the previous array-based reaction from the agarose surface (Scheme 2) to the diblock copolymer surface.

Scheme 2. Site-Selective Click Reaction on an Agarose Coated Array



In this previous study, an acetylene precursor was placed onto the agarose surface by each of the electrodes in the array.[24] The [3 + 2] cycloaddition between the surface bound acetylene and an azide in solution was then accomplished by using selected electrodes in the array to reduce a Cu(II) precursor to the Cu(I) catalyst needed for the reaction. Air was used as the "confining agent" to oxidize any Cu(I) catalyst that migrated away from the electrodes employed for the reaction. A fluorescent tag was placed on the azide so that the quality of the reaction could be assessed with the use of fluorescence microscopy. For the reaction, a checkerboard pattern of electrodes was used in order to probe the success of the reaction over the entire surface of the array. As can be seen in the image provided, the reaction and the confinement strategy both worked well. The cycloaddition also proceeded nicely when an azide was placed on the array and an acetylene used in solution.

RESULTS AND DISCUSSION

Surprisingly, the same reaction run on an array coated with the borate-ester based surface led to the images shown in Scheme 3. For this experiment, a thiol substituted azide was added to the surface of each electrode in the array using the Chan-Lam coupling reaction illustrated in Scheme 1. An acetylene labeled

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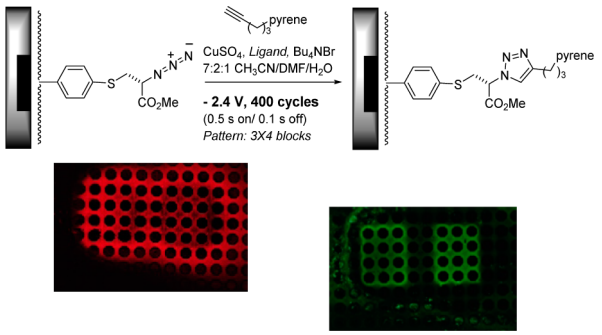
Scheme 3. Initial Attempt at a Cu(I)-Catalyzed Click Reaction with a Borate Ester-Base Polymer Surface on the Array

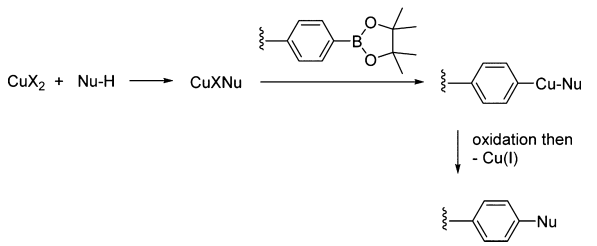


with a pyrene group was used as the solution phase substrate. As can be seen, the reaction did not show the expected fluorescent spot by each electrode used for the "click reaction." In this case, the pattern used on the arrays would have placed the molecules down in blocks of 12 electrodes each (the pattern needed for subsequent signaling studies). Instead, the surface of the array surrounding the electrode was fluorescent and the electrodes dark. Examination of the array using a green filter for the fluorescent light (a wavelength of light that is not ideal for the pyrene exciplex and hence only shows up with very low concentrations of the dimer) showed that the reaction did occur at a faster rate closer to the electrodes used for the generation of Cu(I), but it did not occur on the electrode. So what makes the "click reaction" on the borate ester surface so different, and how do we solve this problem so that we can best take advantage of peptide ligands already modified for "click reaction" based applications?

One problem that can occur during an array-based reaction is a very rapid background reaction between the fluorescent substrate in solution and the unfunctionalized polymer coating the array. Such reactions occur everywhere on the array except where the solid phase substrate has taken the place of the borate esters on the polymer. The result is a fluorescent surface with "holes" over the electrodes just like the images shown in Scheme 3. Three questions immediately arise about this suggestion. What is the background reaction? Why did it occur at a faster rate closer to the electrodes being used as cathodes? And why did the desired Cu(I)-catalyzed [3 + 2]-cycloaddition not occur on the electrodes?

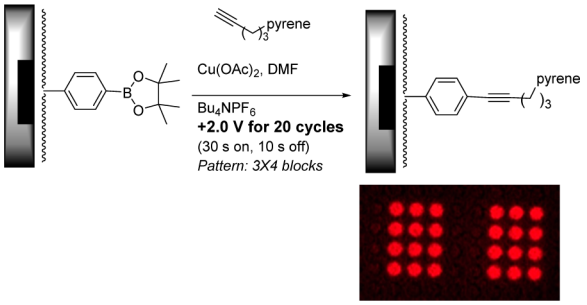
Alternative Copper-Based Peptide Placement Reactions. One possible answer to the first question is that the Cu(II) added as a precursor for the reductive generation of Cu(I) catalyzed a Chan-Lam type coupling between an acetylene nucleophile and the borate ester surface. In the mechanism for the Chan-Lam coupling reaction (Scheme 4), a nucleophile (Nu-H) adds to the Cu(II) reagent, which then undergoes transmetalation with the arylborane prior to oxidation and a reductive elimination reaction. It was certainly possible that an acetylene might serve as the nucleophile for this reaction.

Scheme 4. Chan-Lam Reaction



This suggestion was easy to test by taking advantage of the Cu(II) confinement strategy already developed for the thiol-based Chan-Lam coupling reaction. To this end, an array coated with the borate ester polymer was treated with a DMF solution containing Cu(I) (generated from the reduction of $CuSO_4$ by the acetylene in solution),[28,29] an excess of the pyrene labeled acetylene, and Bu_4NBF_4 as an electrolyte. Blocks of 12 electrodes (again, the pattern needed for subsequent signaling studies) each were then used as anodes in order to site-selectively generate Cu(II) from the Cu(I) in solution. The resulting array was examined by fluorescence microscopy. As shown in Scheme 5, the experiment did result in the site-selective placement of the acetylene nucleophile proximal to the electrodes used for the oxidation. Since no reaction was observed on the array remote from the electrodes used, the reaction is not catalyzed by the Cu(I) precursor in solution, nor is it the result of a "non-specific" interaction between the solution phase substrate and the polymer surface. In this way, the array reaction serves as its own control. Clearly, the reaction only occurs in the presence of the Cu(II) mediator.

Scheme 5. Addition of an Acetylene Nucleophile to the Surface of the Array



Support for the reaction shown in Scheme 5 was gained by examining a similar solution phase reaction. In this case, the deprotected boric acid (**1**) was used in place of the borate ester because the boric acid moiety will undergo a Cu(II) catalyzed reaction more quickly. This is important for the solution phase reaction because array reactions are typically greatly accelerated.[13] In an array reaction, the "catalyst" is generated at the surface of the electrode in large excess relative to the substrate present. Suzuki and Heck reactions that take hours at reflux in solution run in minutes on an array. Many of the previous array reactions using the borate ester surface only proceed in solution when the borate ester is deprotected. In fact, this is the case for the reaction shown in Scheme 5. While a solution phase reaction with a borate ester substrate led to no reaction, the faster solution phase reaction with the deprotected boric acid substrate proceeded nicely (Scheme 6). Two things about the reaction are particularly relevant to the array reaction shown in Scheme 5. First, the reaction did lead to the desired coupling product (**2**, NMR yield based on the acetylene limiting reagent) with the Cu(II) catalyst mediating the coupling of the acetylene to the arylboronate. Second, the major product from the reaction was an expected homocoupling product from the acetylene (**3**, NMR yield based on the acetylene limiting

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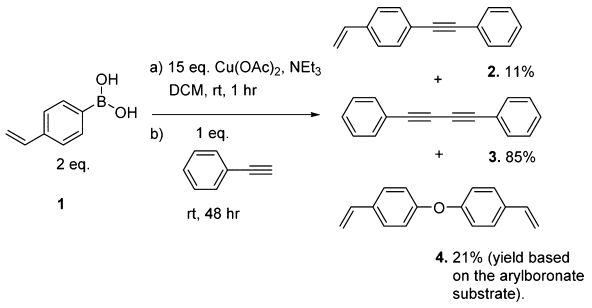
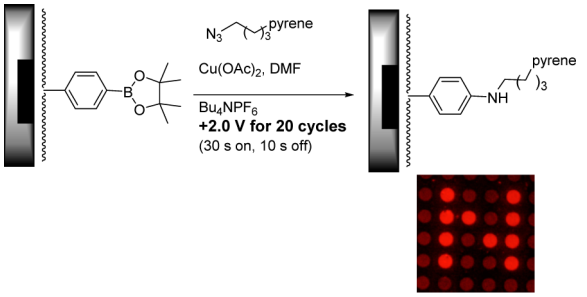
Scheme 6. A Solution-Phase Acetylene Coupling Reaction



Scheme 7. Addition of an Azide Nucleophile to the Surface of the Array



reagent).[28,29] This reaction not only leads to the initial generation of Cu(I) in solution (for oxidation at the anodes in the array) but also is the reaction behind why the array reaction in Scheme 5 was so well confined to the selected electrodes. For confinement on an array, the reagent generated at the electrodes must be quickly consumed in solution before it can migrate to remote sites on the array. The oxidative homocoupling reaction of the acetylene substrate accomplishes this task perfectly because it reduces the copper(II) reagent generated at the anodes in the array. Hence, the solution phase reaction demonstrates both of the reactions necessary for a successful array reaction. As a sidenote, a third product from water addition to the excess aryl boronate used in the reaction was also observed (**4**, NMR yield based on the aryl boronate). This product is the result of the starting boronic acid being wet.

The success of the array reaction shown in Scheme 5 was intriguing because it indicated that an acetylene substituted peptide can be placed directly on the surface of a microelectrode array without any need to prefunctionalize the array with the azide. In this way, the failure of the reaction shown in Scheme 3 led to an even easier method for the placement of an acetylene functionalized peptide onto an array (Scheme 5). As a sidenote, the new method is also advantageous over the previously developed Diels–Alder methods that also require prefunctionalization of the array with a dienophile or diene.[20,23]

The success of the reaction with the acetylene led us to wonder if the azide partner for the "click reaction" might also serve as a nucleophile for the Chan-Lam coupling reaction. Similar solution phase reactions have been shown to afford aryl amines.[30] To this end, Cu(II) was site-selectively generated by an N-pattern of electrodes in a microelectrode array (the N was used for "nitrogen" so that the array could be distinguished from the one shown in Scheme 5) coated with the borate ester polymer. The reaction conditions were identical to those used in Scheme 5 except for the use of a pyrene labeled azide as the nucleophile (Scheme 7). Once again, the reaction worked nicely and led to the selective placement of the pyrene by each electrode used for the reaction.

The second question asked about the images shown in Scheme 3 is not easy to answer. Since the background reaction is clearly catalyzed by Cu(II) and not Cu(I), why did it occur more rapidly close to the electrodes used to reduce Cu(II) to Cu(I)? We can only speculate on a reason for this observation. It is known that passing current through an organometallic reaction can dramatically increase the rate of the reaction.[31] This is thought to occur by the continual regeneration of fresh catalyst by the reduction and oxidation reactions that take place at the electrodes. For example, Pd(0) catalyzed reactions often suffer from oligomerization of the catalyst. When current is passed through the reactions, it is thought that the Pd(0) oligomers are oxidized to Pd(II) at the anode and then the Pd(II) species generated reduced back to new, monomeric Pd(0) catalyst at the cathode. The result is constant regeneration of the active Pd(0) catalyst. If the reduction of Cu(II) to Cu(I) at the cathodes selected for a "click reaction" is followed by rapid reoxidation of the Cu(I) species by oxygen to generate a more reactive Cu(II) reagent, then the Cu(II) background reaction would occur more rapidly close to the electrodes. Of course, more work needs to be done to demonstrate if this is indeed the case.

Revisiting the Click Reaction. The third question asked about the images shown in Scheme 3 wondered why there was no fluorescence on the electrodes themselves. In spite of the background reaction, the electrodes were used as cathodes to generate Cu(I), and it was surprising that the expected [3 + 2] cycloaddition reaction did not occur at those locations. With the success of the reaction illustrated in Scheme 7, it was fair to ask if the azide competed with the thiol nucleophile during the Chan-Lam procedure used to make the starting array. If so, then there would be no azide present on the surface of the electrode and no subsequent cycloaddition.

If the cysteine derivative had been placed on the array with the azide instead of the thiol, then there should be free thiol on the surface of the electrodes. This possibility was tested. Thiols can be detected on the surface of an array by treatment of the array with Cu(II) and a solution phase thiol that is labeled with a fluorescent group. The Cu(II) reaction catalyzes the formation of a dithiane that fluorescently labels the surface of the electrode. Two examples are shown in Scheme 8. In the first (a), a bis-thiol compound is placed on the array (in an S-pattern for sulfur) using the chemistry shown in Scheme 1, and then the free thiol of the surface labeled as a control to show that the reaction works. In the second (b), the experiment is repeated with the cysteine derivative containing the azide. No fluorescence was observed when the cysteine derivative was used, an observation that indicated that the thiol was indeed the nucleophile in the Chan-Lam reaction in spite of the presence of the azide. So why was there no click reaction on the electrodes in Scheme 3?

A second possibility was that the Cu(I) precursor for Cu(II) in the array-based Chan-Lam reaction reduced the azide to an amine following addition of the thiol to the array. The result would be a free amine on the surface of the array and no azide available for the click reaction. To test this possibility, an azide substituted cysteine was placed onto the array using the Chan-Lam coupling conditions using a "block pattern" of electrodes, and then the resulting array incubated with a fluorescently labeled acid fluoride which readily reacts with amines.[32] The reaction did label the electrodes used for the Chan-Lam

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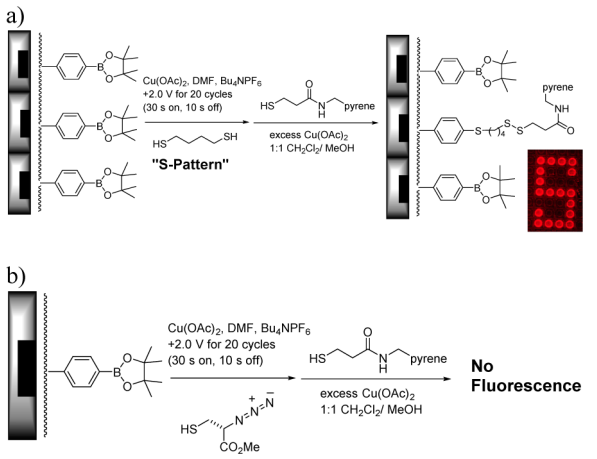
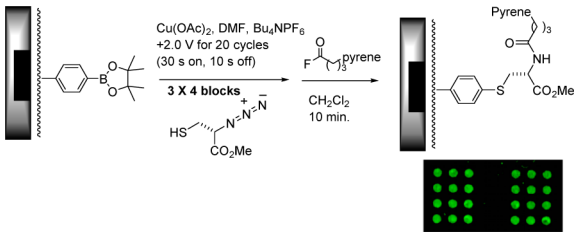
Scheme 8. (a) Control Experiment Showing the Detection of a Thiol on the Surface and (b) Chan-Lam Coupling with the Azide Functionalized Cysteine Leading to No Free Thiol



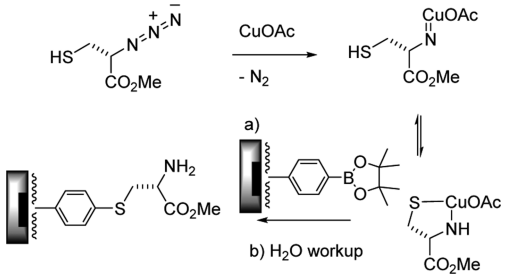
reaction, indicating the presence of a reactive amine at those sites (Scheme 9).

Scheme 9. Detection of an Amine



The presence of the amine readily explained the failure of the "click reactions" in Scheme 3. Reduction of the azide meant that there was no surface bound substrate for the reaction. It also suggested that the Chan-Lam coupling reaction using the azide substituted cysteine proceeded through a pathway like the one illustrated in Scheme 10. In this mechanism, the Cu(I)

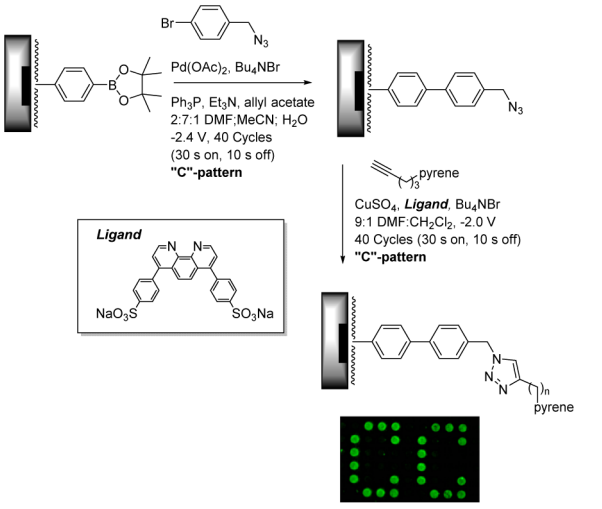
Scheme 10. Mechanism of Azide Reduction and Cysteine Placement on the Array



would undergo an oxidative addition to the azide followed by a ligand exchange to place the thiol onto the resulting Cu(II) intermediate. Transfer of the thiol to the borated ester surface would lead to the free amine on the surface of the electrode. We have found that Chan-Lam reactions with thiol nucleophiles proceed much faster than similar reactions with amines.[27]

At this point, it became clear that the failure of the "click reaction" in Scheme 3 was due to problems with the Chan-Lam reaction used to place the substrate on the array and not necessarily a problem with the reaction itself. For this reason, a site-selective Suzuki reaction was used to place an azide on the array (Scheme 11).[33] The entire array was then treated with a

Scheme 11. A "Click-Reaction" Conducted on a Borate Ester Coated Array



DMF/CH_2Cl_2 solution that contained $CuSO_4$, a ligand developed for optimization of the [3 + 2]-cycloaddition reaction,[34] electrolyte, and a fluorescently labeled acetylene. Electrodes in a "C-pattern" (C- for "click" to distinguish the reaction from the other azide and acetylene placement reactions) were used as cathodes to reduce the Cu(II) to Cu(I) and trigger the "click reaction." Air was used as the confining agent for the Cu(I) generated. As can be seen in the image provided, the reaction worked very well, and the product was generated exclusively at the electrodes used. Clearly, the "click reaction" can be used to add molecules to the borate ester surface as long as the Chan-Lam reaction is avoided as a means to place the azide substrate onto the array.

Direct Esterification of the Borate Ester Surface. Since pinacol-protected borate ester surfaces undergo reactions with water to form boric acid groups and alcohols, we wondered if these groups could be directly coupled to the C-terminus of a peptide through an esterification reaction. This would provide an alternative method for placing an existing peptide on the arrays. For such reactions, the C-terminus of the peptide is typically converted to an activated ester and then coupled to an alcohol in the presence of a base catalyst. In past array reactions, N-succinimide esters have been used to site-selectively place peptides onto agarose and sucrose coated arrays with the use of an electrogenerated base.[14]

The possibility of the same chemistry being used on the new borate ester surface was tested by treating a borate ester coated array with a pyrene labeled *N*-hydroxysuccinimide ester (Scheme 12). As in earlier array esterification reactions, the coupling of the NHS-ester to the surface of selected electrodes in the array was accomplished by using the electrodes to reduce vitamin B12 and in so doing generating a base. Tetramethylammonium nitrate was used as the electrolyte for the reaction, and blocks of 12 electrodes each were used for the reduction. As image a shows in Scheme 12, the coupling reaction did occur at the selected electrodes. However, the esterification lost confinement, and fluorescence can be also be observed at

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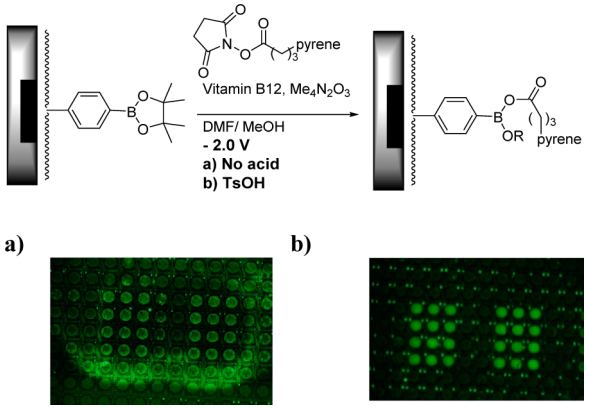
Scheme 12. Esterification Reactions: (a) No Added Acid and (b) with Toluene Sulfonic Acid

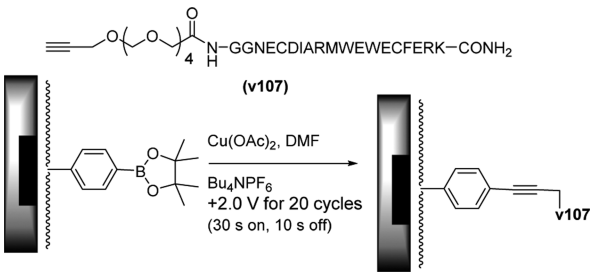


neighboring regions of the array. The loss of confinement typically occurs when generation of the reagent at the electrodes overwhelms neutralization of the reagent in the solution above the array. The problem can be resolved by increasing the concentration of the confining agents. In the current experiment, the reagent generated at the electrodes is a base. Therefore, acid is needed as the confining agent. When the concentration of acid above the array was increased with the addition of toluene sulfonic acid, the esterification was nicely confined to the electrodes used for the generation of base (Scheme 12b). In this way, the NHS esters developed for use on sugar based surfaces could be employed directly on the borate ester coated array.

Probing VEGF–v107 Binding. The reactions developed above are important because they expand the utility of the arrays as platforms for evaluating the biological activity of molecules. For example, one problem that is of interest to us is the interaction between the v107-peptide analog, v107 L19K (GGNECDIARMWEWECFERK-NH_2/cysteines are bridged with a disulfide bond) and its vascular endothelial growth factor (VEGF) target.[35−37] VEGF is important in tumor angiogenesis and other disease states, and in many cases overexpression of VEGF can be correlated to various stages of disease with higher levels of VEGF being correlated to later stages of the disease.[38] The v107-peptide and its L19A analog where the leucine at position 19 is replaced with an alanine are known weak ligands (K_d ca. 1 μM) for human VEGF.[35,36] Previous work has improved this binding interaction *via* covalent binding to a specific lysine residue on VEGF, which serves as a potential lead compound for the development of Positron Emission Tomography imaging agents that will selectively target VEGF expression.[37,39] This technique requires substituting v107 L19 with a lysine (L19K) followed by conjugating with amine-reactive cross-linkers for covalent binding to a specific lysine residue on VEGF. To date, the binding affinity of v107 L19K without the covalent cross-linker has not yet been reported. It would be nice to know this affinity so that v107 L19K could serve as a baseline peptide for developing higher affinity analogs that did not possess the covalent cross-linker. Additionally, the binding epitope to which the peptide binds appears conserved in human and murine VEGF-A homologues (please see the Supporting Information). Determining binding affinities of v107 analogs to both VEGF homologues could be useful in evaluating the biodistribution of these peptides in mouse models of angiogenesis, especially when both VEGF homologues are present *in vivo*.

In principle, the chemistry developed above makes it easy to probe the binding of v107 analogs with both VEGF receptors. The acetylene modified v107 peptide shown in Scheme 13 is

Scheme 13. Placement of a v107−Peptide on a Microelectrode Array



desirable for its compatibility with making bioconjugates in connection with the development of PET imaging agents where "click reactions" are used to add radiometal binding ligands or radiolabeled prosthetic groups to the peptide targeting group. Using the chemistry highlighted in Scheme 5, this same molecule can be placed by specific electrodes in an addressable array, and then the electrodes used to monitor the binding interactions in "real-time" without any need to utilize a labeled receptor or develop an immunological assay or develop a separate coupling strategy. The surfaces used on the arrays are stable, so new peptide analogs of v107 can be added to the array for comparison with the original derivatives, and in so doing an addressable library of v107 analogs assembled.

However, while such studies are easy to propose, the proof-of-principle analytical studies conducted on the arrays to date have all utilized binding interactions between molecules and receptors with nanomolar affinities.[13−15] So, how useful are the arrays for detecting weaker interactions? The v107−VEGF interaction provides a perfect opportunity to answer this question.

To this end, an acetylene functionalized v107 peptide (custom synthesized by CPC Scientific) was placed proximal to 10 blocks of 12 electrodes each in an array that contained 12 544 electrodes/cm^2 (Scheme 13). A second set of 10 blocks of 12 electrodes each was functionalized with a linear hexapeptide (DRDGSP) that served as a negative control. This peptide was placed on the array using the serine side-chain alcohol group using the Chan-Lam coupling reaction described above. Alcohol and thiol nucleophiles have been shown to work equally well in this chemistry.[27] In addition, 10 blocks of electrodes that were coated only with the borate ester surface were selected for comparison with the functionalized surfaces.

It should be noted that the acetylene functionalized v107 peptide used for the chemistry shown in Scheme 13 is a much more complex substrate than the substrate used in Scheme 5, and it is possible that the coupling reaction did not exclusively involve the acetylene and the borate on the surface. However, the binding properties of the array placed peptide in the studies below are consistent with coupling of the molecule to the surface at a location in the molecule remote from the v107 peptide sequence needed to bind the VEGF active site, and therefore the biological studies themselves provide evidence that the coupling reaction proceeded as planned. In addition, it should be noted that the binding studies also provide evidence

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that the disulfide bridge in the v107 is intact. This is not a surprise. While the disulfide linkage may be reduced by the Cu(I)-precursor employed for the Chan-Lam reaction (as was the azide in Scheme 9), such a reduction is reversible. So both the Cu(II) generated at the anode during the Chan-Lam reaction and the net oxidative environment of the signaling experiment where the array is used as an anode would reform the disulfide bridge and ensure its presence during the analytical experiment.

Following placement of the peptide on the array, the array was treated with a 1×PBS buffer solution containing 8 mM potassium ferricyanide/potassium ferrocyanide in a 1:1 ratio. A CV was run at the electrodes in the array and then the solution replaced. This process was repeated three times until the polymer surface on the array was saturated with the redox mediator and the current stabilized. At this point, the array was treated with the 1×PBS buffer solution containing 8 mM potassium ferricyanide/potassium ferrocyanide and 5.0×10^{-9} M of the murine VEGF. A CV was recorded at three of the blocks of electrodes functionalized with v107 (chosen at different sites of the array), three functionalized with the control linear hexapeptide, and three of the blocks functionalized with only the polymer surface for each concentration of VEGF (Gold Biotechnology) used. The solution above the array was then replaced with a new solution having a higher concentration of VEGF and the CV experiments repeated. This was done until a CV was recorded at each set of electrodes used for each concentration of the target protein. The cyclic voltammetry data obtained for one of the blocks of electrodes functionalized with the v107-peptide is shown in Figure 2.

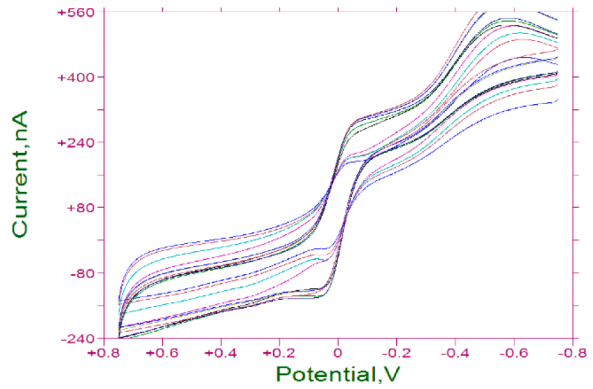

Figure 2. CVs taken for one block of electrodes functionalized with v107 with varying concentrations of VEGF.

The largest CV wave recorded was obtained for the lowest concentration of VEGF (most iron reaching the electrode located beneath the v107-L19K peptide ligand), and the smallest CV wave recorded was obtained for the highest concentration of VEGF used, indicating that the array could be used to detect the interaction between the peptide and its VEGF target even with its low K_d value.

The data obtained from the experiment is summarized in Figure 3. For this figure, the current measured for each CV wave (peak current for the oxidation wave−peak current for the reduction wave) shown in Figure 2 was plotted relative to the concentration of VEGF used. For the electrodes functionalized with the v107-peptide, this gave rise to the blue curve. The error bars provided show the spread in data for three blocks of the v107-peptide functionalized electrodes. The orange line in

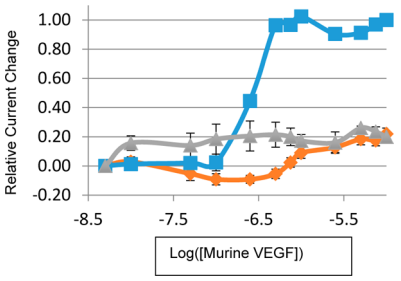

Figure 3. Summary of murine VEGF−v107 peptide binding study. Blue = v107; orange = DRDGSP; gray = unfunctionalized polymer.

Figure 3 summarizes the data obtained for the linear hexapeptide control. No significant binding occurred between this peptide and murine-VEGF. The gray line in Figure 3 showed that VEGF did not bind to the unfunctionalized polymer. From the data, a 50% value for the change in current would correspond to a K_d value of approximately 30 μM, a value consistent with the literature.[35,36] Clearly, the arrays can be used to detect the binding event in spite of the weaker affinity of the peptide for murine VEGF.

A similar conclusion was reached when the v107 peptide was tested for its binding to human VEGF (Figure 4). The

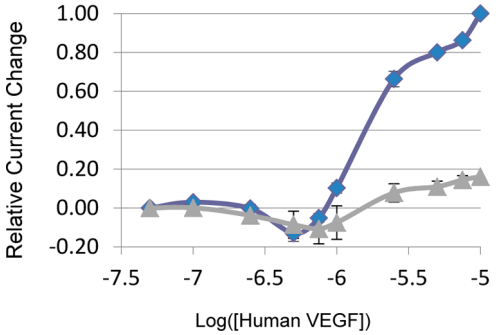

Figure 4. Summary of the human VEGF−v107-peptide binding study. Blue = v107; gray = unfunctionalized polymer.

experiment was conducted in a fashion identical to the one shown in Figure 3 other than the change in receptor. The gray line again showed that the human VEGF did not bind to the unfunctionalized surface of the array. The blue curve showed that the binding event between the v107 peptide and the human VEGF was very similar to the interaction between the peptide and murine VEGF (K_d ca.1 μM). As with the murine homologue, the array-based analytical experiment had no trouble detecting the micromolar binding event.

Conclusions. We have found that molecules having thiol groups,[27] alcohols,[27] acetylenes, azides, and NHS esters can all be added directly to a borate ester containing diblock copolymer surface on a microelectrode array. The reactions can be conducted in a manner that places the molecule by any electrode or set of electrodes in the array. In this way, molecules functionalized for use in Michael reactions, "click reactions," and esterification reactions can all be placed on the arrays without any need to resynthesize the molecules or prefunctionalize the surface of the array. This development should allow the microelectrode arrays to be used in combination with other analytical methods without the additional barrier of having to further functionalize or modify the molecules being studied. The utility of the method for

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functionalizing an array and setting the stage for a bioanalytical study was shown in connection with the binding of v107 to both human- and murine-VEGF targets.

METHODS

Materials were purchased from Sigma-Aldrich and used without further purification unless otherwise indicated. Amino acids were purchased from Advanced Chemtech and used without further purification. 4-Bromobenzylazide was purchased from Sigma. The acetylene tagged v107-peptide was purchased from CPC scientific, and the VEGF-A proteins were purchased from Gold Bio in St. Louis.

Fluorescence microscopy was carried out with a Nikon Eclipse E200 microscope connected to a Boyce Scientific M-100 burner and a Nikon D5000 camera. Optical filters used: CFW-BP01-Clinical-000 (Semrock) filter cube excitation 380−395 nm/emission 420−470 nm, ET−GFP (FITC/Cy2) (Chroma) filter cube excitation 450−490 nm, emission 500−550 nm, and TxRed-A-Basic-000 (Semrock) filter cube excitation 540−580 nm, emission 590−670.

Sample Procedure for Spin-Coating Arrays with the Block Copolymer. The microelectrode arrays were coated with a spin-coater MODEL WS-400B-6NPP/LITE. The chip was inserted into a socket in the spinner and adjusted to be horizontal, then three drops of 0.03 g/mL PCEMA-*b*-pBSt solution (4:1.5 DMF/THF) were added onto the chip in order to cover the entire electrode area. The chip was then spun 1000 rpm for 40 s. The coating was allowed to dry for 15 min and subjected to irradiation using a 100 W Hg lamp for 20 min before use.

Array-Based Synthetic Procedures. Site-selective reactions on the arrays were performed using the published procedures,[13] including the Cu(II)-mediated[27] and Cu(I)-catalyzed methods.[24]

Microelectrode array binding studies were also performed using the already published procedures.[13,14,33]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00685.

Synthetic procedures and spectral data are included for all new compounds along homology modeling for the human and murine VEGF binding epitopes (PDF)

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Notes

The authors declare no competing financial interest.

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