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Moving Known Libraries to an Addressable Array: A Site-Selective Hetero-Michael Reaction

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Abstract

A two-step, Michael reaction-based strategy for site-selectively placing molecules by unique electrodes in an addressable microelectrode array has been developed. The strategy is compatible with the use of polypeptide nucleophiles and works with microelectrode arrays having either 1,024 electrodes/cm² or 12,544 electrodes/cm². The chemistry should allow for the transfer of existing molecular libraries to microelectrode array devices for analysis.

INTRODUCTION

Microarray technology allows for the placement of a molecular library in a small area and simultaneous screening of the library members for binding to a receptor.^{1–4} Our group is interested in using addressable microelectrode arrays as a platform for these efforts.^{5,6} The goal is to build molecular libraries so that each unique member of the library is located next to unique, individually addressable microelectrode in the array. The electrodes can then be used to monitor the binding of molecules in the library with biological receptors as the events happen (in “real-time”).⁷

With this in mind, we have been developing synthetic methodology for site-selectively building molecules on microelectrode arrays.^{8–12} In these efforts, the array is first coated with a porous polymer. The microelectrodes in the array are then used to covalently attach substrates to the polymer proximal to every electrode. The substrates are then site-selectively manipulated by using the microelectrodes to generate reagents that effect chemical reactions involving the polymer-bound substrates. The site-selectivity is achieved by covering the entire array with a solution containing a second substrate that destroys the reagent generated at the microelectrodes. The result is a competition between generation of a reagent at the microelectrodes and destruction of the reagent in solution. Reactions involving the polymer-bound substrate near a microelectrode occur when generation of the reagent at the electrode overwhelms the solution phase reaction. In this way, the location of reactions on the array is controlled by the selection of electrodes used.

In addition to building molecules on the microelectrode arrays, we would like to have the capability to move existing small, targeted molecular libraries to the arrays so that their binding to biological receptors can also be monitored in “real-time”. Accomplishing this goal requires transferring the library to the microelectrode array in a manner that places each individual member of the library proximal to a single microelectrode. For this purpose, we selected the known coupling between a thiol and either a maleimide or acrylate Michael acceptor. This

reaction is often used to attach peptides, sugars, or DNA to a functionalized surface because it occurs quickly with simple incubation at neutral pH in aqueous buffer.^{13–15}

But how can this coupling be performed site-selectively on a microelectrode array having either 1024 electrodes/cm² (1K-chip) or 12,544 electrodes/cm² (12K-chip), the chip used for analytical screening?⁷ In this case, the overall strategy involves a two step sequence (attachment of the Michael acceptor to the polymer and then thiol addition (Scheme 1)) and hence two possible steps for effecting site-selectivity. Since the Michael reaction will occur without a catalyst,^{16,17} it appeared that the best step for controlling the site-selectivity of the process was the step placing the Michael acceptor onto the polymer. We report here, that this step can be controlled with an extremely high degree of site-selectivity.

We began our investigation with two different Michael acceptors, N-acryloxysuccinimide **1** and N-succinimidyl 3-maleimidopropionate **2** (Figure 1). In the first experiment, N-acryloxysuccinimide **1** was placed on the surface of an agarose coated 1K-chip using the electrochemically generated base conditions employed previously for placing substrates by all of the microelectrodes in the array. However, in this case, a checkerboard pattern of electrodes was selected for the reaction. The base was generated by using the microelectrodes as cathodes to reduce vitamin B₁₂, catalyzing a transesterification between the activated ester substrate and the hydroxyls on the surface of the agarose coating the chip (Scheme 1).

The site-selectivity of the process was controlled by using a high concentration (20 mM) of activated ester in the experiment. Deprotonation of an alcohol by the radical anion of vitamin B₁₂ can be expected to occur quickly. Hence, any migration of base away from the region surrounding a selected electrode would involve the formation of methoxide, either by direct deprotonation of the methanol solvent by the vitamin B₁₂ radical anion or as a result of equilibration between the alkoxides generated on the surface of the agarose by the radical anion and methanol. In either case, the excess activated ester in solution would be expected to react with the methoxide to generate a methyl ester and prevent migration of the base to remote locations on the array.

The success of the reaction was determined by conducting a subsequent Michael reaction using the same electrogenerated base conditions and the same checkerboard pattern of selected electrodes. Pyrene-thiol **3** was used as the nucleophile for this test because of its fluorescence properties. Along these lines, imaging the microelectrode array with a fluorescence microscope showed a bright checkerboard pattern (Figure 2A). Clearly, the two step procedure led to the desired site-selectivity.

A control experiment confirmed that placing the Michael acceptor on the agarose was indeed necessary for attachment of the thiol to the agarose polymer. When an agarose coated chip without the Michael acceptor was treated with the pyrene-thiol **3** and electrochemically generated base, there was no reaction between the thiolate generated and the agarose polymer; no fluorescent spots were observed.

The argument that the site-selectivity of the two-step process originated with placement of the Michael acceptor and not the Michael reaction itself was supported with a second control experiment. In this study, the Michael acceptor was placed by every microelectrode in an array and then the thiolate nucleophile for the Michael reaction was generated in a checkerboard pattern. The result was the generation of Michael product and the appearance of bright fluorescent spots by every electrode in the array.

Since the site-selectivity of the process does not depend on the Michael reaction, the thiolate addition does not need to be electrochemically catalyzed. For example, an acrylic acid-patterned chip can be incubated in a solution containing the pyrene-thiol **3** to add the pyrene

to the surface of the microelectrode array. Although these conditions do work, most organic solvents damage or dissolve the agarose polymer used to fix the substrates to the surface of the array during the time frame needed for the coupling reaction. Hence, for libraries that are soluble in organic solvents it is still best to use the electrochemical conditions to accelerate the coupling step and minimize damage to the agarose polymer.

To screen libraries for binding to a receptor using a microelectrode array, the libraries need to be placed on a 12K-array.⁷ While moving to a microelectrode array with a larger density of electrodes can present a problem for the site-selectivity of a reaction, in the current case the optimal conditions with the acrylate linker that gave confinement on a 1K-chip (95 μm electrode diameter) also gave confinement to a single electrode on a 12K-array (45 μm electrode diameter) (Figure 2B). When the maleimide linker **2** was used, the reaction was not confined to a single electrode. Instead, the fluorescent spot observed was roughly the same size as an electrode on a 1K-array. Since the use of the acrylate linker led to cleaner reactions, it was selected for use in subsequent studies.

Having established that the hetero-Michael reaction can be used to put a small organic molecule onto the surface of a microelectrode array, we turned our attention to the placement of water soluble biomolecules such as peptides, proteins, or DNA onto the microelectrode arrays. In these cases, it is best to site-selectively functionalize the polymer coating the array with the Michael acceptor and then incubate the functionalized array with an aqueous solution having a pH consistent with a thiol being more nucleophilic than alcohols or amines,¹⁷ a scenario that favors the cysteine moiety serving as the nucleophile for the hetero-Michael reaction. For example, consider the placement of a short RGD-based peptide onto a microelectrode array. Peptides containing a RGD sequence are known to bind to integrins, cell-surface receptors that mediate cell attachment.^{18–20} The peptide H-K(FAM)GGRGDS-PC-NH₂, with 5(6)-carboxyfluorescein attached to the amine of the lysine side chain (**4**), was synthesized using Fmoc solid phase peptide synthesis. The acrylate acceptor **1** was placed on the surface of a microelectrode array in a checkerboard pattern using 10 electrodes of a 1K-microelectrode array. The chip was then incubated in a 7.6 mM solution of the peptide in 5 $\times$ PBS, pH 6.0. Following this step, the acrylate placement was repeated this time to give a box and dot pattern. The peptide was then placed onto the newly selected microelectrodes by incubating the array in a 7.6 mM solution of peptide in 10 $\times$ PBS, pH 4.0 while using the selected electrodes as cathodes.²¹ The change in pH for the second step was made in order to maximize the stability of the peptide already placed onto the array. The use of the selected microelectrodes as cathodes effectively raises the pH of the solution proximal to the electrodes thereby assisting the addition step in those regions. Following the second addition, the microelectrode array was imaged using a fluorescence microscope (Figure 3A). Clearly, multiple reactions can be performed with this coupling strategy, allowing attachment of different thiol-containing library members to the polymer above selected electrodes in the array.

One control experiment is very important for supporting this conclusion. In this experiment, the sequence of events used to generate the image in Figure 3A was repeated. However, in this case the initial peptide substrate used to make the checkerboard pattern was replaced with 10 mM 2-hydroxyethane thiol. The second step to make the box and dot pattern was repeated just like before using peptide **4**. The microelectrode array was then imaged using a fluorescence microscope as before (Figure 3B). A faint checkerboard pattern could be observed, although it is not observable unless the initial region of the chip is exposed for longer time periods (Figure 3C). Quantitative analysis of the spots revealed that the “bleed-over” of the peptide to the original set of electrodes was minimal.

The small amount of “bleed-over” observed could be explained by either the presence of unreacted Michael acceptor after placement of the 2-hydroxyethane thiol on the microelectrode

array, or an equilibration of the product on the array following the second step. In order to address this issue, a second control study was performed. In this experiment labeled peptide was placed on the array using either the maleimide- or acrylate-based hetero-Michael reaction. A second step was then used to place a Michael acceptor at remote sites on the array. The resulting chip was incubated for 45 min. in the buffer solution used for the Michael reaction, and then the array imaged to see if the peptide migrated from its original location to the remote sites. In this experiment, use of the maleimide acceptor did lead to a small amount of peptide migration. However, no such migration was detected when the acrylate acceptor was employed. Hence, the “bleed-over” observed during the previous study was due to a small amount of unreacted Michael acceptor in the first step, a situation that can most likely be avoided by optimizing the percent conversion of the first Michael reaction.

Peptides placed on the surface of the array using the chemistry described are available for binding ligands in solution. This was demonstrated with the electrochemical impedance experiment illustrated in Figure 4. In this experiment, a 12K-microelectrode array was functionalized with two peptides each being placed next to 10 microelectrodes. The first, peptide **4**, is known to bind the integrin receptor well.²² The second, peptide H-K(FAM) GGRADSPC-NH₂ (**5**), is known to bind weakly to the integrin receptor. After placing the peptides onto the array, the array was inserted into an electrolysis solution containing potassium ferricyanide along with a Pt wire auxiliary electrode. Current was then passed through the solution using the microelectrode array as the anode and the Pt wire as the cathode. The current associated with the oxidation of ferrous ion in solution (made at the cathode) was measured and the red line in both Figure 4A and Figure 4B recorded.

To the solution was then added an integrin receptor (Integrin $\alpha_{IIb}\beta_{III}$ (GPIIb/IIIa)). In an impedance experiment, the binding of a receptor to molecules on the surface of the microelectrode array blocks the ferrous ion from the neighboring microelectrode causing a dropoff in the current measured. The binding between the solution phase integrin receptor and the microelectrode array bound **4** can be seen in Figure 4A by observing the dropoff in current associated with the blue line (measured for the oxidation of ferrous ion after addition of the integrin receptor) relative to the initial red line. The significantly smaller dropoff observed in Figure 4B indicates the weaker binding event occurring next to the electrodes associated with the second peptide.

In conclusion, we have found that the base catalyzed placement of Michael acceptors on a microelectrode array can be accomplished in a highly site-selective fashion. The placement of peptides onto the array can then be accomplished in a selective fashion with a minimal amount of cross over (5%) between sites on the array. The resulting array can be used to monitor binding events between the peptides placed on the array and a receptor in solution. Work to optimize the selectivity of the placement reactions and establish the utility of the microelectrode arrays for monitoring the binding behavior of molecules in a library with a biological receptor is underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Molecules used in this study

A)

B)

Figure 2.

A) Scheme 1, on a 1K-chip. B) Scheme 1, on a 12K-chip.

Figure 3.

A) Two patterns with acrylate linker **2** and peptide **4** as the thiol source. B) Control experiment using 10 mM 2-hydroxyethane thiol as the initial nucleophile for the checkerboard pattern. C) Blow-up of the checkerboard region of the array in Figure B at longer exposure time.

A)

B)

Figure 4.

Red line – no receptor; blue line – with receptor. A) CV data for the best binding peptide H-K(FAM)GGRGDSPC-NH₂ (4). B) CV data for the weaker binding peptide H-K(FAM)GGRADSPC-NH₂ (5). In each case, 10 microelectrodes on a 12K-microelectrode array were used for the experiment. The experiments used 8 mM K₃Fe(CN)₆ in 30% glycerol, 100 mM NaCl, 20 mM Tris-HCl, 1 mM CaCl₂, and a scan rate of 10 mV/s.

Scheme 1.
Site-selective Michael reaction