Fabrication of an electrochemical DNA sensor array *via* potential-assisted "click" chemistry†

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Received 4th February 2010, Accepted 24th March 2010
First published as an Advance Article on the web 23rd April 2010
DOI: 10.1039/c002371j

Here we report the fabrication of a 3-pixel electrochemical DNA sensor array *via* potential-assisted "click" chemistry. We found that the sensors in the array exhibit close to identical sensor performance when compared to sensors constructed *via* conventional "click" chemistry.

Since its initial introduction to the research community in 2001, Sharpless "click" chemistry has become one of the most versatile and beneficial chemistry tools in organic synthesis and, recently, in surface modifications.[1–5] Previous research has suggested that this chemoselective and site-specific modification technique can be implemented in the fabrication of sensors[6] and we have successfully employed this technique in the fabrication of a folding-based electrochemical-DNA (E-DNA) sensor (Fig. SI-1†).[7] In a standard "click" chemistry reaction, sodium ascorbate is used to reduce Cu(II) to Cu(I), and the ligand tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]-amine (TBTA) is used to stabilize the Cu(I) catalyst (Fig. SI-2†). A less explored but equally advantageous application of this technique is to use electrochemistry to activate the copper catalyst directly on a self-assembled monolayer (SAM)-modified electrode. This allows precise control of the site-specific modification of closely spaced electrode elements with target molecules.[8–10] While other site-specific electrode modification techniques have been utilized in sensor-related applications,[11,12] the potential-assisted "click" chemistry approach, however, has not been employed in the fabrication of biosensors to date. Of note, since the generation of Cu(I) is potential-dependent, potential-assisted "click" chemistry can also be utilized in the fabrication of a multi-pixel sensor array by varying the potential applied to each pixel. This approach can potentially be implemented in the fabrication of biosensor arrays for simultaneous detection of multiple clinically-relevant targets for point-of-care diagnosis.

Here we report, for the first time, the fabrication of a 3-pixel E-DNA sensor array with each pixel functionalized with a different stem-loop DNA probe utilizing potential-assisted "click" chemistry. Our strategy was to first optimize the parameters of potential-assisted "click" chemistry on our SAM-modified electrodes *via* a surrogate probe and subsequently use these parameters to fabricate the sensor array. DNA hybridization efficiency and kinetics were used to characterize the sensors and results were compared to the E-DNA sensors constructed *via* conventional "click" chemistry.[7] First, we investigated the electrochemical reduction of Cu(II) to Cu(I) in the presence of TBTA using a glassy carbon electrode (GC) and a SAM-modified gold disk electrode in the solvent system later used in the fabrication of the E-DNA sensor array. The SAM used in this study was a monolayer consisting of 11-mercapto-1-undecanol (C11-OH) and 1-azido-11-undecanethiol (C11-N_3), similar in composition to the azide-containing SAM used in our previous study.[7] In order to determine the reduction potential of Cu(II) in presence of TBTA, cyclic voltammetry was performed in a deacerated solution of $CuSO_4$ and TBTA in 1 : 1 dimethyl sulfoxide (DMSO)/H_2O supplemented with 0.1 M NaCl. As observed, a reversible voltammetric wave with a half-wave potential of +50 mV is evident for the bare GC electrode within the scan range of 450 mV to −175 mV, indicating the reversible reduction of Cu(II) to Cu(I) in the presence of TBTA (Fig. 1). These results validate the previously reported electrochemical reversibility of the Cu(II)/Cu(I) reaction in DMSO.[13] In a separate study, a half-wave potential of +60 mV for a similar copper complex stabilized by a more water-soluble TBTA derivative has been reported.[14] The cyclic voltammogram obtained with the SAM-modified gold electrode[15] exhibited an irreversible reduction wave with maximum current observed at the limit of the scan range (~−550 mV). A gradual increase in current from −200 mV to −550 mV was observed, suggesting the reduction of Cu(II) to Cu(I) in presence of the TBTA ligand.[16] The absence of an oxidation peak in the anodic scan is presumably due to the insulating effect of the SAM.[17,18] The difference in the electrochemical response between the GC and SAM-modified gold electrode is expected since a C11-OH/C11-N_3 SAM is insulating enough to impede interfacial electron transfer.[17,18] Despite the observed differences, it is apparent that in the solvent system utilized in our study, an adequate amount of Cu(I) catalyst can be electrochemically generated through a C11-OH/C11-N_3 SAM-modified gold electrode.

Having established the feasibility of potential-assisted generation of Cu(I) in our solvent system, we proceeded to investigate the "click" potential dependency on probe capture. A surrogate probe, an alkyne-modified methylene blue (MB) (C_7-MB), was used in this study (Fig. SI-3†). Here we varied the applied potential while keeping all other experimental conditions constant, with the goal of determining an optimal potential for probe immobilization. We then calculated the probe density at each applied potential utilizing a previously established method.[19,20] The computed probe densities at −300, −400 and −500 mV (mV *vs.* Ag/AgCl reference electrode) were 6.88×10^{11}, 1.02×10^{12} and 1.88×10^{12} molecules cm^{-2}, respectively.[21] Despite the apparent trend that more negative

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† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/c002371j

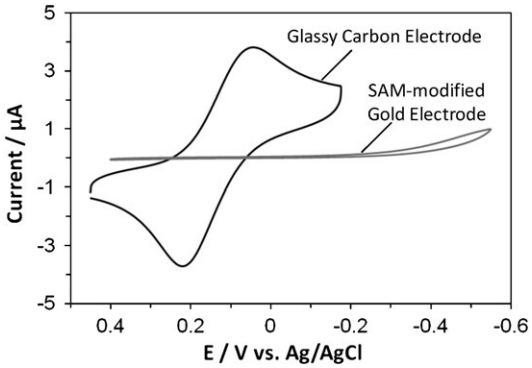

Fig. 1 Cyclic voltammograms of the Cu–TBTA complex obtained in a deaerated 1 : 1 DMSO/H_2O solution containing 150 mM TBTA, 75 mM $CuSO_4$ and 0.1 M NaCl, using a C11-OH/C11-N_3 SAM-modified gold electrode and a bare glassy carbon electrode.

applied potentials yielded higher probe densities, no attempt was made to utilize potentials more negative than −500 mV to prevent reductive desorption of the monolayer. For our system, the computed C_7-MB probe density at −500 mV was almost 3 times higher than that at −300 mV, a previously reported applied potential for electrochemical-assisted "click" chemistry.[8–10] Based on this study, −500 mV appeared to be a good compromise between probe coverage and SAM stability, and was thus used in the remaining studies. Of note, control experiments conducted at open circuit potential (OPC) yielded a notably lower probe density (9.28×10^9 molecules cm^{-2}),[21] suggesting that a relatively negative potential is required for the generation of Cu(I) catalyst and thus the "click" reaction.

Owing to the successful results obtained with the surrogate probe, we proceeded to "click" a MB-labeled DNA probe (*K-ras* probe) onto an azide-containing SAM-modified electrode and compared the results with those obtained with the C_7-MB model system. We found that the probe density of C_7-MB is significantly higher than that of the *K-ras* probe (Fig. SI-4†). While we are aware of the possible differences in electron transfer efficiency between the C_7-MB probe and the *K-ras* DNA probe, it is conceivable to have such drastic probe coverage differences. Specifically, this difference could be attributed to the difference in molecular sizes in which the larger DNA probe produces more steric hindrance when compared to the smaller C_7-MB molecule, leading to the lower probe density (Fig. SI-4†). Another possible reason is the difference in charges: C_7-MB is singly positively charged while the *K-ras* probe is highly negatively charged and susceptible to electrostatic repulsions upon application of the negative "click" potential, thus contributing to the overall low "clicking" efficiency.

With the goal of increasing the DNA probe coverage, a crucial aspect for optimal sensor performance, we studied the effect of ionic strength on probe coverage. We proceeded to optimize the salt (*i.e.*, sodium, magnesium) concentration in the "click" mixture[22] to ensure adequate shielding between the negatively charged phosphate backbone, thus minimizing probe–probe repulsion and probe–electrode repulsion.[23,24] Our results show that the presence of sodium and low levels of magnesium significantly increases DNA "clicking" efficiency, whereas high concentration of magnesium impedes probe attachment (Table 1). While a low concentration of magnesium, in presence of sodium, aids in the reduction of electrostatic repulsion between the probe and the electrode, a higher concentration of magnesium (*i.e.* 100 mM) presumably induces probe aggregation, thus limiting "clicking" efficiency, as indicated by the low probe density.[25,26] Based on these results, the optimal salt concentration for DNA probe immobilization was 0.1 M NaCl with 2.7 mM $MgCl_2$, and this salt combination was used in the remaining studies.

Table 1 *K-ras* DNA probe density as a function of the ionic strength of the "click" mixture.[22] The reported values are averages of two separate experiments

Salts added to the "click" mixture	Probe density (molecules cm^{-2})
None (deionized water)	8.87×10^9
100 mM Na only	3.97×10^{11}
100 mM Na + 2.7 mM Mg	5.16×10^{11}
100 mM Na + 5.9 mM Mg	3.03×10^{11}
100 mM Mg only	4.04×10^8

After having successfully optimized both the "click" potential and the ionic strength, the two main challenges in potential-assisted "click" chemistry, we applied this approach to the fabrication of a 3-pixel E-DNA sensor array.[11] The three MB-modified DNA probes used in this study were designed to target specific cancer-related gene sequences (*i.e.*, the *Brca2*, *p53* and *K-ras* gene). To fabricate the sensor array, we incubated the C11-OH/C11-N_3 SAM-modified electrode array in a "click" mixture[27] containing the specific DNA probe while applying a potential of −500 mV to the desired pixel (*i.e.*, Pixel 1) and holding the other two pixels at OPC. All three pixels were exposed to the "click" mixture, but only the pixel at which the potential was applied was modified with DNA probes; pixels that were held at OPC remained unaltered. Using this approach, the 3 pixels in the array were sequentially modified with their respective DNA probes prior to target interrogation (see Supplementary Information†). Pixel 1 was modified with the *Brac2* probes, whereas Pixels 2 and 3 were modified with the *p53* and *K-ras* probes, respectively. Sensor interrogation with the full complement target DNA was conducted one at a time to determine possible cross-reactivity (*i.e.*, specific conjugation of DNA probes onto pixels that were held at OPC). In this study, Pixel 3 was the last to be modified with probe DNA (*K-ras*), but as observed, no substantial signal suppression (*i.e.*, indication of hybridization) was evident when challenged with target DNA complementary to the other two probes, suggesting minimal cross reactivity during the functionalization steps (Fig. 2, Fig. SI-5†).

With regard to sensor performance, we found that the sensors modified with *Brca2* and *p53* probes responded faster (saturation time ~13 min)[28] and showed larger signal suppression (~72%) when compared to the sensor functionalized with the *K-ras* probe, which showed a signal saturation time[28] of ~55 min and signal suppression of ~54% (Fig. 2, Fig. SI-5†). The faster and better sensor response observed with the *Brca2* and *p53* probes is probably due to the design of the probe in which both are normal stem loop probes; the *K-ras* probe, however, has a mini-loop within the major loop region (Fig. SI-6†). Thus, target hybridization to the *Brca2* and *p53* probes would be both kinetically and thermodynamically more

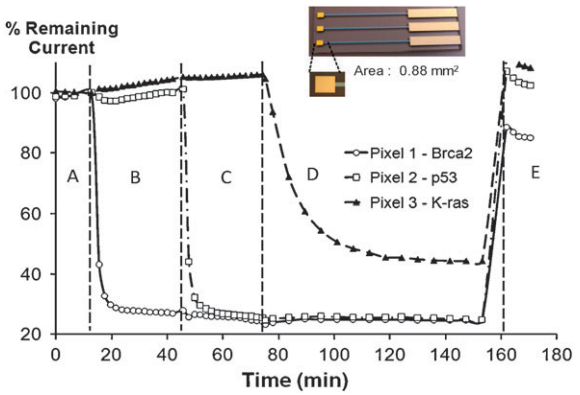

Fig. 2 Equilibration–interrogation–regeneration cycles of the 3 DNA sensors in the array. Area A indicates the time used to reach sensor equilibration prior to target interrogation. Areas B, C and D indicate sensor interrogation with *Brca2*, *p53* and *K-ras* target DNA, respectively. Area E indicates sensor regeneration. Inset: the microfabricated 3-pixel array used in this study.

favourable when compared to the *K-ras* probe. Of note, the slower hybridization kinetics and lower signal suppression observed with the *K-ras* probe are evident even when immobilized on a gold disk electrode using conventional "click" chemistry with sodium ascorbate as the reducing agent.[7] We also noticed that the *Brca2* probe did not regenerate (~84% regeneration) as well as the *p53* (~101% regeneration) and *K-ras* (~106% regeneration) probes.[29] The lack of complete sensor regeneration with the *Brca2* probe could be attributed to the differences in the probe sequence or features which will require further investigation. The observed signal recovery in excess of 100% presumably arises because the AC current observed with the E-DNA sensors, in particular, those fabricated on thin-film gold electrodes, is rather sensitive to the force with which the washing step is conducted.[30] Of note, despite the slight differences in sensor behaviour among the three probes, E-DNA sensors fabricated *via* potential-assisted "click" chemistry can be used repeatedly and have shown sensor stability of ~8 h.[31]

In conclusion, we have fabricated the first E-DNA sensor array *via* potential-assisted "click" chemistry. The sensors exhibit comparable attributes to sensors fabricated *via* the conventional "click" chemistry approach.[7] This versatile sensor array fabrication approach could presumably be adapted to the fabrication of other folding-based electrochemical biosensors and these prospects are currently being investigated in our lab.[32,33]

The authors acknowledge the University of Nebraska-Lincoln and NSF MRSEC (Grant No. DMR-0820521) for financial support. The authors also want to thank Dr Kevin Plaxco and Dr Hyongsok Tom Soh for the electrode arrays.

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