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Electrochemical Modification of Glassy Carbon

by Amethyst S. Finch and James J. Sumner

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14. ABSTRACT With the sequencing of whole genomes now a reality, the push to synthesize genomes de novo is of great interest. Currently, state-of-the-art oligonucleotide synthetic methods allow for synthesis of up to 200 base pairs. In order to realize increased lengths of an order of magnitude or more, the development of new synthetic strategies and coupling mechanisms is necessary. This project aims to provide an alternative methodology for the synthesis of very long (>200 base pairs) deoxyribonucleic acid (DNA) oligonucleotides. This technique involves using a glass carbon rod as the support for the DNA synthesis and requires development of experimental techniques for irreversibly linking an organic molecule with an aniline-type moiety to the glassy carbon. By in situ conversion of the aniline functional group to its diazonium derivative, we demonstrate direct electrochemical modification of the glassy carbon rod. These preliminary results allowed for the transfer of these materials to collaborators at the Food and Drug Administration for use in their research on alternative methods for automated synthesis of oligonucleotides.					
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1. Introduction

The field of biology has greatly pushed the envelope over the past decade with the ability sequence whole genomes in a matter of days (1). Many different competing technologies have been developed that allow for the sequencing of organism genomes and each of these technologies has its strengths (2). These sequencing technologies will allow for applications that go beyond de novo sequencing by tying genomics into lines of research on things such as chromatin structure, epigenomics, RNA transcription, immunology, and genomes of microbial communities.

In contrast, the field of deoxyribonucleic acid (DNA) oligonucleotide synthesis has changed very little over the last decade. The same methodologies that were developed in the 1960s and 1970s are still in use for solid phase oligonucleotide synthesis today. While these methodologies are very well studied and each step proceeds with high yield when the steps are combined to make longer oligonucleotides, the intrinsic error is additive, prohibiting this technology from progressing towards longer sequences. Additionally, for DNA synthesis to reach the level of whole genome synthesis, great leaps in scientific understanding and alternative technologies are necessary. In order to move the field to the next level, there is a need to develop ultra high fidelity methodologies that will allow for the synthesis of ultra long oligonucleotides.

This project aims to provide a scaffold that will allow for a revolution in DNA synthetic technology, ultimately allowing for the synthesis of very long (>200 base pairs) DNA oligonucleotides. This technique involves using a glassy carbon (GC) rod in a flow cell device as the support for the DNA synthesis and requires development of experimental techniques for irreversibly linking an organic molecule with an aniline-type moiety to the glassy carbon. By in situ conversion of the aniline functional group to its diazonium derivative, we demonstrate direct electrochemical modification of the GC rod. These preliminary results allowed for the transfer of these materials to collaborators at the Food and Drug Administration for use in their research on alternative methods for automated synthesis of oligonucleotides.

2. Materials and Methods

All chemicals were purchased from Thermo Fisher or Sigma-Aldrich and were of the highest grade available. Working, counter, and reference electrodes as well as a polishing kit were purchased from BASi (West Lafayette, IN). GC rods and diazonium linker moiety (DLM) were provided by Serge Beaucage and Cristina Ausin of the Food and Drug Administration (FDA). All electrochemical characterization measurements were carried out using a CH Instruments

660A electrochemical workstation (Austin, TX) and data were processed using standard (OriginPro V. 8.5) graphing software.

2.1 Alternating Current (AC) Voltammetry

AC voltammetry experiments were run in a three-electrode cell with a GC working, a platinum wire counter, and a saturated calomel reference electrode. Phosphate buffered saline (PBS) pH 7.4 (Sigma) was used as the electrolyte. One tablet of PBS was dissolved in 200 mL of double-distilled water (ddH₂O) to make a working solution of 10 mM potassium phosphate, 137 mM sodium chloride (NaCl), and 2.7 mM potassium chloride (KCl). Solutions of diaminonaphthalene (500 mM) were prepared in high performance liquid chromatography (HPLC)-grade acetonitrile. Diaminonaphthalene solutions (20 μ L) were then mixed with 10 mL of PBS to final concentration of 1 mM in 0.2% acetonitrile. The following parameters were set for on the AC voltammetry workstation for all experiments:

- Init E (V) = 0
- Final E (V) = 0.7
- Incr E (V) = 0.004
- Amplitude (V) = 0.025
- Frequency (Hz) = 10
- Sample Period (s) = 1
- Quiet Time (s) = 2
- Sensitivity (A/V) = 1e-5

2.2 Cyclic Voltammetry (CV)

Experiments were carried out in a three-electrode cell with a GC working, a platinum wire counter, and a saturated calomel reference electrode. A 1 M stock solution of the DLM was prepared by dissolving 0.6 mg in 1 mL of HPLC-grade acetonitrile. This stock solution was used to make the working solutions of 1 and 2 mM in 0.25 M KCl in 0.1% acetonitrile. The following parameters were set for CV on the workstation for all experiments:

- Init E (V) = -0.4 to 0
- High E (V) = 0.7
- Low E (V) = -0.3
- Init P/N = P
- Scan Rate (V/s) = 0.05

- Segment = 2
- Sample Interval (V) = 0.001
- Quiet Time (s) = 2
- Sensitivity (A/V) = 1e-5 to 1e-7

2.3 Electrode Testing

Coverage DLM on standard electrodes and GC rods was determined via fluorescence experiments conducted at the FDA.

3. Results and Discussion

3.1 Previous Results

Figure 1 illustrates the compounds used in initial CV experiments for determining E_{ox} of diaminonaphthalene (DAN) and DAN derivatives (figure 1) using CV. This proved to be problematic due to polymer film formation of DAN upon the surface of the GC and platinum electrode (3). This polymer was not conductive under aqueous conditions; therefore, the reverse scan and subsequent scans showed diminishing current. In order to determine that instrumentation was not the cause of the erratic scans and polymer formation, E_{ox} determinations were repeated under the CV conditions outline in reference 3 (3a). The data were successfully reproduced using AC voltammetry coupled with an exhaustive process of cleaning and polishing the electrode surface after each scan (figure 2) (4).

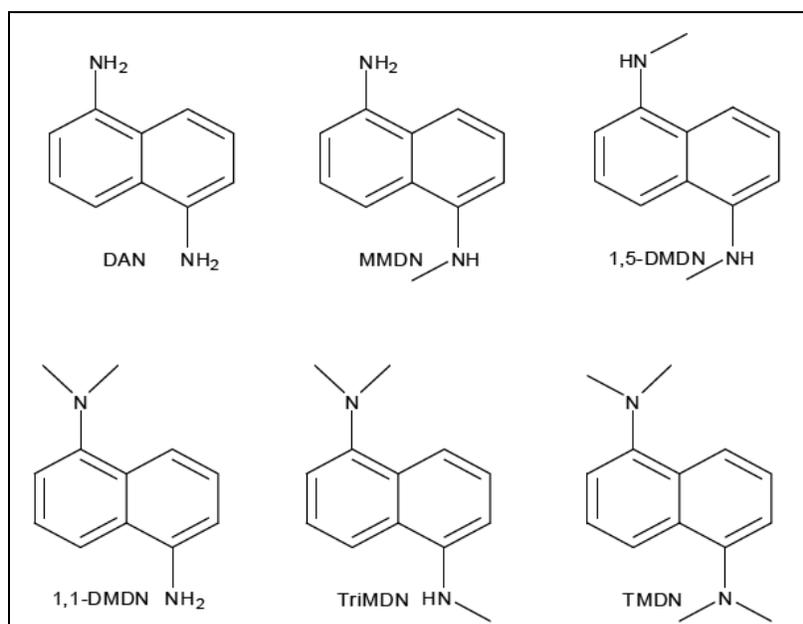


Figure 1. Diaminonaphthalene derivatives.

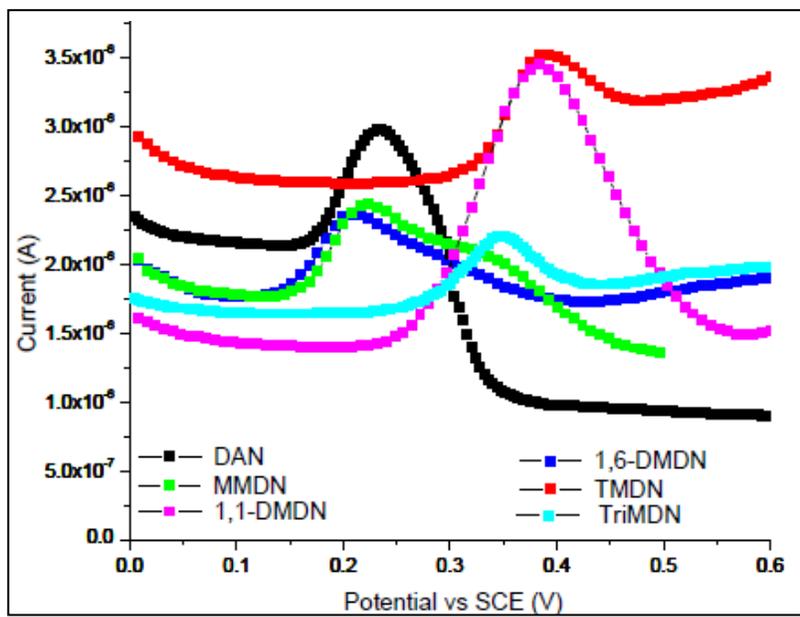


Figure 2. Representative composite AC voltammogram of DAN derivatives. All experiments were conducted at ambient temperature in a three-component electrode cell with a platinum working, a platinum wire counter, and a saturated calomel reference electrode.

This polymer film formation was a hindrance in E_{ox} determinations; however, for the purposes of linking the DLM to the GC electrode surface, this film formation was seen as an advantageous occurrence. The methodologies used for these experiments were translated to the electrochemistry on the DLM compound with GC electrodes.

3.2 Linker Moiety

The DLM compound (figure 3) was synthesized and provided in its diazonium form by the FDA. The DLM was dissolved directly into acetonitrile and used in all subsequent electrochemistry experiments.

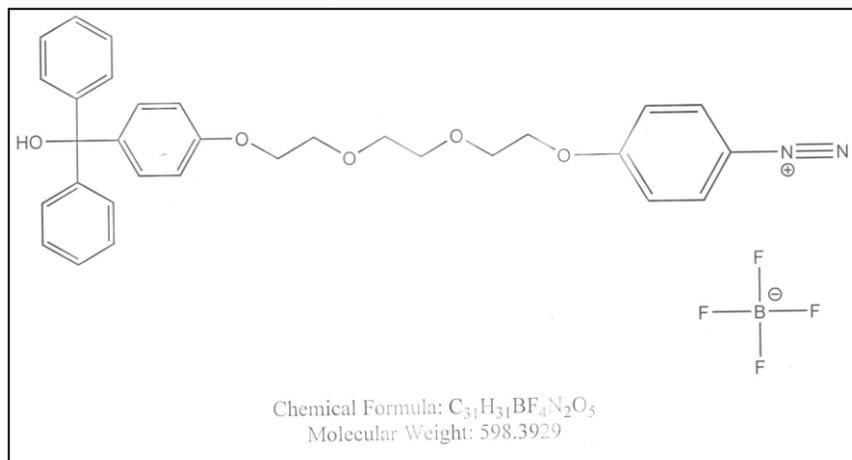


Figure 3. DLM provided by the FDA for coupling to GC electrodes via electrochemical methods.

3.3 Standard Glassy Carbon (GC) Electrode Experiments

Initial experiments were conducted on standard GC electrodes as supplied by the manufacturer. These electrodes (figure 4) are manufactured in a solvent-resistant chlorotrifluoroethylene (CTFE) plastic body (7.5 cm length x 6 mm outer diameter [OD]), which is embedded with highly polished 3.0-mm GC disks.

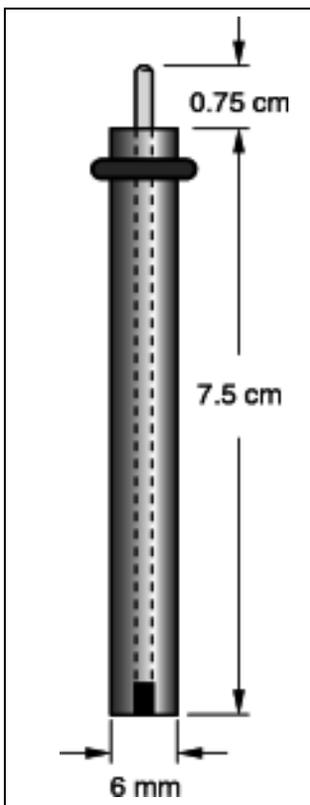


Figure 4. Schematic of standard GC electrode (BASi Inc.) (5).

Three standard electrodes were scanned under varying conditions in order to determine the optimal scanning parameters. Electrode 1 was scanned a single time while electrodes 2 and 3 were each scanned five times. The plot of the single scan on electrode 1 is illustrated in figure 5. Note the redox activity around 0.8 V riding the background with a dramatically increasing slope.

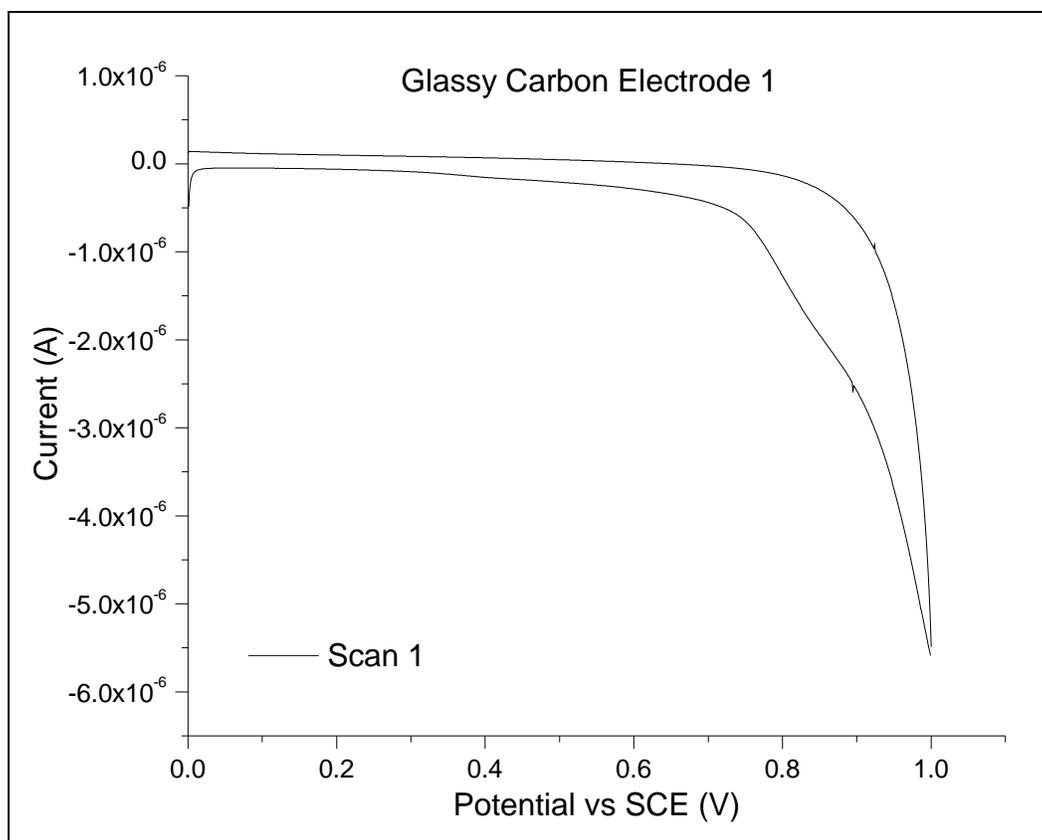


Figure 5. Representative CV of DLM scanned one time with a standard GC electrode. All experiments were conducted at ambient temperature in a three-component electrode cell with a GC working, a platinum wire counter, and a saturated calomel reference electrode in 0.25 M KCl (pH 7.0) and 0.1% acetonitrile with an DLM concentration of 1 mM.

Electrode 2 was scanned a total of five times. The scan range was optimized from electrode 1. The initial and third scans were conducted under the same parameters as electrode 1 and there was little or no change between these plots from that of electrode 1. Scan 3 (figure 6) was conducted in an increased potential window from -0.4 to 1 V and scans 4 and 5 were conducted in a reduced potential window from 0 to 0.6 V. A representative CV of scans 4 and 5 are illustrated in figure 7. Figure 6 shows a scan in which the potential window is too large. There is hysteresis near -0.3 V and the rapidly changing slopes at both extremes indicate solvent or electrode breakdown. These scans suggest that the most optimal window for scanning may be between 0 to 0.7 V.

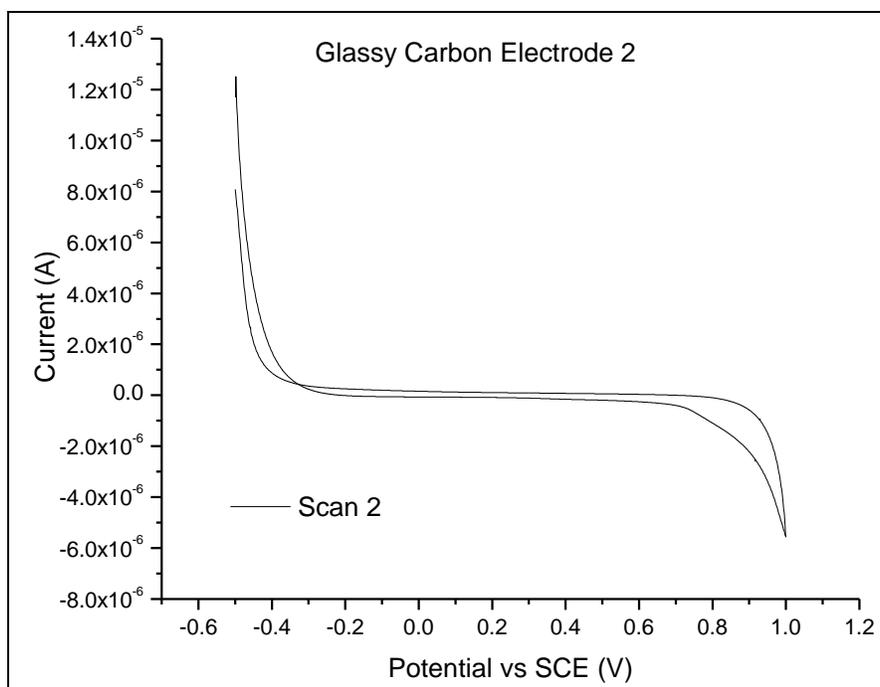


Figure 6. Representative CV (scan 3) of DLM scanned with a standard GC electrode. All experiments were conducted at ambient temperature in a three-component electrode cell with a GC working, a platinum wire counter, and a saturated calomel reference electrode in 0.25 M KCl (pH 7.0) and 0.1% acetonitrile with an DLM concentration of 1 mM.

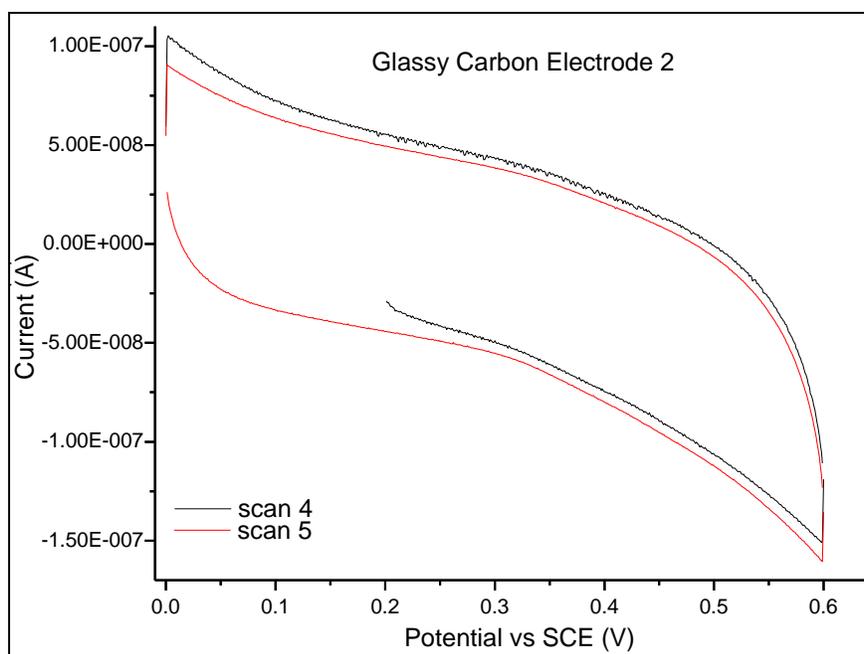


Figure 7. Representative CV (scans 4 and 5) of DLM scanned with a standard GC electrode. All experiments were conducted at ambient temperature in a three-component electrode cell with a GC working, a platinum wire counter, and a saturated calomel reference electrode in 0.25 M KCl (pH 7.0) and 0.1% acetonitrile with an DLM concentration of 1 mM.

Electrode 3 was scanned five times all under the same parameters from 0 V to 0.7 V. Figure 8 shows the results of the 5 scans. These scans clearly show redox activity at 0.45 V and each subsequent scan illustrates that there is less chemistry occurring on the surface of the GC electrode. This was due to the polymerization on the electrode surface.

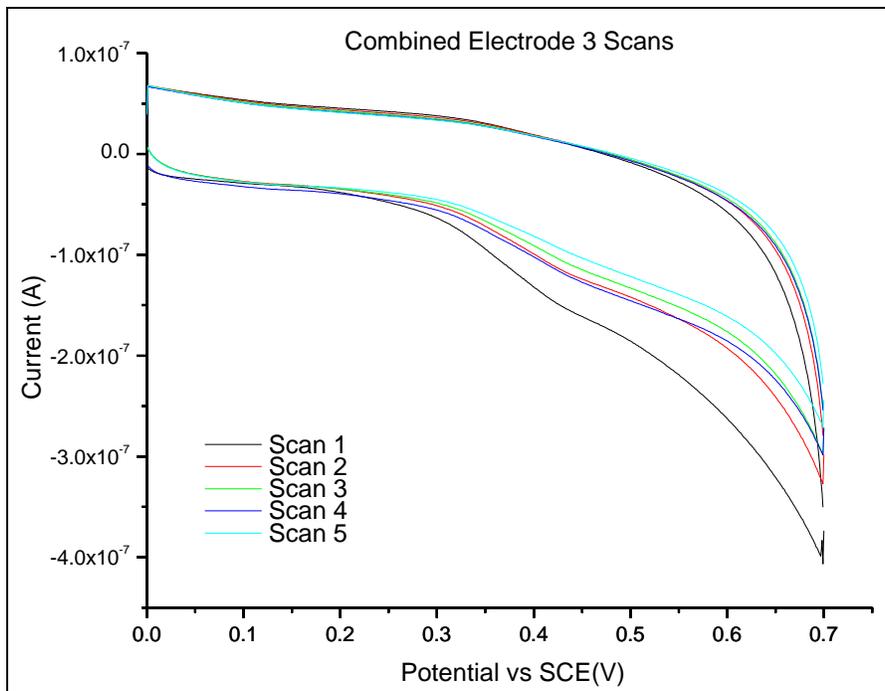


Figure 8. CV of DLM scanned five times with standard GC electrode 3. All experiments were conducted at ambient temperature in a three component electrode cell with a GC working, a platinum wire counter, and a saturated calomel reference electrode in 0.25 M KCl (pH 7.0) and 0.1% acetonitrile with an DLM concentration of 1 mM.

All three DLM passivated standard GC electrodes were provided to our collaborators at the FDA, where they conducted standard fluorescence measurements to determine extent of coverage on the GC surface. Initial results indicate that that all electrodes were covered, although second and third electrodes provided the most uniformity by fluorescence experiments. These preliminary results allowed for us to move the technical methods determined from these control experiments to the full GC rods.

3.3 Modified GC Rod Experiments

Prior to testing the electrochemical coupling strategy on the GC rods, a methodology for protecting the ends rods needed to be developed. This methodology was necessary because the design of the flow cell required the GC rods to be fully functionalized except on the ends of the rods. This was to prohibit chemistry on the ends of the rods, which was hypothesized to be lower yielding due to steric constraints. Additionally, because of the cost of the ultra high purity and polished GC rods, the initial studies were conducted on less expensive graphite rods. The

graphite rods were first wrapped with conductive wire (platinum) in order to create an electrical connection, wrapped with parafilm, and capped with shrink wrap tubing. This entire assembly was immersed in a 0.25-M KCl solution for testing of the passivation technique. The graphite rods were tested for their conductivity to determine the ability of the rods to act as electrodes after capping and checked to ensure the rod remained dry underneath the cap. Upon successful testing of the capping methodologies on the graphite rods, the subsequent experiments were conducted on GC rods.

GC rod 1 was capped by the method described above and the sample was scanned five times from 0 to 0.7 V. Figure 9 illustrates these scans. After the first scan, there does not appear to be much change in CV, indicating that there is little or no change in the surface chemistry of the GC rod. The capping was removed and the GC rod 1 was then stored in a microfuge tube filled with fresh 0.25 M KCl.

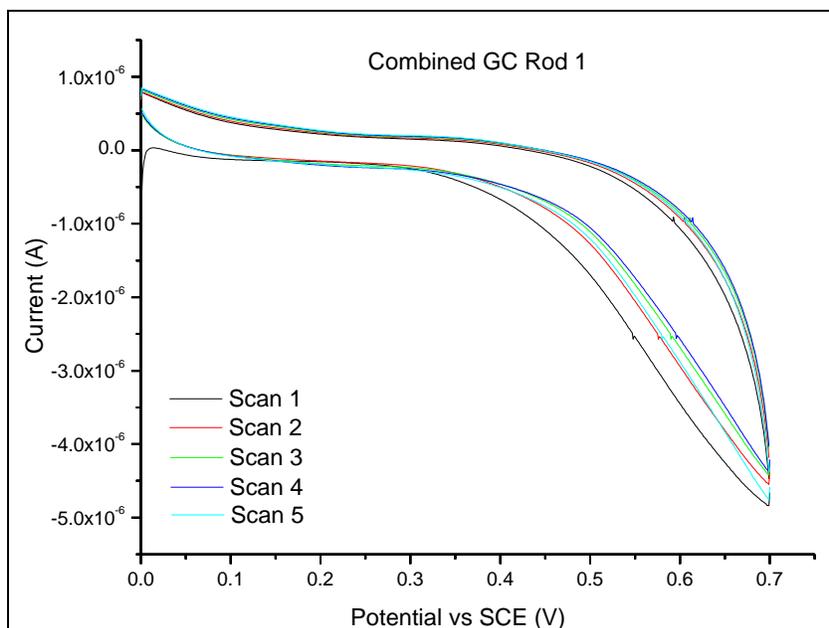


Figure 9. Representative CV of DLM scanned five times with a GC rod 1. All experiments were conducted at ambient temperature in a three component electrode cell with a GC working, a platinum wire counter, and a saturated calomel reference electrode in 0.25 KCl (pH 7.0) and 0.1% acetonitrile with an DLM concentration of 2 mM.

GC rod 2 was also capped by the method described previously and this sample was scanned five times with an increased potential scan window. The initial scan was conducted with a range of -0.4 to 0.7 V and subsequent scans (2–5) were conducted from -0.3 to 0.7 V. Figure 10 illustrates the results of these sets of scans. As before, there appears to be little or no change in the CV after the initial scan. The end capping was removed and GC rod 2 was stored in a microcentrifuge tube filled with fresh 0.25 M KCl.

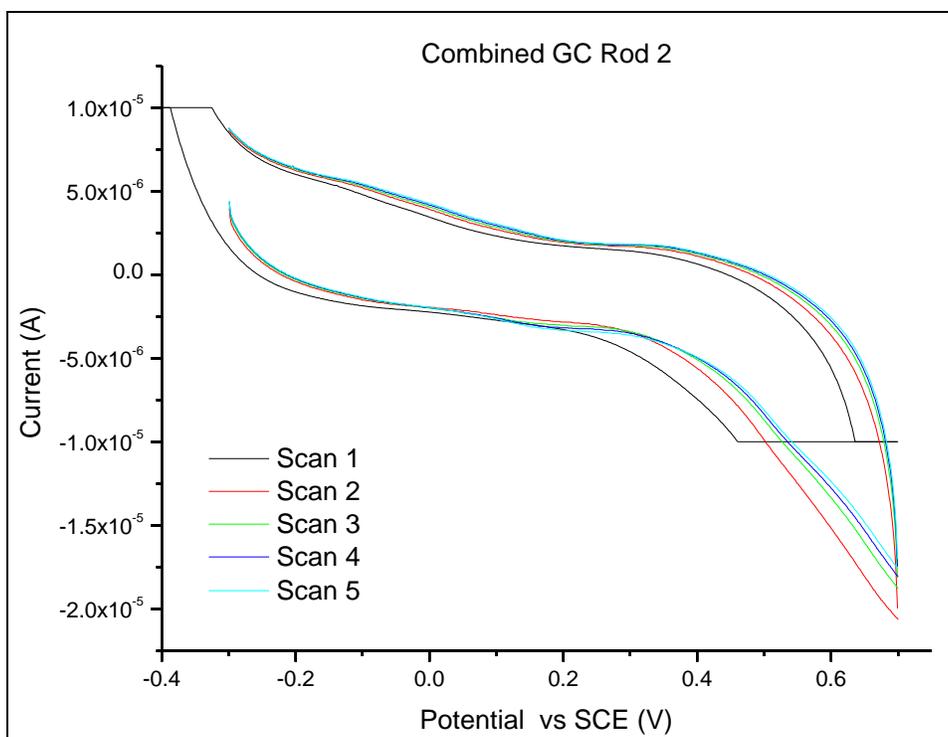


Figure 10. Representative CV of DLM on scanned five times with a GC rod 2. All experiments were conducted at ambient temperature in a three component electrode cell with a GC working, a platinum wire counter, and a saturated calomel reference electrode in 0.25 M potassium chloride (pH 7.0) and 0.1% acetonitrile with an DLM concentration of 2 mM.

Both GC 1 and 2 rods were then provided to the FDA for further characterization via fluorescence measurements. The rods were tested by the FDA and both were adequately coated with the DLM. Our FDA collaborators were then able to attach a nucleoside (~1 nmol based on fluorescence measurements) to each rod and manually synthesize a dinucleotide. They also checked the recyclability of one of the rods, by cleaving the dinucleotide from the rod and successfully reattaching a new nucleoside afterwards with consistent yields. Currently, we are in the process of optimizing the design of the synthesis cell and will soon proceed with the automated synthesis of oligonucleotides.

4. Conclusions

In conclusion, we were able to quickly determine an electrochemical coupling strategy that can be translated to a variety of linker arms similar to DLM for passivation of GC surfaces. We transitioned GC rod passivated with DLM samples to the FDA for further testing and all the experiments were successful. The ease and versatility of this methodology provides a strong framework for future work.

5. References

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List of Symbols, Abbreviations and Acronyms

AC	alternating current
CTFE	chlorotrifluoroethylene
CV	cyclic voltammetry
DAN	diaminonaphthalene
ddH ₂ O	double-distilled water
DLM	diazonium linker moiety
DNA	deoxyribonucleic acid
FDA	Food and Drug Administration
GC	glassy carbon
HPLC	high performance liquid chromatography
KCl	potassium chloride
NaCl	sodium chloride
OD	outer diameter
PBS	phosphate buffered saline

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