Chapter 7

Development of Glassy Carbon Flow-through Cells for Biomolecule Analysis

M. G. Hill contributed to the design and construction of the flow-through cell.

Introduction

Biomolecule detection involving DNA charge transport (DNA CT) has enabled the analysis of a variety of targets, including nucleic acids,[1, 2] DNA-binding proteins,[3-5] and epigenetic modifications.[6] In fact, such platforms have enabled the sensitive and selective analysis of the human methyltransferase, DNMT1, from crude tumor lysate.[7] As this assay has the potential to function as a clinical diagnostic, increasing the efficiency and ease of use will greatly increase its utility. One major component of increasing the efficiency of this system is to decrease the amount of sample necessary for detection. To achieve detection from smaller sample volumes without significantly altering detection assay itself, it is necessary instead to modify the electrode platform utilized for detection through the incorporation of microfluidics into the platform.

Microfluidics, the manipulation of fluids over micrometer to millimeter length scales, is ideal for biomolecule analysis, as it requires minimal reagent and provides a large surface area-to-volume ratio for measurements.[8] Microfluidics platforms enable accurate, rapid operation with multiplexing for the simultaneous analysis of multiple clinical samples and targets.[9] Biological applications of microfluidic devices range from high-throughput pharmaceutical analysis[10] to lab-on chip analysis of blood glucose levels.[11] Such platforms are advantageous, as they enable sample preparation and analysis in a single device, minimizing the amount of sample and the level of sample preparation required. Additionally, electrochemical detection is compatible with microfluidics platforms; common electrochemical methods incorporated into microfluidics platforms include potentiometry,[12] amperometry,[13] and conductometry,[14] in addition to electrophoretic separations.[15]

Although a multitude of biomarkers have been connected to tumorogenesis, DNA methylation has recently garnered significant interest because of its role in gene expression.[16-21] Specifically, DNA methyltransferase activity detected electrochemically is a viable method to monitor potential cancerous transformations. Tight control over DNA methylation in cells is vital, as methylation patterns strongly affect gene expression.[22] DNA methyltransferases maintain a specific genomic methylation pattern by the covalent methylation of cytosine at 5′-CG-3′ sites. Aberrant activity of DNA methyltransferases can cause hypermethylation, silencing tumor suppressor genes and promoting cancerous transformations.[23-26] The most abundant mammalian methyltransferase is DNMT1, a maintenance methyltransferase that preferentially methylates hemimethylated DNA using the cofactor *S*-adenosyl-L-methionine (SAM).[27, 28] In the Barton lab, we have recently developed an electrochemical assay for the specific detection of DNMT1 activity from crude cultured cell and human tissue lysate.[7] This assay employs a methylation-sensitive restriction enzyme to convert the methylation state of the DNA into an electrochemical signal, thereby enabling the detection of a methyl group, even though methylation itself does not significantly affect DNA CT.

Although our two working electrode platform requires a minimal amount of lysate for detection, all of the preparation of the lysate must be performed macroscopically external to the platform. Furthermore, while nucleic acid sensors have been incorporated into microfluidic platforms,[29] the electrodes involved in detection remain planar, limiting the surface area to volume ratio. Additionally, the sensitivity, specificity, and selectivity of DNA-mediated charge transport (DNA CT) have yet to be applied to microfluidics-based biomolecule detection. We have developed an electrochemical flow-through cell

in which the electrodes themselves form the fluidics channels. The devices, fabricated from glassy carbon, silver, and Kel-F, have a total volume of less than 5 μL with two working electrodes, each with an area of 4.7 mm^2.

Methods and Materials

Unless otherwise stated, all reagents were purchased from Sigma Aldrich.

Glassy Carbon Flow-Through Cells

Glassy carbon cells were fabricated in a stepwise manner. Glassy carbon working electrodes and silver pseudoreference electrodes, as well as Kel-F endcaps, were fabricated in the Caltech machine shop. Glassy carbon electrodes were milled from 10 cm x 10 cm x 1.5 mm glassy carbon sheets (Bayville Chemical) to final dimensions of 6 mm x 6 mm x 1.5 mm with a 1 mm diameter hole drilled through the center. A diamond grit drill bit (National Diamond Lab) with wet drilling was used to form the hole in the electrodes.

A 10 cm x 10 cm x 1 mm sheet of silver foil was used to form the silver pseudoreference electrode. The silver electrodes were formed to 6.2 mm x 6.0 mm x 1 mm, again with a 1 mm hole in the center. The Kel-F pieces were 6 mm^3 with a 1 mm hole in the center.

Before fabrication, the faces containing the holes of both the glassy carbon and the silver electrodes were coated with Super Corona Dope (MG Chemicals). One face of the Kel-F pieces was roughed with P50 grit sandpaper to ensure that the epoxy remains affixed. The holes were then aligned, and the pieces are sandwiched together and affixed with 5-minute epoxy. One Kel-F piece, followed by a glassy carbon and a silver piece were added, followed by the second glassy carbon electrode and a final Kel-F piece were connected. The epoxy was allowed to harden with the devices in a vice.

To form electronic connections between the potentiostat and the electrodes, silver epoxy was painted in a thin strip on the top of each electrode (two glassy carbon working electrodes and the silver pseudoreference electrode). 0.25 mm diameter silver wire was connected to each electrode with the silver epoxy. It is vital at this point that the silver epoxy and the wires are isolated to a particular electrode. If any crossover occurs, the device will short circuit.

To create a flow cell through this piece, 1 mm outer diameter HPLC tubing (IDEX Health and Science, LLC) was inserted into the Kel-F until it reached the glassy carbon working electrode. A sharp was then inserted into the tubing to enable facile connection to a syringe. Electrochemical readout with this platform was performed by connecting the flow-through cell to a potentiostat via each of the silver wire leads previously attached to the cell. The silver in the center acts as a pseudoreference electrode, and a Pt counter electrode was inserted into the HPLC tubing output from the device.

Synthesis of 4-azidobenzene Diazonium Tetrafluoroborate

Protocol adapted from Evrard et al. 2008.[30] An aqueous solution of $NaNO_2$ (90 mg, 1.3 mmol) in 0.5 mL H_2O at 4° C was added dropwise to a solution of 4-azidoaniline hydrochloride (200 mg, 1.18 mmol) in 2 mL 1 M HCl on ice. The resulting solution was stirred at 4° C for 1 h. 1.5 mL of a saturated aqueous solution of $NaBF_4$ was then added, forming a light brown precipitate. The solid was filtered and rinsed with 2 x 6 mL diethyl ether. The product was purified by recrystallization in diethyl ether from

acetonitrile. The product is an off-white powder, which was stored under vacuum. ESI-MS: calc'd: 145.18 found: 145.8 $[M]^+$.

Glassy Carbon Rod Electrode Modification

Preliminary experiments with aniline attachment to glassy carbon were performed on glassy carbon rod electrodes (3.0 mm diameter) (Bioanalytical Systems, Inc.). Glassy carbon electrodes were prepared by polishing the electrodes with 300 grit sandpaper, followed by 0.3 micron diamond polish on a cloth polishing pad, and finally 0.05 micron silica polish. Electrodes were then sonicated in aqueous solution for 5 minutes.

Aniline and its derivatives were attached to glassy carbon either using an *in situ* diazonium formation method[31] or with activation of a pre-formed diazonium salt. For *in situ* formation, either 1 mM or 10 mM total aniline was dissolved in 0.1 M tetrabutylammonium tetrafluoroborate containing 4% 1M HCl. The solutions were chilled to 4° C and degassed on an ice bath, followed by the addition of 1 mM or 10 mM sodium nitrite with continued argon bubbling on ice. The diazoium was tethered to the glassy carbon through electrochemical activation, either by cyclic voltammetry (CV) or by constant potential amperometry (CPE). Generally, CV's were scanned from 500 mV to -1000 mV v AgCl/Ag for various numbers of scans. Additionally, CPE was run at -750 mV v AgCl/Ag for 120 seconds.

Electrochemical surface modification of glassy carbon electrodes using 4-azidobenzene diazonium tetrafluoroborate[30] was performed in a similar manner to the *in situ* method. 1.3 mM 4-azidobenzene diazonium tetrafluoroborate in 0.1 M HCl was degassed and cooled to 4° C. Electrodeposition was achieved through either CPE for 120

s at -800 mV v AgCl/Ag or cyclic voltammetry from 500 mV to -800 mV v AgCl/Ag at 50 mV/s for four scans.

Passivation against both methylene blue and ferricyanide were tested by scanning the modified glassy carbon electrodes in phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) containing various concentrations of methylene blue or $[Fe(CN)_6]^{3-}$. Multiple combinations of 4-azidoaniline with aniline, p-nitroaniline, 4-aminobenzoic acid, or p-phenylenediamine were tested for passivation against methylene blue and $[Fe(CN)_6]^{3-}$.

Coupling of ethynyl ferrocene to surfaces was effected using copper-catalyzed click chemistry. Copper catalyst was activated by ascorbic acid. Ascorbic acid dissolved in water was added to ethynyl ferrocence and thoroughly degassed. $CuSO_4$, $[Cu(bathophen)_2]^{2+}$ (bathophen = Bathophenanthrolinedisulfonic acid), $[Cu(phendione)_2]^{2+}$ (phendione= 1,10-Phenanthroline-5,6-dione), or $[Cu(TBTA)]^{2+}$ (TBTA= Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) was independently degassed. Just before clicking to the electrode, copper solution was added to the ascorbic acid and ethynyl ferrocene. The final concentrations of all components in 2:1:1 H_2O: DMSO:EtOH were 300 μM ascorbic acid, 25 μM copper, and 25 μM ethynyl ferrocene. Rod electrodes were soaked in 25 μL of the clicking solution for varying times between one and two hours, followed by rinsing with water. Residual copper was removed by soaking the electrodes in 1 mM EDTA in pH 8.0 phosphate buffer (5 mM PO_4, 50 mM NaCl, pH 8.0).

Glassy Carbon Flow-through Cell Modification

Glassy carbon flow-through cells were modified with diazonium salts in the same manner as the rod electrodes. Briefly, aniline derivatives were attached to glassy carbon using an *in situ* diazonium formation method.[31] 1 mM azidoaniline was dissolved in 0.1 M tetrabutylammonium tetrafluoroborate containing 4% 1M HCl. The solution was chilled to 0° C and degassed on an ice bath, followed by the addition of 1 mM sodium nitrite with continued argon bubbling on ice. The diazoium solution was added to the device *via* syringe, and was tethered to one of the glassy carbon working electrodes through electrochemical activation by CV. CVs were run by scanning from 500 mV to -850 mV for between 2 and 4 scans.

Electrochemical click on the flow-through cell was performed through the addition of 25 μM $[Cu(bathophen)_2]^{2+}$ and 25 μM ethynyl ferrocene in 1:1 water : ethanol. A 0.25 mm diameter silver wire was inserted into the cell, and a -350 mV potential was applied to the wire for 10 minutes.

Results and Discussion

Fabrication of Flow-through Cells

A basic four-electrode set-up contains two working electrodes, a reference electrode, and an auxiliary electrode. A flow-through cell combines these elements, along with a path for liquid flow. An overview of the setup is shown in Figure 7.1. Briefly, the set-up contains a path for fluidics that runs through a sandwich structure comprised of insulating material, glassy carbon working electrodes, a silver pseudoreference electrode, and a Pt wire counter electrode. This overall schematic allows for incredibly small sample volumes compared to macroscopic electrodes. While a single inlet and outlet are shown, these devices can be multiplexed and combined in parallel to perform detection of multiple analytes simultaneously.

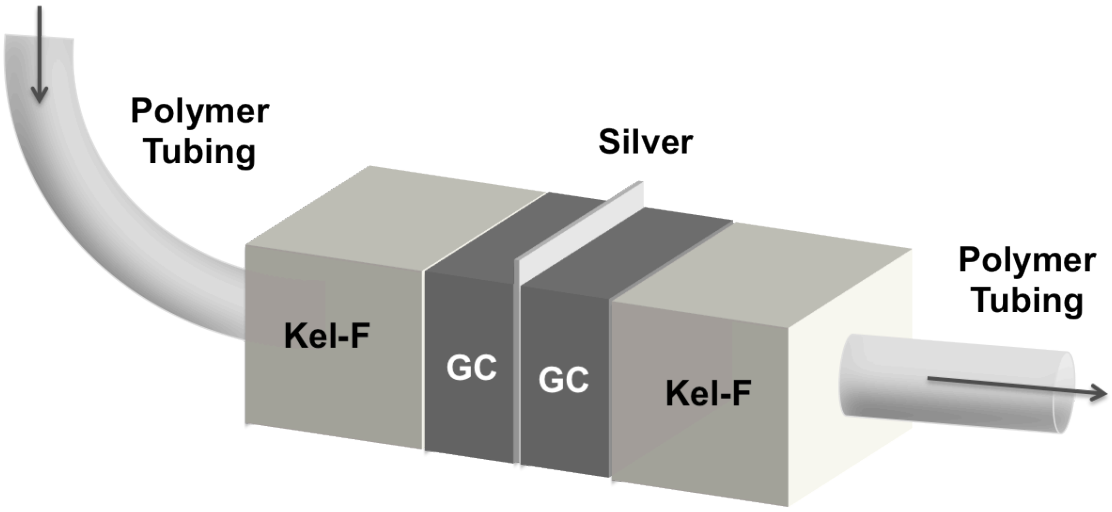

Figure 7.1 Flow-through cell configuration. A 1 mm diameter hole is created through all of the materials to form a channel through which analyte solution is added for detection. The glassy carbon electrode flow-through cell is comprised of two Kel-F ends, two glassy carbon working electrodes, and a silver foil pseudoreference electrode sandwiched between the two working electrodes. Analyte is added to the device through polymer tubing attached to the Kel-F pieces.

Working Electrode Fabrication

A microfluidic detection device should use materials that are cost-effective, easily manipulated, and well characterized. Glassy carbon (GC) was chosen as the electrode fabrication material for several reasons. The relative cost of GC compared to precious metals is extremely low. Additionally, it can be easily milled into a variety of shapes, including the incorporation of cylindrical interior surfaces, something that is not easily accomplished with other carbon materials such as HOPG and carbon paste. Yet, GC remains a robust material with a multitude of surface modification methods available.[31-37]

The GC working electrodes were individually designed to maximize the surface area-to-volume ratio, while maintaining fluidics dimensions compatible with commercially available supplies, including drill bits and tubing. With that in mind, the electrodes were optimized to a small square of dimensions 6 mm x 6 mm x 1.5 mm, with a 1 mm diameter hole through the 6 mm x 6 mm face. This yields an electrode with a surface area of 4.7 mm^2, but a volume of only 1.2 μL. Additionally, this 1 mm diameter hole is on the order of the smallest commercially available diamond drill bits.

Silver was chosen to act as a pseudoreference for similar reasons; AgCl/Ag references are an industry standard reference electrode, and the cost of silver foil is relatively low compared to other precious metals. The silver foil was milled to the same dimensions as the GC electrodes, with one edge slightly longer. This extra length creates an overhang of the silver foil upon assembly of the flow-through cell, which helps to prevent the working electrodes from making electrical contact with the silver foil upon attachment of silver wires with silver epoxy. The silver wires connected to the individual electrodes in the flow-through cell enable their connection to a potentiostat. Kel-F was

chosen to seal the ends of the flow-through cell because of its durability and resistance to a variety of solvents.

Flow-through Cell Assembly

When constructing electrodes, it is vital to prevent electrical contact between the electrode surfaces. Both GC electrodes and the silver electrode were coated in Super Corona Dope, an electrical insulator used to both insulate and protect electronic components on circuitry boards. The device is fabricated from one Kel-F piece attached to one GC electrode, followed by the silver electrode, a second GC electrode, and finally, the second Kel-F cap. These pieces were adhered to one another using 5-minute epoxy and allowed to dry in a vice to prevent leaking and to ensure that the hole in each material remained properly aligned.

After drying, silver wires were connected to each of the electrodes in the flow through cell using silver epoxy to enable connection with the potentiostat. It is important to note that the silver epoxy must be isolated to a particular electrode. If the epoxy makes electrical contact with multiple electrodes, the cell will short circuit. Finally, to complete the device, polymer tubing with a 1 mm outer diameter and a 0.58 mm inner diameter was inserted into the Kel-F piece until it reached the GC electrode. A sharp was inserted into the distal end of the tubing to enable facile connection of a syringe. Photographs of the setup are shown in Figure 7.2.

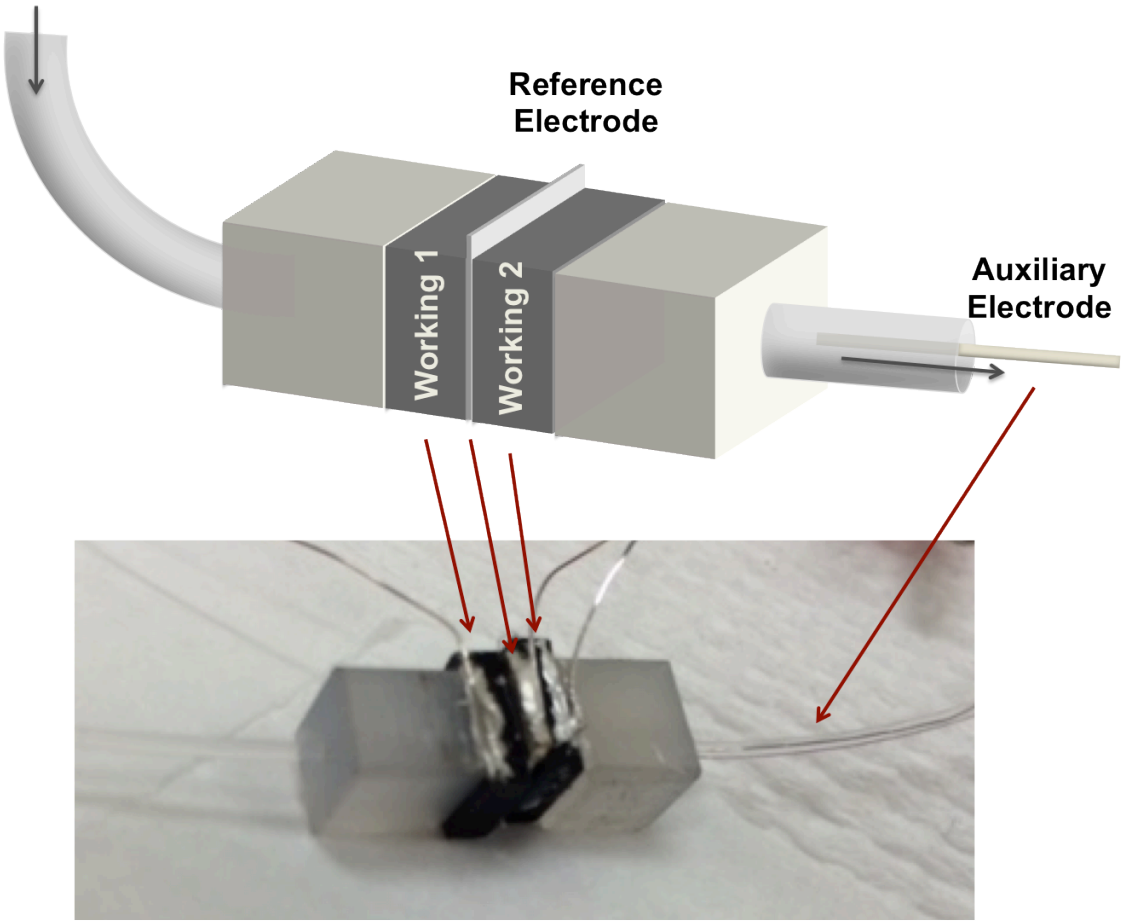

Figure 7.2 Assembled glassy carbon flow-through cell. A cartoon of all components of the cell are shown *(top)*, with the two glassy carbon working electrodes and an integrated silver pseudoreference electrode between them. A platinum auxiliary electrode is inserted into the terminal polymer tubing. A photograph of the assembled cell (*bottom*) shows all of the flow-through cell components with silver lead wires attached to each electrode *via* conductive silver epoxy.

To mimic previously designed platforms that incorporate a secondary working electrode for detection, two working electrodes were incorporated into the flow-through cell.[5, 7] This platform design enables DNA-mediated electrocatalysis between methylene blue and ferricyanide at the first working electrode. Methylene blue is reduced to leucomethylene blue, and can, in turn reduce ferricyanide in solution to ferrocyanide, regenerating the oxidized methylene blue in the process. A second working electrode incorporated into a fluidics device enables the ferrocyanide to be transported to the secondary working electrode, where it can be reoxidized to ferricyanide. The reoxidation of ferrocyanide at the secondary electrode will generate a current proportional to the amount of ferrocyanide in solution, which is a direct measure of the amount of DNA CT occurring at the primary electrode. Before DNA CT-based detection is attempted with this platform, however, the fundamentals of glassy carbon surface modification must be established.

Surface Modification of Glassy Carbon Rod Electrodes

Aniline-based modifications are the most prevalent and well established glassy carbon modification methods.[30-32, 34, 36, 37] The general procedure for covalent aniline attachment to carbon surfaces is to convert the aniline to a diazonium, followed by electrochemical generation of a radical through the loss of N_2 (Figure 7.3).

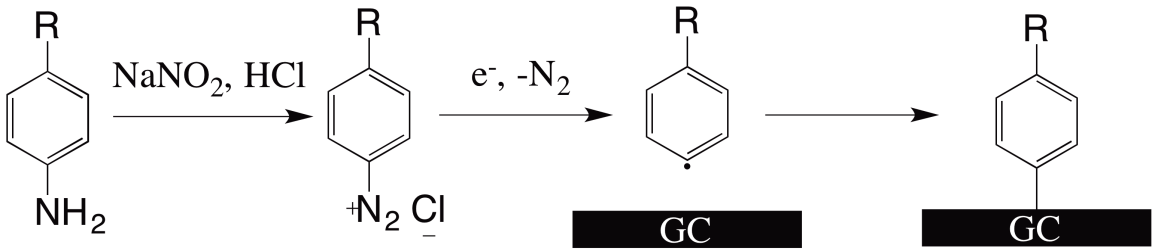

Figure 7.3 Diazonium formation and glassy carbon surface modification. An aniline derivative is first converted to a diazonium salt upon treatment with sodium nitrite under acidic conditions. Following diazonium formation, an aryl radical is formed electrochemically, which is capable of covalent attachment to carbon surfaces.

This aryl radical then reacts to form a carbon-carbon bond with the surface. Two main methods exist to generate the diazonium: it is either formed and isolated[30] before electrodeposition or it is generated *in situ*.[31] Both techniques involve the formation of the diazonium with sodium nitrite at 4° C, followed by conversion of the diazonium to an aryl radical electrochemically.

Many conditions are reported for the electrochemical deposition of anilines onto carbon electrode surfaces,[31-33, 35-37] but the most common techniques are cyclic voltammetry (CV) and constant potential amperometry (CPE).[30] Using CV, aniline derivatives were activated under various conditions to verify the activation in the flow-through device (Figure 7.4). By CV, multiple irreversible reductive peaks are observable. On GC rod electrodes (Figure 7.4, top left) with *in situ* diazonium formation, these peaks occur at -0.4 and -0.8 V (vs AgCl/Ag), and with pre-formed diazonium salts (Figure 7.4, bottom left), the peaks occur at -0.2 V and -0.5 V (vs AgCl/Ag). While these peaks occur at slightly different potentials, this is likely due to differences between the solvents and the other components present in solution for activation. On the flow-through electrodes (Figure 7.4, top right), peaks are observed at -0.1 V and -0.5 V (vs AgCl/Ag). Importantly, after the first scan, the intensities of these peaks decrease significantly, and the potential of remaining peaks shifts slightly negative. This phenomenon is consistent with the formation of a grafted azidobenzene layer on the carbon electrode,[30] indicating that our device is capable of electrochemical aniline deposition.

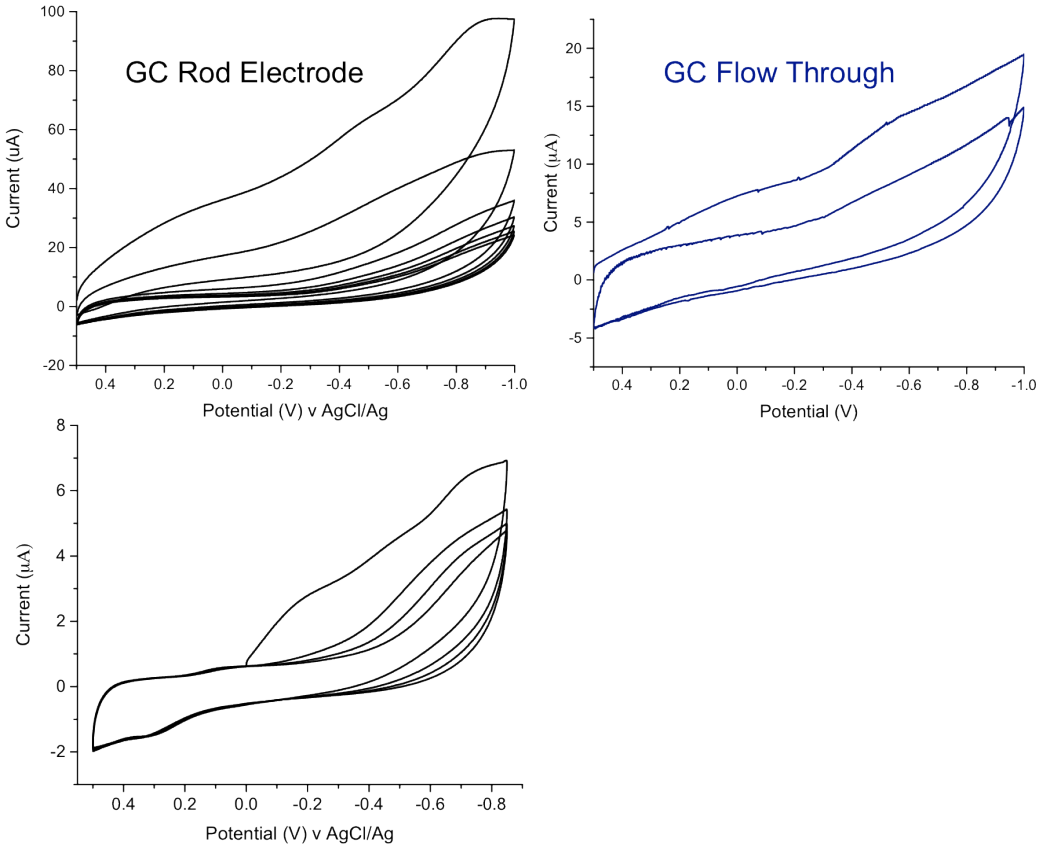

Figure 7.4 Electrochemical modification of glassy carbon surfaces with 4-azidobenzene diazonium salt. Shown are activations on rod electrodes (*left*) and on flow-through cells (*right*). *In situ* diazonium formation (*top*) yields two very similar sets of cyclic voltammograms for the rod electrodes and the flow-through cells. The pre-formed diazonium salt used to modify a glassy carbon electrode (*bottom*) has slightly different peak characteristics as compared to the *in situ* formation. However, in all cases, after the preliminary scan, a significant decrease in current is observed, indicating coupling to the carbon surface. For *in situ* diazonium formation, the electrochemistry was obtained with degassed 1 mM aniline in 0.1 M tetrabutylammonium tetrafluoroborate containing 4% 1M HCl at 4° C, with 1 mM sodium nitrite added. In the case of the pre-formed diazonium salt, surface modification is performed with 1.3 mM aniline in degassed 0.1 M HCl at 4° C. Scan rate: 0.1 V/s.

Modified Glassy Carbon Rod Electrode Passivation against Ferricyanide and Methylene Blue

With the eventual goal of performing DNA CT-based detection on these flow-through cells, it is vital that monolayers formed be passivated against electrocatalytic components. A family of aniline derivatives (4-azidoaniline, aniline, 4-aminobenzoic acid, p-phenylenediamine, and 4-nitroaniline) (Figure 7.6) was tested for passivation against the redox-active species commonly used for DNA CT electrocatalysis, methylene blue and ferricyanide.[38] All of the aniline derivatives yielded modified surfaces that were passivated against ferricyanide in excess of 500 μM (data not shown), a significantly higher concentration than is necessary for DNA-mediated electrocatalysis. However, the same was not true for passivation against methylene blue.

Methylene blue titrations against the same family of aniline derivatives were performed (Figure 7.5). On electrodes modified with 4-azidoaniline and aniline, methylene blue had a higher binding affinity than on bare GC electrodes, with electrochemical signals apparent at a concentration of 500 nM (methylene blue signals are detectable on bare GC at 1.5 μM). 4-aminobenzoic acid modified electrodes were well passivated against methylene blue until 2 μM DNA, which is below the necessary concentration for DNA binding. Finally, surfaces modified with either 4-nitroaniline or p-phenylenediamine were extremely well passivated against methylene blue with no detectible binding below 10 uM (Figure 7.5).

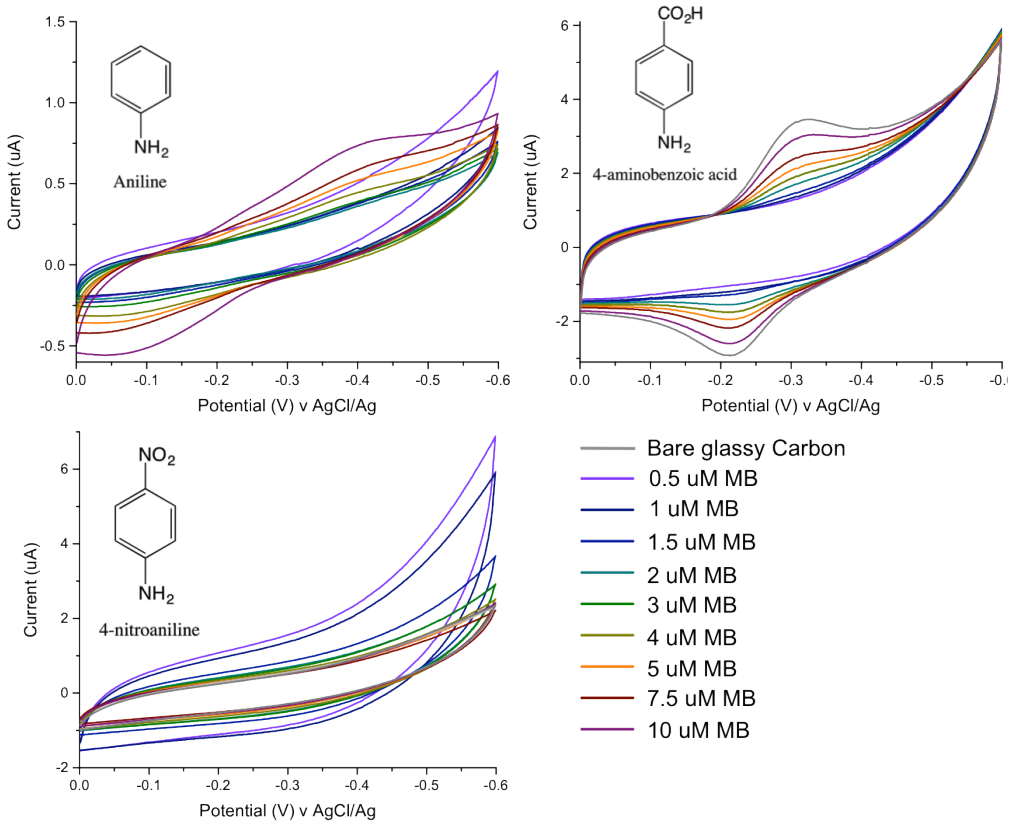

Figure 7.5 Methylene blue titration with modified glassy carbon electrodes. Shown is an electrode modified with aniline alone (*top left*), on which methylene blue signals are observed at 500 nM concentrations. 4-aminobenzoic acid is slightly better passivated, with methylene blue signals apparent at 2 µM (*top right*). Finally, the best passivation is observed with 4-nitroaniline (*bottom left*), which is passivated against methylene blue at concentrations in excess of 10 µM. All titrations were performed in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6). Scan rate: 0.1 V/s.

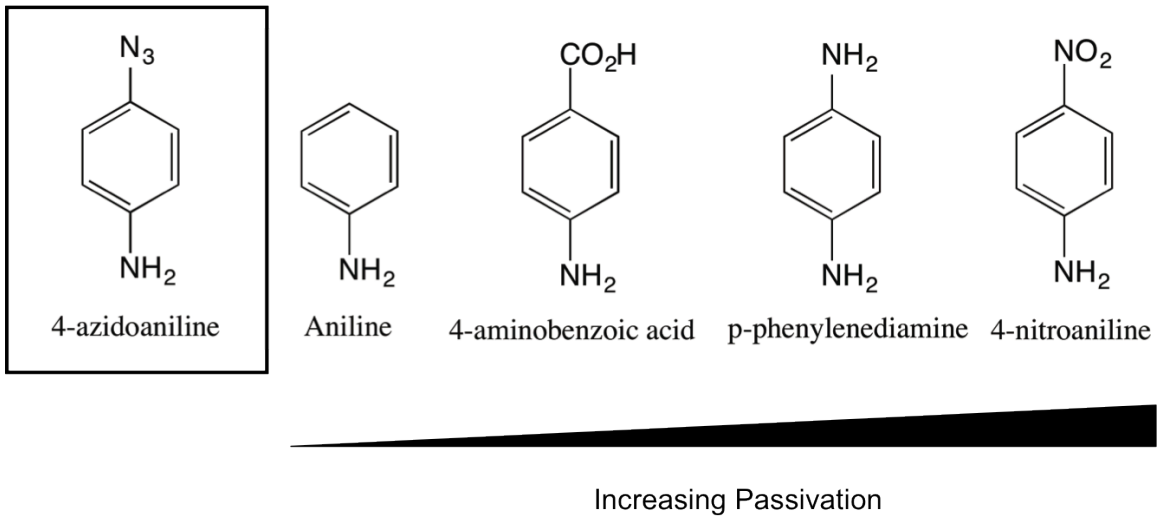

Figure 7.6 Aniline derivatives evaluated for methylene blue passivation ability. Each compound is used to functionalize a glassy carbon electrode. Methylene blue is titrated onto the monolayers to determine the maximum concentration at which the modified electrodes are passivated. The order of passivation, from least passivated to most passivated, is shown from left to right.

It is important to note, however, that we were unable to click ethynyl ferrocene onto the GC electrodes modified with a mixture of azidoaniline and either *p*-phenylenediamine or 4-nitroaniline, the two compounds that yielded passivation against methylene blue. This result will be discussed further in the following section.

Functionalization of Modified Glassy Carbon Rod Electrodes

To form DNA-modified glassy carbon electrodes, DNA must be covalently tethered to azidoaniline-modified GC electrodes. To evaluate the ease of performing the click reaction on such monolayers, ethynyl ferrocene was used as a model alkyne complex, as it is inherently electrochemically active, and provides a large electrochemical signal upon successful coupling. The general method of ethynyl ferrocene covalent attachment to modified GC electrodes is shown in Figure 7.7.

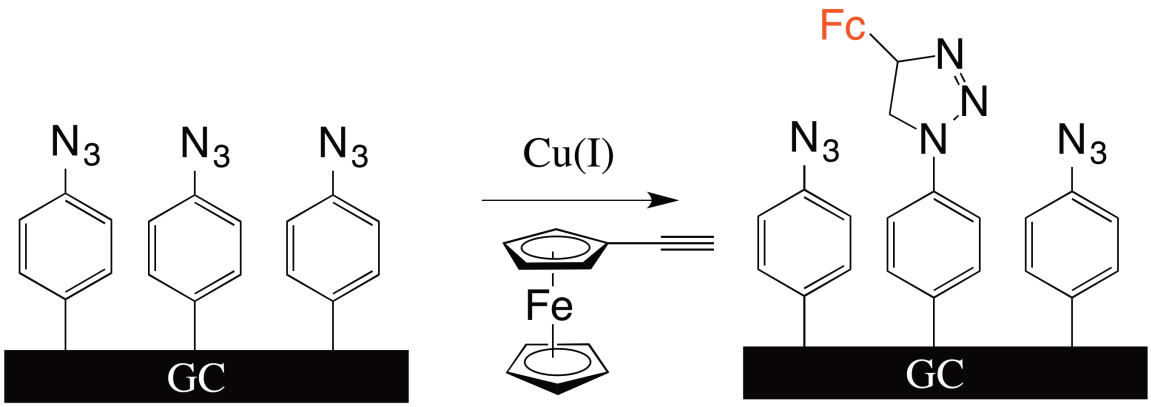

Figure 7.7 Functionalization of modified glassy carbon electrodes with ethynyl ferrocene (Fc). In the presence of a copper (I) source, ethynyl ferrocene is covalently tethered to glassy carbon electrodes modified with azidoaniline through click chemistry.

Preliminary attempts to tether ethynyl ferrocene to modified GC utilized mixed aniline modified electrodes. 50% of the aniline in solution for attachment was 4-azidoaniline, and 50% was an alternative aniline derivative shown in Figure 7.6. The ability to tether ethynyl ferrocene was found to be inversely proportional to the ability of the aniline derivative to block methylene blue. This result suggests that free methylene blue cannot be used for electrocatalysis with this flow-through device because glassy carbon modifications are insufficiently passivated against the redox-active molecule, resulting in direct surface reduction of methylene blue instead of DNA-mediated reduction. Therefore, a covalent redox probe may be necessary for electrocatalysis with this platform; many are compatible with both DNA CT and electrocatalysis.[4, 39]

Ethynyl ferrocene was successfully tethered to a mixed aniline and 4-azidoaniline surface using click chemistry. To directly compare the efficacy of copper(I) sources for this click reaction, GC electrodes were either modified with 50% aniline and 50% 4-azidoaniline or 100% 4-azidoaniline. Four copper sources were tested for their ability to catalyze the click reaction between the electrode surface and ethynyl ferrocene: $[Cu(phendione)_2]^{2+}$, $[Cu(bathophen)_2]^{2-}$, $CuSO_4$, and $[Cu(TBTA)]^{2+}$. The copper compounds were reduced with ascorbic acid, and ethynyl ferrocene coupling to surfaces was allowed to proceed for 90 minutes. The results of these coupling attempts are shown in Figure 7.8. In all cases, ethynyl ferrocene coupling is observed. The lighter color for all copper compounds is coupling to the 50% aniline/50% 4-azidoaniline modified surface, while the darker is coupling to the 100% 4-azidoaniline modified surface.

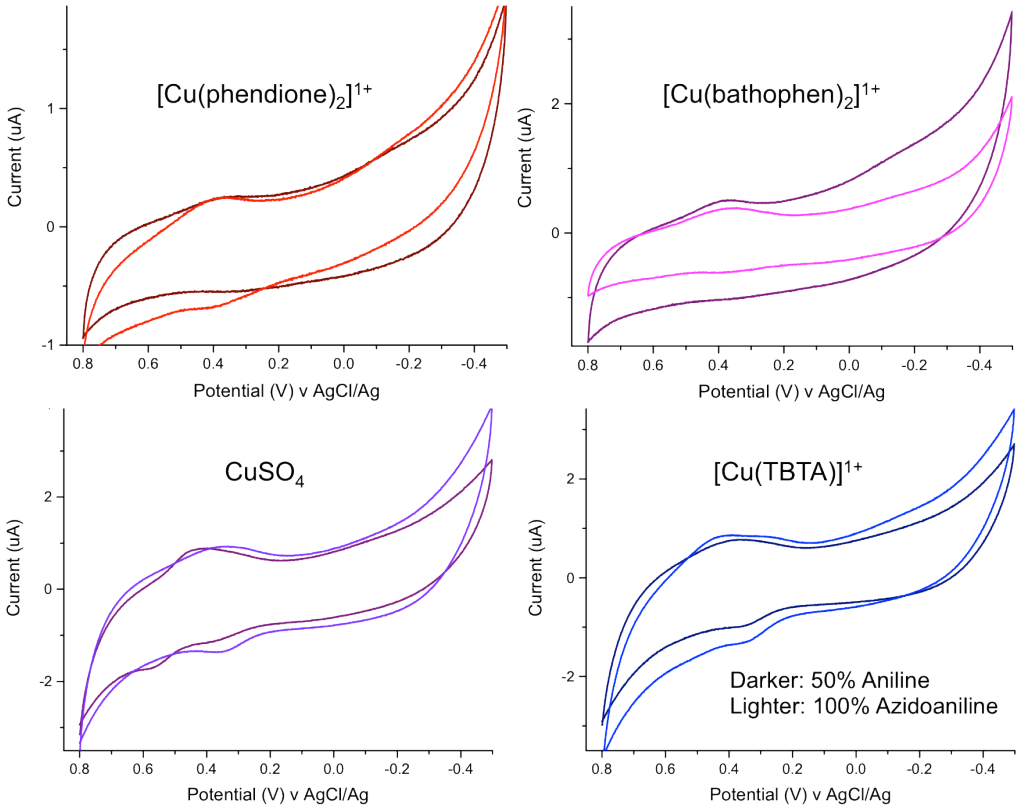

Figure 7.8 Ethynyl ferrocene coupling to modified glassy carbon electrodes. In all cases, the light color is the result of coupling to an electrode modified with 50% aniline/50% azidoaniline, while the darker is the result of coupling to a 100% azidoaniline-modified electrode. As can be seen, more efficient coupling is observed on the 50% aniline-modified electrodes. Scans were obtained in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6). Scan rate: 0.1 V/s.

Two issues arose with attempts to couple ethynyl ferrocene to modified GC surfaces using ascorbic acid-reduced copper compounds: first was optimization of the total amount of azidoaniline coupled to the modified GC surface, and second was optimization of the copper compound used for ethynyl ferrocene attachment. Based on the amount of ethynyl ferrocene coupled, the 50% aniline/50% azidoaniline-modified electrodes enable more efficient surface coupling with all of the copper sources. This result is consistent with what we have previously observed: more access to surface modifications through better spacing enables more efficient interaction between the surface modifications and the molecules in solution.[5, 7, 40]

Additionally, though, issues arose with the removal of copper complexes from the GC surface following activation. Some copper compounds are not as easily removed from electrode surfaces following coupling, and can result in electroactive species that remain on the electrode surfaces. As can be seen in Figure 7.9, copper signals are apparent with almost all compounds, except $[Cu(TBTA)]^{2+}$. This result is problematic for both eventual DNA attachment and electrochemical detection with monolayers formed using this technique. However, upon treatment of electrodes with EDTA (Ethylenediaminetetraacetic acid), a strong chelator of copper, the electrochemical copper signals were no longer observable. Results from a 20 minute EDTA treatment are shown in Figure 7.9.

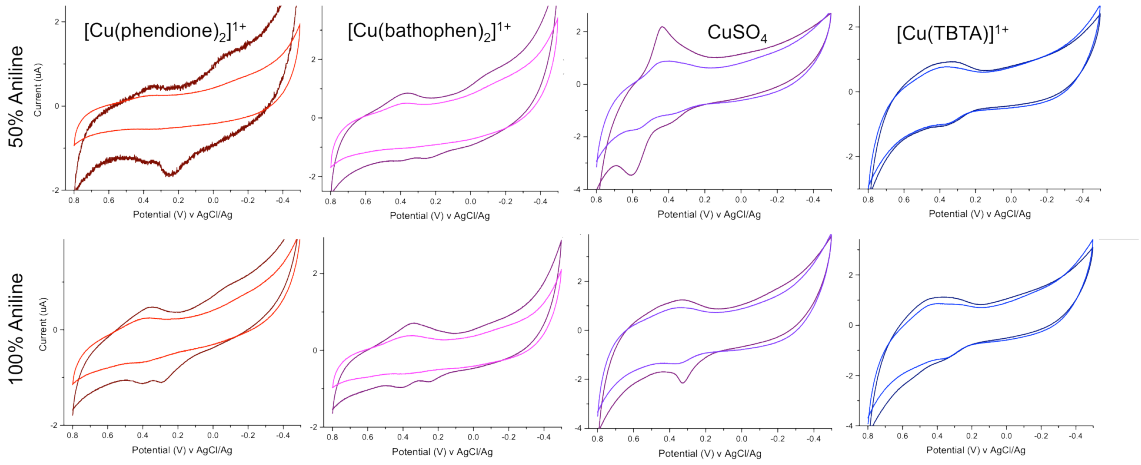

Figure 7.9 Ethynyl ferrocene-modified glassy carbon electrodes upon treatment with EDTA. Four sources of copper are evaluated for the ability to tether ethynyl ferrocene to an electrode modified with 50% aniline/50% azidoaniline (*top*) and coupling to a 100% azidoaniline-modified electrode (*bottom*). Before EDTA treatment (*darker colors*), copper contamination is apparent on the majority of electrodes. Following EDTA treatment (*lighter colors*), the copper signals are no longer present, indicating chelation and removal by EDTA. Scans were obtained in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6). Scan rate: 0.1 V/s.

Further study will optimize the glassy carbon modification procedure for applications on the flow-through cell. Similarly, conditions for copper-catalyzed click will be evaluated to determine the optimal method for DNA coupling.

Conclusions

For clinically relevant biomolecule detection, not only should a sample be easy to prepare and analyze, but small volumes are key to minimize invasiveness. We have previously established platforms for the detection of methyltransferases from crude tissue lysate. However, to apply this assay clinically, both the ease of sample preparation and speed of detection must be increased. The most efficient technique to meet both of these requirements is microfluidics. We have developed a device that uses inexpensive commercially available materials. This device enables flow through within the electrodes, minimizing the sample volumes required, while also maximizing the surface area-to-volume ratio of the electrodes, which are made of glassy carbon. This flow-through cell differs from traditional nucleic acid electrodes because it is cylindrical, as opposed to common planar electrodes.

To verify that the structure of these cylindrical electrodes does not affect performance, aniline was electrochemically deposited on both glassy carbon rod electrodes and the flow-through device, and the two different electrode shapes were found to behave similarly. The flow-through cell additionally incorporates the ability to control the density of surface functionalization through mixed aniline surface modification. A second working electrode is incorporated into the flow-through cell, facilitating detection in the same manner as the two working electrode detection platform previously shown to enable especially sensitive detection. A family of aniline derivatives was used to test passivation against common electrocatalytic agents. Surfaces are fully passivated against 500 μM ferricyanide; however, they are not passivated against even

very low concentrations (500 nM in some cases) of free methylene blue, indicating the need for a covalent electrochemical probe.

Click attachment of ethynyl ferrocene, and subsequently DNA, to these modified surfaces is being optimized on glassy carbon rod electrodes and will be applied to the flow-through device. This flow-through device behaves similarly to rod electrodes while significantly reducing the sample volume required for the electrode areas. This device is easily manipulated to enable multiplexing, and the cylindrical GC electrode surface is easily modified for rapid attachment of aniline derivatives. The ease of modification of this device will enable a multitude of new detection platforms with covalently-tethered duplex DNA attached to the surface to facilitate DNA-mediated detection of particular analytes from sub-μL sample volumes.

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