Chapter 9

Summary and Perspective

Detection of biologically relevant targets, including small molecules, proteins, and nucleic acids, is vital for both fundamental research as well as clinical and field diagnostics. Sensing strategies that feature biological substrates for analyte capture provide a natural foundation for bioassays, owing to the inherent molecular-recognition nature of substrate-ligand binding. Nucleic acid-based platforms in particular comprise an especially robust and flexible class of sensors, capable of detecting a variety of small-molecule, protein, and DNA/RNA targets.[1-5]

Protein targets, in particular, have garnered significant interest because of their roles in the initiation and development of many diseases, including cancer. While DNA arrays using fluorescence-based technology have changed the way we probe gene expression and the variations therein that lead to disease, they generally cannot directly detect proteins. Additionally, they inherently require expensive optical systems for readout and exhibit significant signal variability.

DNA-based electrochemistry, in contrast, enables fast, facile detection of a variety of biomolecules, with high sensitivity, low cost, and the capacity to incorporate multiplexing.[1-5] All of these features are vital for the clinical applicability of a detection platform. Many DNA-modified electrodes employ the same strategies for detection as fluorescence-based sensors, but with the added sensitivity and lower complexity of electrochemistry (i.e., hybridization with labeled targets,[6-8] structural changes to a DNA hairpin to turn on a signal,[2, 5, 9] and impedance measurements[10, 11]). These similarities in detection strategies lead many of these platforms to have the same issues as the fluorescence-based assays, including non-specific hybridization interactions, yielding false positive signals and difficulty directly detecting protein activity.

If the intrinsic structural and electronic properties of DNA are harnessed, the sensitivity and selectivity of DNA-based electrochemical detection is significantly increased.[12, 13] One especially sensitive method of detection relies on the phenomenon known as DNA-mediated charge transport (DNA CT). DNA CT is based on the ability of electrons to flow through the stacked, aromatic bases that comprise the core of DNA. The structure of the stacked DNA bases is very similar to the structure of the known conductor, graphene; the vertical rise between the aromatic bases, as between the layers of graphene, is 3.4 Å, which is sufficiently small to enable the π orbitals of the DNA bases to overlap, creating a conduit for electron flow. The ability of DNA to conduct charge through its core was initially shown with excited-state solution experiments,[14, 15] and this process was found to be efficient over long distances on fast time scales. Importantly, this process is extremely sensitive to anything that disturbs the π stacking of the DNA bases.[16-20]

For DNA CT to be an effective method of biomolecule detection, the DNA must be anchored to provide a handle for detection. DNA-modified gold electrodes provide a solid support and simple electrochemical readout to facilitate detection by DNA CT. Conventionally, DNA-modified electrodes are formed from the self-assembly of thiolated DNA duplexes on gold electrode surfaces. These monolayers have enabled single-base mismatch and lesion detection, irrespective of sequence context.[17, 21] DNA-mediated electrochemistry also offers a sensitive means to monitor protein activity at low concentrations. Proteins that perturb the base stack or chemically modify DNA are easily detected by DNA CT-based platforms, including methyltransferases, photolyases, and transcription factors. Selectivity is governed by the sequence-specificity of the protein.[22-

[24] Self-assembly of thiolated DNA for DNA film formation has enabled the detection of a variety of biomolecules, but little control is afforded with this method over the spacing between individual DNA helices.[25] Increased access for biological targets to bind, particularly in congested, crude lysate samples, is a critical parameter for sensitive, relevant biomolecule detection. The importance of interhelical spacing was previously demonstrated by the effect of DNA deflection angles on nanostructured microelectrodes. Increasing the deflection angle between helices enabled significantly lower detection limits of oligo targets.[1-3]

In this thesis work, we have improved i) control over the morphology of DNA monolayers and ii) platforms to facilitate sensitive biomolecule detection. We first utilized copper-free click chemistry to better control the density and homogeneity of DNA monolayers. Subsequently, we applied click chemistry-based formation of low-density DNA monolayers to the patterning of DNA arrays. The platform developed for DNA array formation also incorporated a secondary electrode for detection, which, when coupled with electrocatalysis, enabled especially sensitive protein and nucleic acid detection. This platform was subsequently multiplexed for simultaneous detection from multiple biological samples. When a signal-on assay for methyltransferase detection was combined with this electrochemical platform, methyltransferase activity from crude tissue lysates was detected. Differential activity between tumor tissue and healthy adjacent tissue was consistently observed when measured with this platform. We have increased the control over DNA monolayer morphology and combined this control with improved sensitivity to facilitate detection of an important cancer biomarker from clinical samples.

Extensive characterization of conventional thiolated DNA monolayers has confirmed their inhomogeneity. Some control over the total amount of DNA assembled is obtained by varying the ionic strength of the assembly solution. Thiolated DNA monolayers contain some regions of very high DNA density and other regions with little to no DNA, making biomolecule detection difficult, as different regions of the surface will respond differently to the addition of biomolecules.

To improve the morphology of DNA monolayers, we first developed a method of monolayer formation using copper-free click chemistry, which enables control over the amount of DNA assembled. Extremely low-density DNA monolayers, with as little as 5% total surface coverage of DNA, have been formed. These DNA-modified electrodes (DMEs) were fully characterized both visually and electrochemically, and were found to be significantly more homogeneous than traditional thiol-modified DNA monolayers. Furthermore, these monolayers support greater accessibility to individual DNA helices, resulting in more sensitive detection of the transcriptional activator TATA-binding protein (TBP), with detection limits on the order of the dissociation constant of the protein. The improved DNA helix accessibility, and therefore sensitivity, afforded by these DNA monolayers reflects their general utility for electrochemical sensor development.

We further developed click chemistry-based DNA monolayer formation to create patterned DNA arrays, which facilitate direct comparisons between multiple sequences of DNA on a single electrode surface. These arrays are electrochemically formed and addressed with a two-electrode platform. Electrochemical activation of a copper catalyst at a particular location on a bare secondary electrode precisely places multiple sequences

of DNA onto a single, substrate electrode surface. The incorporation of a secondary microelectrode with electrocatalytic signal amplification for electrochemical readout provides improved sensitivity with spatial resolution of the DNA array on the primary electrode surface. We have applied this two-electrode platform to form DNA arrays that enable differentiation between well-matched and mismatched sequences, detection of TATA-binding protein, and sequence-selective DNA hybridization, all with greater control than was previously available.

Multiplexing of this two working electrode platform has enabled simultaneous and specific detection of multiple proteins. In this platform, which contains two complementary electrode arrays, DNA duplexes are patterned onto the primary electrode array, while the secondary array is used for DNA patterning and biomolecule detection. Particular sequences of DNA are specifically placed on the primary electrode array using electrochemically-activated click chemistry. We have found that catalyst activation at the bare secondary electrode array is essential to maintain the integrity of the DNA monolayer. The deleterious effects of catalyst activation at the DNA-modified electrode have been confirmed by impedance spectroscopy, cyclic voltammetry, and constant potential amperometry. Electrochemical readout at the secondary electrode has further eliminated the need for large background corrections, as current is only generated at the secondary electrode when DNA CT occurs at the primary electrode. We expanded our protein detection capabilities with this platform through the sensitive detection of the transcription factors TATA-binding protein and CopG on the same electrode array with sequence specificity.

Clinically relevant detection with this two working electrode platform was established by incorporating an electrochemical signal-on methyltransferase activity assay. Epigenetic modifications, namely DNA methylation, have been found to necessitate tight regulation in the cell to prevent the development of cancer.[26-28] As such, abnormal DNA methyltransferase activity has been connected to the development and progression of cancer. However, detection methods to assess methyltransferase activity from crude tumor samples are limited because they rely on radioactivity or fluorescence and require bulky instrumentation.[29, 30] We developed an electrochemical platform for the label-free detection of the activity of the most abundant human methyltransferase, DNMT1, that enables measurements from crude cultured colorectal cancer cell lysates (HCT116) and biopsied tumor tissues. We specifically and selectively measured DNMT1 activity within these congested cellular samples using this platform and, based on differences in DNMT1 activity, we can distinguish colorectal tumor tissue from healthy adjacent tissue.

As differences in DNMT1 activity between tumor tissue and healthy adjacent tissue were measureable with our platform, we evaluated ten sets of tumors to determine the clinical relevance of this technology. With our multiplexed, two working electrode platform, we have found a direct correlation between higher DNMT1 activity and tumorous tissue. In the majority of samples analyzed, the tumorous tissue had significantly higher DNMT1 activity than the healthy adjacent tissue. No such correlation was observed in measurements of *DNMT1* expression by quantitative PCR, DNMT1 protein abundance by Western blotting, or DNMT1 activity using a radiometric

DNA labeling assay. Our electrochemical platform provides a direct measure of DNMT1 activity in crude, clinically relevant samples.

DNA CT-based electrochemical sensors offer many advantages over other nucleic acid sensors. The inherent sensitivity of CT to small perturbations in the base stack of DNA has enabled the detection of proteins, nucleic acids, and even epigenetic DNA modifications. With conventional DNA-modified electrodes, however, we have been limited to the detection of purified biomarkers. It has been especially rewarding to have developed platforms that enable detection from clinically relevant crude samples, a great step toward real-world applications of these DNA sensors. The platforms described here offer a basis for further development, with the goal of methyltransferase analysis on a single-cell level, detection of the epigenetic modification hydroxymethylation, and the further application of this technology to the development of assays that could provide better diagnostics for disease.

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