Conjugation of Unmodified Nucleic Acid Probes to Electrode Surfaces for Electrochemical Biosensors

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Abstract

Electrochemical deoxyribonucleic acid (DNA) sensors have emerged as a promising clinical diagnostic technology for infectious disease and cancer. DNA biosensors can exploit DNA recognition events and convert them into an electrochemical signal. Rapid response, selectivity, good sensitivity, and ease of miniaturization are a few advantages of electrochemical biosensors. Chapter 1 describes the various classifications of electrochemical biosensor techniques (e.g., impedimetric, amperometric, and others). We also discuss recent strategies for DNA immobilization on the surface of electrodes including covalent and non-covalent bonding. Sensors are described for DNA, RNA, protein and small molecule targets. Chapter 2 discusses DNA immobilization by DVS conjugation for the electrochemical detection of complementary DNA. Conjugation of DNA on the surface of the electrode through DVS is a new method for detection of complementary DNA. Graphene oxide was used is an immobilization platform to improve the charge transfer. This DNA biosensor avoids the need for modified oligonucleotides with synthetic attachment chemistry. We also analyzed the mechanism of DNA conjugation through mass spectrometry. In chapter 3, we describe a low-cost method for detecting nucleic acids with more conventional attachment chemistry. Carbodiimide chemistry was used for conjugation of DNA on the surface of electrode. Cyclic voltammetry and electrochemical impedance were used to detect DNA.

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1.1. Introduction

Nucleic acids must be detected to diagnose genetic and infectious diseases. The nucleic acid is a key type of macromolecule found in all living organisms. Nucleic acids are polymers with a sugar-phosphate backbone. Specific interactions between nitrogenous bases allow nucleic acids to express and transmit genetic information[1]. Conventional methods for detection of a nucleic acid include polymerase chain reaction (PCR)-based fluorescence assays, quantitative PCR (qPCR), and northern blotting [2, 3]. These methods each have limitations such as the limited sensitivity of northern blotting. New methods like RNAseq produce huge datasets but require very expensive, specialized sequencing equipment. Polymerase chain reaction (PCR) needs precise temperature control, and good primer design[4]. While qPCR and reverse transcriptase qPCR (RT-qPCR) remain the gold standard for laboratory-based DNA diagnostics, they have not yet met the requirements of POC (point of care) clinical diagnostics. The complexity of qPCR has mostly restricted its use to centralized testing laboratories[5].

Electrochemical sensors have proven useful for point-of-care and consumer use (e.g., blood glucometers)[6]. Electrochemical biosensors are constructed from three units as shown in Figure 1, a bio-receptor (also called the recognition element: an enzyme, antigen, antibody, nucleic acid, etc.), a transducer element (magnetic, electrochemical, optical, colorimetric, etc.), and a signal processor. DNA biosensors (sometimes called geno-sensors) are devices

that convert a biological recognition event (bio-receptor binding to target) into a measurable signal[7]. DNA hybridization biosensors usually rely on the immobilization of a single-stranded (ss) oligonucleotide probe onto a transducer element to recognize a target by hybridization. The electrochemical biosensing field holds promise for cheaper diagnostics, and faster response time in patient care. The common advantages of DNA biosensor-based electrochemical methods are easy miniaturization, ease of use, fast response, high sensitivity, high portability, and compatibility with scalable microfabrication methods[8]. Because of all these merits, electrochemical biosensing for nucleic acid diagnostics is a rapidly expanding field with strong near-term applications.

Electrodes mediate charge transfer and support immobilized DNA. Electrodes convert a chemical signal (interactions with the analyte) to an electrical signal. Different materials (e.g., polymers, carbon nanomaterials, semiconductors, and gold nanoparticles, etc.) enhance the immobilization of DNA on the electrode. Although carbon structures vary widely, many carbon materials enhance performance and reduce cost in electrochemical biosensors[9]. Carbon electrode materials are conductive, and some (especially carbon nanoparticles) transfer electrons efficiently. Many carbon materials have a large specific surface area and can be functionalized easily (i.e., modified to improve and enrich their functions in electrochemical biosensors)[10].

Gold electrodes are a common alternative to carbon[11]. This review will discuss and compare the limitations and strengths of gold and carbon structures as immobilization platforms for electrochemical biosensors. We will compare different biofunctionalization methods that have been studied and reported including non-covalent physical adsorption

and covalent cross-linking. We hope that this review will provide an overall summary of the current standing of electrochemical nucleic acid biosensors based on different conjugation methods to carbon structures.

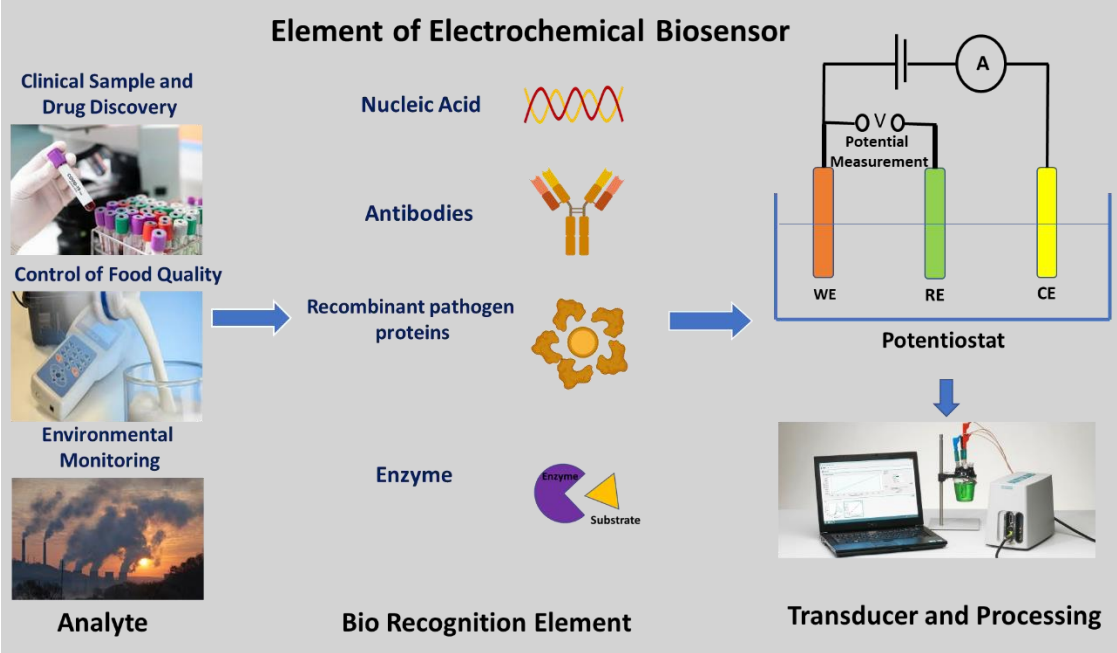

Figure 1: Schematic showing the major components of biosensors to highlight the role of nucleic acids as biorecognition elements.

1.2. Classification of Electrochemical biosensor techniques

Biosensors may be divided according to the mode of signal transduction or the biological specificity-conferring mechanism, or both[7]. Examples of specificity conferring mechanisms include antibody-antigen interactions[12], aptamer-target interactions[13], or base pairing[14]. Once a target has been specifically captured, it changes the electrochemical properties of the electrode. This generates a signal. Electrochemical sensors may monitor the current (amperometric or voltammetric), the open cell potential (potentiometric), the electrochemical impedance (impedimetric), or conductivity (conductometric)[15]. In the

following sections, each of these methods is discussed along with representative examples of each and its respective advantages.

1.3 Impedimetric NA detection

Electrochemical Impedance Spectroscopy (EIS) can detect biomolecules at low concentrations without using covalent labels[16]. EIS measures the AC current as a function of frequency[17]. From this data, the charge transfer resistance (Rct) is calculated. Interactions between the biorecognition element and the analyte change the Rct value. Often, an electroactive species in solution (i.e., not on the biorecognition element or target) is reversibly reduced and oxidized to generate an AC current. Analyte interactions at the modified electrode surface change the interfacial electron transfer resistance (faradaic current) between the electrode and this species[18].

Unlabeled biomolecule analytes such as proteins or DNA can thus be monitored with electrochemical EIS using several redox systems: ascorbic acid, Ferricyanide ($Fe(CN)_6^{3-}$), ruthenium hexamine ($Ru(NH_3)_6$), and dopamine are some examples[19, 20]. Each can have advantages in specific circumstances. Ferricyanide is the most common redox species used for this purpose for several reasons: 1. The reduction potential of ferricyanide is conveniently located at approximately +230 mV vs. Ag/AgCl. This is far away from other reactions in an aqueous solution (e.g., water hydrolysis or dissolved oxygen reduction). 2. Ferricyanide is affordable (i.e., compared to ruthenium hexamine). 3. Ferricyanide is stable and safe under most conditions. 4. Ferricyanide has fast charge transfer which improves its sensitivity[21].

The shape of the EIS spectrum reflects the diffusional characteristics and charge transfer kinetics of the electrochemical system. Researchers analyze this shape with an electrical circuit model. The model is a simulated set of capacitors and resistors combined in series or parallel. The most common model is the Randles-Ershler equivalent circuit. This model is composed of charge-transfer resistance (Rct), electrolyte resistance (Rs), double-layer capacitance (Cdl), and diffusional resistance element (Warburg impedance). The Rs parameter is calculated by the distance between the electrodes and the conductivity of the solution. The double-layer capacitance depends on the electrode area and ion concentration. Rct reflects the charge transfer kinetics[22]. The best fit Randles-Ershler equivalent circuit for the EIS spectrum has specific values for these constants. Figure 2 shows an example of the Randles-Ershler equivalent circuit in DNA hybridization on the surface of graphene-modified with gold nanoparticles. The spectrum changes depending on whether the attached DNA is single- or double-stranded. In particular, the semicircular portion of the Nyquist plot shows an increased radius which corresponds to increased Rct[23].

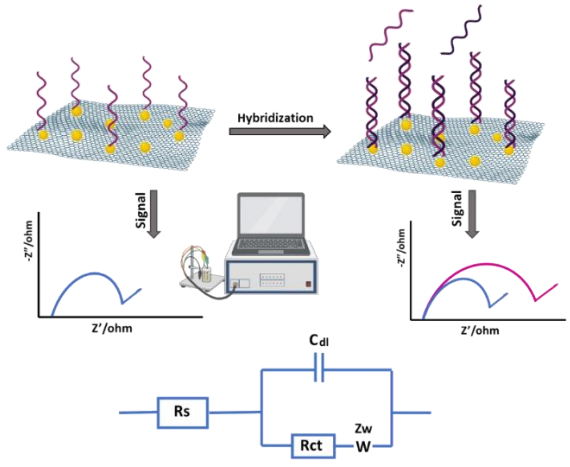

Figure 2. A schematic of a nucleic acid biosensor using EIS to measure hybridization. The spectrum changes (blue to magenta) upon the formation of double-stranded DNA (dsDNA). This can be interpreted with a Randles-Ershler equivalent circuit (circuit diagram) to infer the physiochemical properties of the electrode system.

1.3.1 Hybridization based impedimetric sensors

Hybridization generally refers to combining two complementary ssDNA or RNA molecules to form a double-stranded NA molecule via base pairing. Hybridization-based impedimetric biosensors use the immobilization of an ssDNA onto an electrode. The hybridization event changes the electrode and produces an electrical signal. Figure 2 shows a schematic of hybridization-based EIS detection. This technique has some advantages such as rapid analysis, low cost, and easy miniaturization. By detecting specific NA molecules, the disease can be detected at an early stage. NA biomarkers are a growing part of the field of clinical diagnosis and prognosis[33].

EIS reveals the interface properties of modified surface electrodes. The information extracted from EIS can be used to interpret changes that take place when an analyte binds to a biorecognition element on the surface of an electrode. When NA hybridizes to a surface-bound NA, it changes the surface charge, the steric accessibility, or the proximity of covalently-modified labels. For label-free detection, an electro-active molecule is added to the solution. The charge transfer resistance (Rct) of the electrode is proportionally related to the electrode-DNA hybridized with the target NA. Thus, the quantity of target NA can be measured[34].

Different materials can be used to enhance this method. Quantum dots, gold nanoparticles, carbon nanotubes, carbon-nanotubes/nano zirconium dioxide/chitosan, and graphene oxide/gold nanoplatform can all reduce the initial charge transfer resistance (Rct)[35–37].

The change due to hybridization is therefore larger. This can increase the sensitivity of DNA hybridization detection.

Hybridization-based EIS sensors can detect specific mutations in specific genes. Chen-zhong Li et al. reported an electrochemical biosensor that used a DNA-modified carbon nanotube electrode for monitoring DNA hybridization[38]. They detected DNA with a specific sequence in the breast cancer 1 (BRCA1) gene. Around 1200 different single nucleotide polymorphisms (SNPs) have been found in BRCA1 associated with breast cancers. Detecting a specific mutation can help to accurately predict and diagnose specific forms of cancer.

Kinetic model EIS can also discriminate mutations in DNA targets. Jianyun Liu, et al. studied hybridization kinetics with label-free DNA target oligonucleotides on a mixed monolayer of peptide nucleic acid and 6-mercapto-1-hexanol. EIS revealed the change of Rct with time. The presence of mutations changes the kinetics, and so were measurable by EIS[39].

1.4 Amperometric NA detection

Amperometry is the measurement of electric current between electrodes. A difference potential is applied between the working and counter electrode. This potential is controlled relative to a reference electrode. The current, which is produced at the surface of the working electrode, is measured when a specific potential is applied[15]. In the amperometric NA sensor, electroactive species are reduced or oxidized to generate a current signal. Electroactive species can be generated by an enzyme-labeled NA or a DNAzyme to facilitate the detection of NA analytes.

1.4.1 Amperometric Enzyme-based biosensor

Enzyme-based biosensors contain an enzyme (as a biorecognition element or label), a transducer, and a signal processor. Biosensors based on amperometry and enzyme labels are sensitive, selective, fast, simple, reproducible, and manufacturable[40]. Because of these advantages, there are many applications of amperometric enzyme-based biosensors for biochemical analysis (for a review, see reference [42]).

The Clark oxygen electrode is a historically important amperometric sensor that was used to make the first enzymatic biosensor. The Clark electrode measures oxygen, O_2 by measuring the current resulting from the reduction of O_2 at a controlled potential. The Clark electrode can sense glucose when combined with an enzyme, glucose oxidase (GOx)[41]. GOx is a catalyst for the oxidation of glucose to gluconolactone (shown in the reaction below). This reaction also consumes dissolved oxygen, O_2. When O_2 is consumed by glucose and GOx, the current generated at the Clark electrode changes and so allows glucose to be measured amperometrically[42].

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOx}} \text{Gluconolactone} + H_2O_2$$

The Clark electrode system gave way to modern glucometers which are still based on GOx activity. Detecting glucose is very important because it is used as a biomarker for diabetes and to regulate the dose of insulin[43]. Biofuel cells can also be monitored with a glucose biosensor. Biofuel cells generate electrical energy through microbial digestion. It is hoped that they might power cardiac pacemakers [44].

Some amperometric biosensors use GOx as a label. Jambrec et al. detected DNA with GOx covalently modified with acridine orange. The modified GOx bound to hybridized DNA and produced an amperometric signal. Gang Liu et al. developed a sensor with horseradish peroxidase (HRP) as a label. This DNA sensor uses a DNA probe immobilized at an electrode surface via a biotin−avidin bridge. In the presence of a NA analyte, HRP can bind the probe; HRP activity is detected amperometrically. The E-DNA sensor has a femtomolar limit of detection for DNA targets.

To make an enzyme-based biosensor, an enzyme must be immobilized (i.e., on an electrode surface or as a label on a biorecognition element). There are several ways to immobilize enzymes such as adsorption [45], entrapment [46], cross-linking/covalent bonding [47] [48]. In some cases, researchers added a mediator in the sample or on the surface of the electrode. Mediators are small electroactive species responsible for moving electrons between the electrode and enzyme active sites. Mediators can be covalently immobilized. For example, mediators were covalently linked to a carbon nanotube electrode[49]. The mediator can increase the charge transfer from enzymes to the circuit[42].

1.4.2 Amperometric DNAZyme-based biosensor

Deoxyribozymes or DNAzymes are DNA oligonucleotides with specific sequences that display catalytic activity. A DNAZyme can perform a specific chemical reaction. DNAzymes are often discovered within vitro selection technology[50]. DNAzymes have some advantages versus ribozymes (RNAzymes) and protein enzymes. DNAzymes have high chemical and thermal stability (compared to RNAzymes) and are easy to synthesize and modify (compared to

protein enzymes). DNAzymes can be readily conjugated to a solid surface via any of several synthetic functional groups (e.g., amine, sulfhydryl). The synthesis of DNAzymes is inexpensive compared to recombinant protein production[51].

DNAzymes can be used as catalytic labels to increase sensitivity in biosensors. One of the most investigated DNAzymes is the hemin/G-quadruplex (hGQ) peroxidase[52]. The hGQ DNAzyme can act as a catalyst for the oxidation of substrates by hydrogen peroxide (i.e., the same reaction as horseradish peroxidase). The hGQ DNAzyme is stable against heat treatment and hydrolysis. The hGQ DNAzyme has been used for electrochemical, chemiluminescence, and colorimetric sensors and for the detection of diverse targets[53–55].

Negar Alizadeh et al. designed an electrochemical immunosensor to detect hepatitis B virus. They used nanoparticles to label the target. Antibody-conjugated magnetic nanoparticles (MNPs) captured an analyte (hepatitis B virus surface antigen) on an electrode. A gold nanoparticle (GNP) that supported hGQ peroxidase and antibodies acted as a label in a sandwich assay. The GNP complex is attached to the antigen on the electrode surface. Figure 3. demonstrated the outline of the different steps for fabrication of the immunosensor. Once the GNP complex was attached to the electrode, hGQ allowed them to detect the analyte via amperometry. The hGQ DNAzyme generated a signal by the catalytic reduction of H_2O_2 through oxidation of methylene blue (MB) as a mediator. This was very sensitive as each molecule of analyte on the surface captured many molecules of hGQ DNAzyme via the GNP complex. Moreover, each hGQ DNAzyme could catalyze the production of many molecules of MB. As a result, the signal was very strong and detection limits were f 0.19 $pgmL^{-1}$ [56].

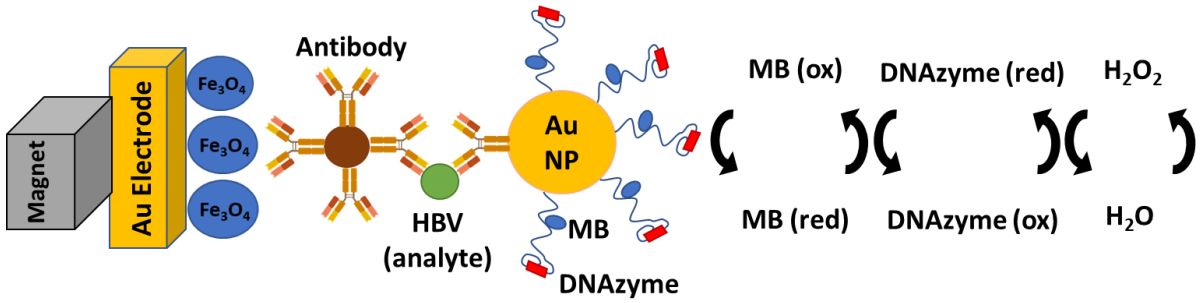

Figure 3: An outline of the MNP/GNP sandwich immunoassay

1.5 Voltametric NA detection

Voltammetry is a common and simple method for electrochemical detection in nucleic acid biosensors. Voltammetry is based on measuring a current during controlled variations of the potential. Voltammetry can be investigated in several methods, cyclic voltammetry (CV), stripping voltammetry, differential pulse voltammetry (DPV), polarography, AC voltammetry, linear sweep voltammetry (LSV), and many more. CV, DPV, and Chronocoulometry methods are most generally used in nucleic acid biosensors[57]. We introduce and give examples of these methods in the following paragraphs.

In CV, a potentiostat records electric current while changing the electrochemical potential (i.e., relative to a reference electrode). The instrument ramps the potential up and down cyclically. CV is a common method because it is low-cost, portable, and relatively simple. Variations of CV use more complicated patterns of potential change (e.g., differential pulse voltammetry, DPV) to increase sensitivity[58]. Using labels like methylene blue and catalytic labels like hGQ, CV can detect diverse analytes with low limits of detection.

Chen Li et al. developed a sensitive, label-free biosensor for deoxyribonuclease I (DNase I) activity. They used technique related to CV, square wave voltammetry (SWV). They used a gold electrode coated with thiol-conjugated DNA plus methylene blue (MB) as an indicator. DNA on the electrode associated with MB. MB oxidation and reduction were monitored with SWV. DNA associated with the surface of the electrode gives a stronger SWV current. DNase I cleaved the DNA attached to the electrode. The DNA-associated MB left the surface. Thus, the SWV signal was reduced in the presence of DNAse I. The signal changes were directly proportional to the logarithm of concentrations of DNase[59].

Differential pulse voltammetry (DPV) is based on fixed potential with a series of pulses. For each pulse current is monitored at two points, the first is before the use of the pulse and the second is at the end of the pulse[57]. The advantage of DPV over CV is sensitivity and makes it possible to use analysis at the ppb (parts per billion) level. Low detection limits and small background current are considered as this technique to one of the best methods in electrochemical nucleic acid biosensors.

Li et al. used the hGQ DNAzyme and DPV to detect an oligonucleotide specific to Escherichia coli O157:H7 (a pathogenic strain). The researchers coated an electrode with a DNA probe specific to E. coli O157:H7. This captured an oligonucleotide specific to E. coli O157:H7 (analyte). The analyte was labeled with a “signal tag” nanocomposite. This signal tag was made from graphene oxide-thionine gold nanoparticles and included both a second, specific probe for E. coli O157:H7 and the hGQ DNAzyme. This complex binds the analyte as well and links many DNAzymes to the electrode surface. Graphene oxide improved the electron transfer and provided a large specific area. Thionine is electrochemically active. The hGQ

DNAzyme produced the electrochemical signal. DPV signal increased with increasing analyte concentrations. This biosensor shows a low detection limit 0.01 nM with a linear range from 0.02 to 50.0 nM E. coli O157:H7 DNA oligonucleotide[60].

Hassan et al. presented a DNA biosensor for detection of carrageenan (a common food additive) with DPV. They immobilized calf thymus double-stranded DNA (dsDNA) on a carbon-based, screen-printed electrode and used methylene blue (MB) as an indicator. The biosensor used the competitive binding of MB with carrageenan and the dsDNA-coated electrode. Figure 4. illustrates the principle of the system. Carrageenan and DNA are both negatively charged, and MB is positively charged. When carrageenan was in solution, MB left the immobilized dsDNA and bound carrageenan. DPV measured the loss of MB from the electrode surface. This study showed a fast, simple, and highly sensitive technique compared to chromatographic, spectrophotometric and potentiometric methods. The biosensor is selective towards carrageenan detection with low detection limit (0.08 mg L^{-1})[61].

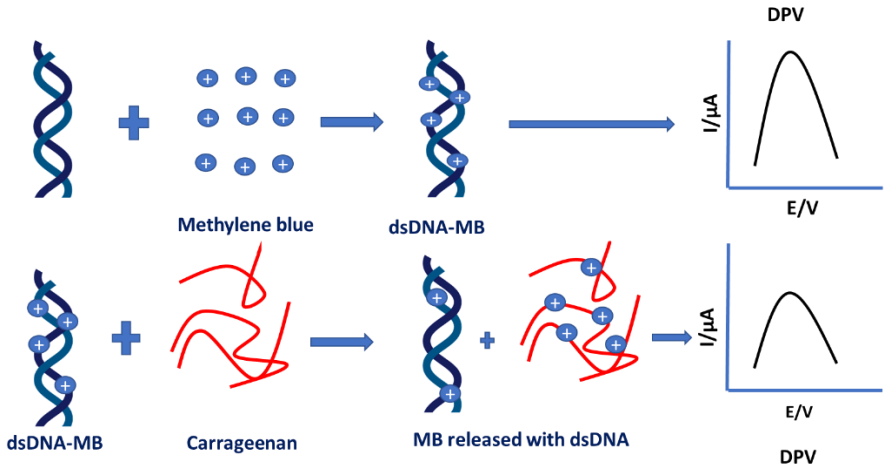

Figure 4. Schematic competitive electrostatic interaction of carrageenan and dsDNA towards binding with MB. (a) High DPV signal obtained after accumulation of MB in the dsDNA. (b) Lower DPV signal produced after immersion of the dsDNA electrode in the carrageenan solution

1.6 Chronocoulometry NA detection

Chronocoulometry is another method used in electrochemical biosensors. This technique is based on monitoring the charge transfer to an electroactive species on the surface of the electrode with respect to the time. Detection and quantification of nucleotide molecules can also be accomplished with chronocoulometry. For chronocoulometric DNA biosensors, researchers usually use hexaammineruthenium (III) chloride as an indicator[62].

Xiao Dong et al. presented a chronocoulometric biosensor to detect a specific DNA target. The biosensor is composed of a glassy carbon electrode modified with gold nanoparticles, multi-walled carbon nanotubes and polydopamine. The researchers attached a thiol-modified ssDNA probe to the gold nanoparticles through a gold-thiol bond. Figure 5. demonstrate the immobilization and hybridization detection of probe DNA. Ruthenium (III)hexamine (RuHex) acts as the electrochemical indicator. Chronocoulometry was used to detect DNA hybridization. This technique measured the change of the signal intensity of RuHex before and after the probe hybridized with the target DNA. RuHex (positively charged) and dsDNA (negatively charged) are attracted through electrostatic forces. When target was present, it formed dsDNA at the surface. When dsDNA was present at the surface it attracted a high concentration of RuHex. The high concentration of RuHex at the surface produced a high electrochemical signal. RuHex current increased when the concentrations of the complementary target DNA increased. The detection limit of DNA biosensor could detect around 3.5 fM[63].

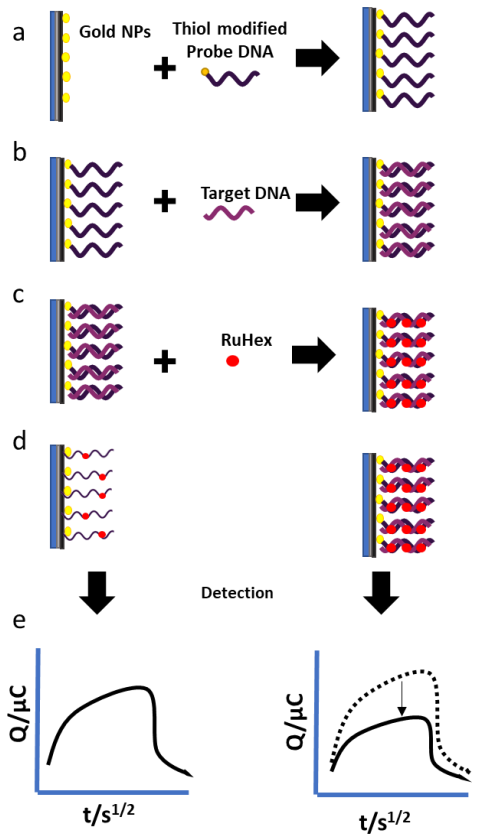

Figure 5. Schematic illustrations of the immobilization and hybridization detection of probe DNA.

1.7 Carbon structures as immobilization platforms in nucleic acid Biosensors

Recently, the unique properties of carbon structures (especially carbon nanomaterials) have led to advances in electrochemical NA biosensors. Carbon materials show high conductivity, large surface area and excellent electron mobility at room temperature. Different allotropes of carbon have been used including: one-dimensional (carbon nanotube), two-dimensional (graphene), and three-dimensional (graphite). In the following sections we discuss NA biosensors made with various carbon materials, with representative examples.

1.7.1 Carbon nanotubes as immobilization platform in nucleic acid Biosensors

Carbon nanotubes (CNTs) are cylindrical molecules made of a hexagonal arrangement of carbon atoms. CNT-based biosensors are emerging as the next generation of sensitive and fast biosensing systems. The high surface area of the CNT accelerates detection of biological analytes and improves detection limits. The surface-to-volume ratio in CNTs is very high due to the extremely small diameter and long length. This property enables immobilization of many biomolecules of the CNT surface. Generally, there are two types of carbon nanotubes: 1. single wall (SWCNTs) made of a single graphite sheet rolled into a tube and 2. multi-wall (MWCNTs) which consist of multiple layers of graphite sheets[64].

Carbon nanotubes (CNTs) can immobilize biomolecules at their surface, and have useful characteristics (physical, chemical, electrical, and optical) for sensors[65]. CNTs show fast electron transfer kinetics. The side walls of CNTs can be rich in oxygen functional groups such as carboxylate. This functional group can serve as an attachment point for different molecules to CNTs. The surface-to-volume ratio in CNTs is very high due to the extremely small diameter and long length. This property enables immobilization of many biomolecules[66].

Carbon nanotubes (CNT) without any defects possess good mechanical properties. However, defects in the CNT structure are important for electrochemical biosensors. CNTs efficiently transfer electrons to electroactive species due to point defects as shown by Compton et. al. They showed that edge-plane sites located at the end and at pentagonal defects generated regions with high charge density. These regions were able to transfer electrons rapidly to electroactive species in solution.[67, 68]

One of the problems for preparation of CNT-based biosensors is the insolubility of CNTs in most solvents. To address this, scientists are using ultrasonication and dispersion of CNTs in alternative solvents and polyelectrolytes. Addition of surfactants, conjugated aromatic material, or polymers is another way to increase the solubility of CNTs[69].

CNTs often form highly tangled ropes that hinder their capacity to form stable films. To solve this problem CNT are dissolved or dispersed and then immobilized on the surfaces of substrates through chemical or physical techniques. Scientists improved immobilization with Nafion, chitosan and surfactant additives. The most common method to create a CNT electrode is to create a suspension of CNTs and additives and then cast the CNT material on a surface (i.e., by evaporating the solvent).

Yanqiong Zheng et al. presented one example of this approach. They prepared an electrochemical DNA biosensor for monitoring phenolic pollutants (phenol, m-cresol and catechol). They evaporated a mix of Nafion and MWNT on a GCE. DNA was then allowed to bind the electrode with a slight positive potential. The interactions between phenolic pollutants and DNA changed the CV signal of the direct oxidation/reduction of DNA. The adenine reduction peak decreased in the presence of phenolic pollutants[70].

In another example, Shuyan Niu et al. developed a DNA biosensor based on MWNT functionalized with carboxyl groups for DNA hybridization detection. A manganese (II) complex (MnL) acted as a new hybridization indicator. CNT enhanced the effective surface area and conductivity of the electrodes. The researchers used EDC (1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide) to attach a 5’-amine-modified DNA probe to carboxyl

groups on the MWNT electrode. Changes in the DPV signal from MnL indicated hybridization between the DNA probe and the target DNA[71].

Saeed Shahrokhian et al. developed a label-free DNA biosensor by using MWCNTs for the detection of a specific DNA analyte (Figure 6). Unlike many CNT-based sensors, this technique did not use a cast/evaporated CNT electrode. The researchers used the strong association between MWCNTs and ssDNA. Immobilized ssDNA probes on the electrode bound MWCNT in suspension. The MWCNT accelerated the electron transfer to ferricyanide in solution. Thus, the addition of MWCNT increased the electrochemical current. When the analyte was present, it hybridized to the ssDNA on the electrode. This produced dsDNA and the MWCNTs were displaced. This resulted in decreased current. The change in CV response was used to measure the concentration of target DNA. The MWCNT system greatly improves signal compared to ferricyanide alone[72].

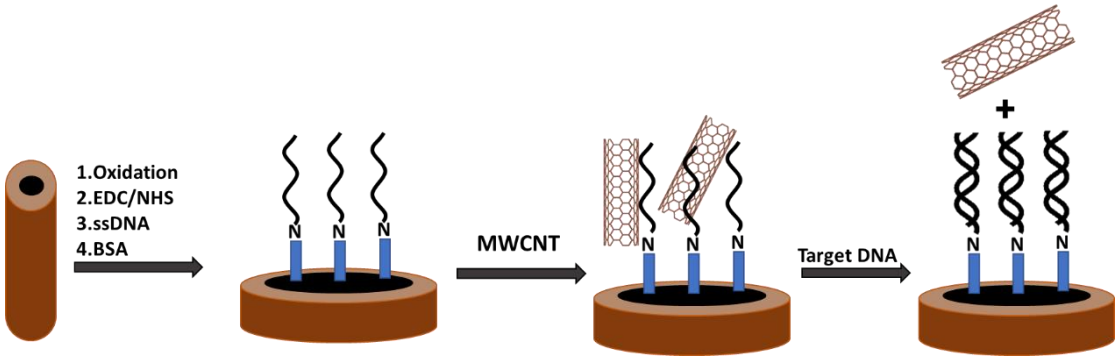

Figure 6. Electrode preparation and DNA hybridization detection

1.7.2 Graphene oxide (GO) and graphene quantum dots (GQDs) as immobilization platform

Unmodified carbon electrodes (e.g., glassy carbon electrode, GCE) have trouble with low sensitivity and selectivity. Electrodes modified with GO and Graphene quantum dots (GQDs) can improve sensitivity and selectivity. GO is a form of graphene containing oxygen functional groups (i.e., carboxylic group, alcohols and epoxides). GQDs are made from graphene nanoparticles with a size less than 100 nm. Graphene oxide [73] and graphene quantum dots (GQDs)[74] can both enhance DNA biosensors because they are biocompatible, low cost, conductive, mechanically strong, chemically inert, and have large specific surface area for immobilizing biomolecules.

Scientists have investigated different methods to improve the conductivity and surface area of graphene. For instance, when oxygen groups are removed from graphene oxide it becomes reduced graphene oxide (rGO). Compared to GO, rGO has better charge transfer and surface area[75]. Doping sulfur, nitrogen, or selenium can improve the electrocatalytic activity and charge transfer of GO[76–78].

Graphene materials have several advantages over CNT. Firstly, graphene materials contain fewer metallic impurities than CNTs[79]. Metallic impurities can interfere with electrochemical methods[80]. The fabrication process of graphene comes from natural graphite which is affordable and commercially available[81].

Moghaddam et al. presented an example of a DNA biosensor using a graphene electrode. This sensor detected the anti-cancer drug Tamoxifen (TMX). TMX has been applied for the treatment and prevention of breast cancer. Moghaddam used a carbon paste electrode

modified with graphene and dsDNA. TMX bound to the DNA-coated electrode. DPV obtained after accumulation of TMX shows increased current because of the high concentration of TMX on the surface. The biosensor was found to be capable of successfully analyzing of human serum, urine samples, and TMX tablets[82].

1.8 Different methods for Immobilization of nucleic acids on carbon structures

Different nucleic acid probe immobilization techniques have been used in electrochemical DNA sensing including non-covalent bonding (adsorption) and covalent bonding (crosslinking). Each technique carries its advantages and disadvantages. These techniques can be applied to carbon nanotubes, graphene oxide, quantum dots, gold nanomaterials, and metal oxides to attach biorecognition elements on electrochemical biosensor electrodes. In this part, we will discuss and compare the limitations and advantages of some of these methods.

1.8.1 Immobilization of nucleic acid on carbon structures based on covalent bonds and crosslinking

DNA can be immobilized covalently on the surface of the electrode. Functional groups can be added to DNA during synthesis for attachment. Such synthetic functional groups include amines (NH2) or thiols (SH) at the 3’ or 5’ end of the DNA probe. Such functional groups mediate attachment on the surface of the electrode through the formation of ester bonds, amide bonds, imine bonds, ether bonds, or gold-thiol (Au-S) bonds[83]. Covalent bonding technique can provide a good vertical orientation where the end of DNA probe was grafted

on the electrode surface which can result in high efficiency of DNA hybridization. By far the most common are amide and Au-S bonds[84].

The formation of Au-S between thiol-modified DNA probes and gold (Au) surface is common example of chemisorption technique. Gold-thiol chemistry is well characterized in the self-assembly monolayer (SAM) literature. There is a strong affinity interaction between the gold surface and thiol group to generate a covalent bond between Au and S. For instance, Wang et al. modified a glassy carbon electrode with graphene oxide sheets and decorated them with gold nano particles. The gold nanoparticles served as attachment points for thiol-modified DNA probe on the surface of the nanocomposite[85].

Carboxylate modified electrode can be functionalized with amine-terminated DNA probe through EDC/NHS chemistry. EDC is a water soluble and relatively inexpensive which is convenient to use in a variety of conjugation techniques. EDC reacts with carboxylates to generate an active ester as an intermediate. This reactive intermediate undergoes nucleophilic substitution in the presence primary amine. Thus, EDC mediates covalent binding between the carboxyl groups and primary amines. The reversion of the intermediate can limit of this reaction. To overcome this limitation, sulfo N-Hydroxysuccinimide (sulfo-NHS ester) has been used to form a more stable intermediate[86]. As noted above, Shuyan Niu et al. developed a DNA biosensor based on MWNT where DNA probes were attached to MWCNT using EDC/NHS chemistry[71].

Covalent bonding techniques have a good lifespan in comparison to adsorption due to the stronger association with the electrode. In DNA biosensors, this means that the DNA probe

on an electrode does not desorb into solution. Covalent bonds to the electrode surface can also orient a DNA probe to enhance hybridization[83].

As an example of a less common covalent attachment, Anu Singh et al. prepared electrochemical DNA biosensor by using graphene oxide-chitosan nanocomposite for detection of typhoid (Figure 7). Typhoid-specific DNA was detected by DPV using a DNA probe on an Indium Tin Oxide (ITO) electrode modified with GO and chitosan. Changes in the CV and DPV of ferricyanide allowed the scientists to measure the analyte DNA . To fabricate this electrode, a 5' amine-modified ssDNA probe was covalently immobilized on the surface of the electrode. In this case, glutaraldehyde mediated the attachment to amines on the chitosan. The addition of GO improved the electron transfer on the surface. Chitosan is an attractive polysaccharide for DNA attachment due to its biocompatibility, cationic nature, and abundant amine groups for attachment[87].

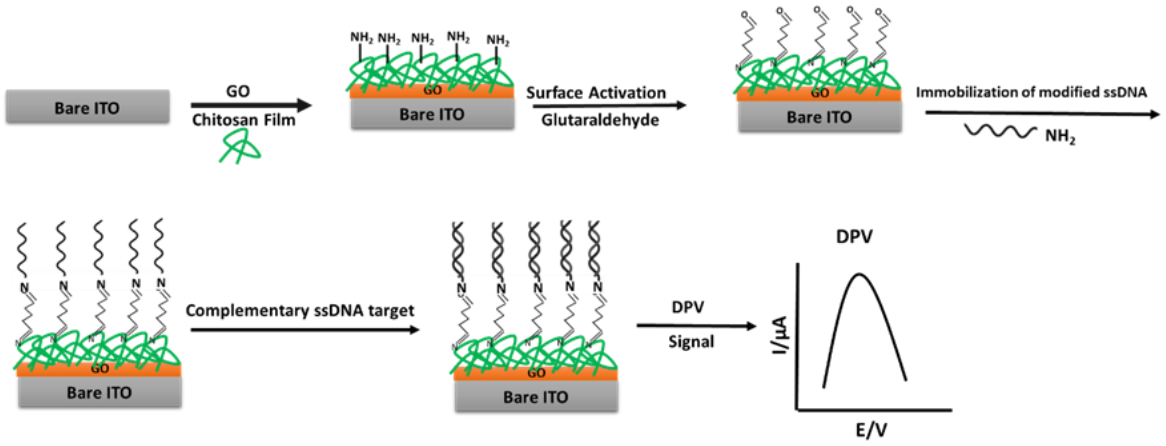

Figure 7. Methodology for fabrication of GO-CHI nanocomposite-based DNA electrode

1.8.2 Immobilization of NA on carbon structures based on non-covalent bonds or adsorption

A second common strategy for immobilization of DNA probes on electrode surfaces is based on non-covalent bonding. DNA (and other biomolecules) can bind to an electrode through different mechanisms. These include hydrogen bonding, hydrophobic bonds, van der Walls interactions, and ionic binding or electrostatic interactions[88].

Non-covalent methods have several advantages. Adsorption is the easiest technique to immobilize NA on electrode surfaces. The chemical structure of an immobilized biomolecule is not changed by non-covalent immobilization (as opposed to crosslinking, which alters the chemical structure)[89]. Chen-zhong Li [38]et. al showed an example of the non-covalent immobilization of a DNA biorecognition element as discussed in Section 2.1.2.

The main disadvantage of non-covalent methods is that biomolecules desorb from the surface. For example, DNA can desorb after it hybridizes. Another problem is poor analytical performance (i.e., higher limits of detection). NA and other biomolecules can adsorb and desorb dynamically. Surface-associated molecules may have a short lifetime on the electrode surface[88].One convenient type of non-covalent immobilization of NAs is based on the formation of an avidin-biotin complex. This interaction is strong and highly specific. Avidin and streptavidin are tetrameric proteins that each bind four biotin molecules with near-covalent strength. Biotin is a small molecule that can be attached to other biomolecules. Biotin-modified biomolecules bind strongly to the surface of an avidin modified electrode.

The strength of the interaction is -20 kj per mol, [90] compared to -350 kj per mol for a typical covalent bond.

A. Bonanni et al. published an example of a sensor using a (non-covalent) biotin-avidin association[91]. In this work, the authors present a label-free DNA biosensor using an avidin-modified graphite-epoxy electrode (Figure 8). The graphite–epoxy composite enhances the electrochemical performance and increases the conductivity of the electrode. Avidin molecules on the electrode mediate fast and simple immobilization of biotin-modified DNA. This biosensor was used to detect the hybridization of specific target oligonucleotides. The biotin-modified DNA probe hybridized to target DNA in solution and was captured on the electrode. The detection of hybridized or non-hybridized DNA was monitored through EIS of ferricyanide in solution (similar to Jianyun Liu, et. al[39] as discussed in Section 2.1.2).

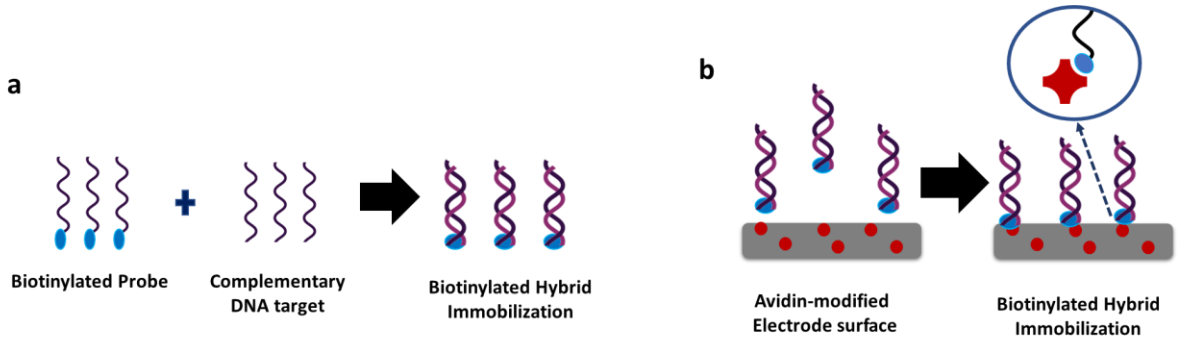

Figure 8. (a) Hybridization in solution between biotinylated-DNA probe and a DNA complementary target. (b) Immobilization strategy

1.9 Conclusion

EIS is emerging as a rapid and low-cost method for the sensitive detection of biomolecules. We have shown that there are many ways to approach DNA biosensors. Of all of these, EIS is the newest and most sensitive. EIS biosensor publications have grown steadily with 2400 listed in Pubmed since 1989, more than half of which were published in the last 5 years. EIS may ultimately compete with expensive optical methods like fluorescence and surface plasmon resonance. As an example, Fariba Khosravi-Nejad et al. used EIS to detect hepatitis B virus DNA with very impressive performance (LOD of the HBV biosensor was 0.1 pM)[92].

Electrochemical biosensors have been widely used in health care and industry. Electrochemical DNA biosensors are of considerable interest for measuring a wide variety of biomarkers (including NA, protein, and small molecule analytes). Detecting biomarkers can assist clinicians in making accurate diagnosis of diseases such as cancer. Sensitive DNA electrochemical biosensors are emerging for early-stage cancer detection. Other DNA biosensors have been used for food safety. Such biosensors can be cheaper, simpler, and faster than traditional methods.

In a recent (2019) example, Somayeh Mousavi et al.[93] developed electrochemical aptasensors using a nanocomposite of graphene oxide and gold nanowires. The electrochemical sensor detected aflatoxin B1 in real pistachio samples. The mold-derived aflatoxin B1 is toxic and carcinogenic. This aptasensor shows an excellent limit of detection of 1.4 pM.

Many scientists have developed DNA biosensors for cancer diagnostics. As a representative example, Lipei Luo et al.[94]developed an electrochemical DNA biosensor based on a locked nucleic acid (LNA) with GCE and polylysine film. In 2020, they detected exosomal microRNAs (miRNAs) derived from cancer cells. This work showed a high sensitivity and accuracy with a limit of detection as low as 2.3 fM.

In this review, we covered a wide variety of electrochemical methods and electrode materials. We showed the advantages and disadvantages of different carbon structures as immobilization platforms. New carbon nanomaterials can improve the stability and sensitivity of electrochemical sensors. Specifically, we highlighted the advantages of graphene as one of the best structures for electrochemical sensor electrodes. Immobilization of DNA through covalent bonds is the most promising immobilization strategy. Covalent bonds can be made between synthetic attachment groups on DNA (i.e., modified DNA) and different functional groups on electrode surfaces. Using non-modified DNA would be advantageous. Non-modified DNA can be synthesized more cheaply than modified DNA or it could be derived from biological sources. The conjugation of unmodified DNA to electrodes is a problem that remains largely unaddressed.

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Chapter 2: Electrochemical Biosensors based on Divinyl sulfone conjugation of DNA to graphene oxide electrodes

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2.1. Introduction

Detecting DNA can diagnose genetic diseases and detect pathogens. Specific DNA can be detected through fluorescent, chemiluminescent, and radioactive techniques,[1–3] which often suffer from high cost and poor stability. Electrochemical DNA biosensors are inexpensive and shelf-stable over long periods of time[4]. The instruments needed to read electrochemical sensors can be more easily mass-produced than optical sensors (e.g., for surface plasmon resonance or fluorescence)[5, 6]. Electrochemical biosensors also offer rapid detection, excellent sensitivity, and do not require covalent modifications (i.e., they can be used for label-free detection)[6, 7]. One type of electrochemical biosensor operates by monitoring current during reduction and oxidation of a solution species (e.g., potassium ferricyanide). When the analyte is immobilized on the electrode surface, a change in current is detected using cyclic voltammetry (CV)[8]. Other types of electrochemical sensors monitor the electrode with electrochemical impedance spectroscopy (EIS) to detect analyte binding[9, 10].

Most EIS and CV based biosensors use gold electrodes, which are inconvenient to fabricate (e.g., electroless or electrochemical deposition of gold, sputtering, etc.)[11, 12]. More importantly, gold has a narrow electrochemical window in water. Gold is conjugated to DNA with thiol linkages, which limit the electrochemical window, as the thiols are reduced and desorbed at approximately -1.0 V vs. Ag/AgCl. A graphite surface with a covalent bond to DNA has a wider electrochemical window. The covalent link to carbon electrodes is more stable, and the hydrogen and oxygen overpotentials are large on carbon surfaces[13].

Graphene electrodes are relatively new and have advantages for CV. Graphene was discovered in 2004[14]. It has high conductivity, reagent compatibility, and functional groups for the attachment of biomolecules. Graphene oxide (GO) has carboxylate groups, quinone groups, and hydroxyl groups on the periphery of the carbon sheets. GO can be suspended in aqueous solutions and various solvents[15]. GO electrodes have a wider electrochemical window than gold or graphite. Unlike gold, GO can be screen-printed or drop-cast[16]. DNA-based biosensors using GO have demonstrated good sensitivity, high electron transfer rate, large surface area, and a wide variety of analytical targets[17].

Graphene oxide has many promising features for biosensors: it is conductive and interacts with DNA. Normally scientists divide the interaction into two types: physisorbed and chemisorbed. Because physisorption is simple and has significant sensitivity, physisorption is the most common. Physisorption includes direct adsorption, competitive adsorption with the use of blocking agents, and inhibited adsorption. Additionally, DNA can be chemisorbed or covalently linked to GO. For a recent review, see Lopez and Liu [18].

DNA and GO bind when they are mixed in an aqueous solution. In this method, salt (such as NaCl) is required to decrease repulsion between the negatively charged surface of GO and DNA. Once this is overcome, the nucleobases can hydrogen-bond to the GO surface. Poly-adenine (poly-A) can be added to a DNA sequence to increase the affinity for GO because purines associate with GO more strongly than pyrimidines. This simple adsorption method suffers from a lack of specificity, as GO binds to many protein and nucleic acids.

Another way to use physisorption is to associate DNA with a polymer on the GO surface. Abhinav Sharma. et al. presented a label-free, sensitive electrochemical biosensor by using a thin film of polyethylenimine on reduced graphene oxide for detecting cardiac myoglobin. The polymer helps to immobilize the DNA aptamer[19].

In an example of a particularly strong physisorption process, Yaqiong Wang *et al.* reported a non-covalent functionalization of Reduced graphene oxide (CRGO) with tetra butyloxyphenylporphyrins bearing mono or tetra-carboxylic groups. The carboxylic acid groups of the mono or tetrakis-(carboxyl phenyl) porphyrin were then conjugated to the ssDNA through an amide link. This allowed for the detection limits in the attomolar range. However, this still required a covalent coupling process between amine-modified DNA and the porphyrin species[20].

DNA can also be directly and covalently attached to the surface of GO. Covalent binding has several advantages. The reproducibility and stability of the surface functionalization are higher than physisorbed DNA. Covalent attachment of DNA also improves the electron transfer rate. Another advantage is covalently linked DNA completely resists nonspecific

displacement. One common method is to apply amino-modified DNA plus 1-ethyl-3-3-dimethylaminopropylcarbodiimide (EDC). This generates an amide bond to the carboxylates on the surface of GO. In one example of the use of EDC, Gong et al. demonstrated an impedimetric DNA biosensor detecting the HIV-1 gene with a detection limit of 3.0×10^{-13} M.[21].

Like EDC, DVS can activate the surface of a carbon nanomaterial such as graphene oxide. DVS can activate various functional groups like thiol, amino, or hydroxy groups. This generates a reactive vinyl group on the carbon surface. The surface can then be linked to phenol, hydroxy, amino, imidazole, or thiol groups[22].

DNA and RNA can be measured using synthetic, complementary DNA probes. Protein and small molecule targets can be measured using aptamers (oligonucleotides evolved to bind specifically and tightly to a target molecule)[23]. A DNA oligonucleotide probe can be conjugated to the surface of graphene. Binding of the analyte to the oligonucleotide probe produces a change in the electrochemical properties of the surface (e.g., electron transfer rate)[24]. This change is interpreted as an analytical signal for electrochemical biosensors. To generate a biosensor, most researchers report conjugating biomolecules to GO with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)[25]. This activates carboxylates on the GO surface and allows for conjugation to a primary amine. DNA can be synthesized with a terminal primary amine for attachment. This has two disadvantages. Firstly, the activated carboxylate intermediate is unstable, and so the conjugation must be carried out rapidly or using a succinimidyl ester intermediate. Secondly, the primary amine terminal modification adds expense and time to the synthesis of the oligonucleotide[26]. We sought simple,

alternative conjugation chemistry that can be applied to conjugate an unmodified oligonucleotide to an electrode surface.

Since the 1970s, DVS has been used as a linker to conjugate biomolecules to different materials[27]. Recently scientists showed divinyl sulfone could act as a crosslinker to bind hyaluronic acid (HA) and collagen hydrogels in an alkaline solution[28]. DVS can react with a hydroxyl functional group with each vinyl group. DVS has been used to conjugate enzymes, antigens, and carbohydrates for purification of protein[29]. Also, DVS can be used to add labels for detection (e.g., biotin and fluorophores), drug delivery, and immunoassay development[30–32].

We generated graphene oxide (GO) via Hummers' method [33] and fabricated electrodes by drop-casting the material in a suspension of Nafion and alcohol. We conjugated unmodified DNA to the electrode with divinyl sulfone (DVS). DVS can irreversibly bind to different compounds displaying a reactive lone pair[34]. This includes amine, hydroxyl, and carbonyl functional groups. DVS can activate a variety of functional groups on the GO surface. The activated surface can then react with DNA to produce a covalent link to the electrode. We explored the possible sites of attachment on the DNA molecule with LC-MS/MS.

We show that the resulting DNA-coated electrode can be used to detect complementary oligonucleotides by EIS, DPV, or CV. Also, to show the availability of single-stranded DNA (ssDNA) for hybridization, we used fluorescent microparticles coated with the reverse complement of a mock-analyte DNA oligonucleotide. This was accomplished with an electrochemically active compound in solution and with an electrochemically active

intercalating dye, methylene blue (MB). This method detects complementary single-stranded nucleic acids directly. The method could also detect the amplification products from an enzymatic amplification reaction (e.g., LAMP, NASBA, SDA, or EXPAR) for ultrasensitive detection[35].

2.2 Experimental section

2.2.1 Chemicals and Reagents

Divinyl sulfone 98% was purchased from Sigma Aldrich. Potassium ferricyanide was purchased from Ward's Science (Rochester, NY). Analytical grade sulfuric acid, sodium hydroxide, potassium permanganate, graphite, and potassium chloride were analytical grade. The stock solutions of the oligonucleotides (5 μM) were prepared in Tris–HCl buffer solution (pH 8.00) and kept frozen at −20 °C. DNA oligonucleotides were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa, USA) and had the following sequences:

GSurf: TAT CGA GGA GGA GGA GGA GTA TCG AGG AGG AGG AGG ATT CGA TTC CTA TGT

Complementary target (GSurf*): ACA TAG GAA TCG AAT CCT CCT CCT CCT CGA TAC TCC TCC TCC TCC TCG ATA

Non-complementary target (mut-GSurf*): TAA GAA TGG GTA GAG GTG GAG GTG AGG GTG AGT GGT AGA GGT GGA GAT GAA

2.2.2 Instruments

Electrochemical measurements were performed using a Pine WaveDriver Galvanostat (Pine instruments, Durham, NC, USA). A conventional three-electrode system was used with glassy carbon as the working electrode, Ag/AgCl (3.5 M KCl) as a reference electrode, and Pt as a counter electrode in 0.1 M potassium chloride (KCl) solution containing 6 mM of potassium ferricyanide as noted. All measurements were performed at room temperature. Analysis of conjugation was performed using a Waters Q-Tof Premier Quadrupole-Time of Flight Mass Spectrometer. The resolution of this instrument is up to 17,000 and a mass range up to 100,000 Da.

2.2.3 Preparation of graphite oxide and Fabrication of GO-modified the carbon paste electrode

Graphene oxide (GO) was prepared from graphite powder by following Hummer's method [33, 36]. Firstly, 9 g of graphite powder and 1.5 g of $NaNO_3$ were dissolved in 25 mL concentrated 98-99% sulfuric acid. Then the mixture was stirred for 3 h in an ice bath. Then, 8 g of $KMnO_4$ as a strong oxidizing agent was slowly added to the mixture at 35°C. The mixture was stirred for 10 hours until the color of the solution was dark brown. After, 25 ml 30% H_2O_2 was added to the solution to quench the reaction and eliminate the excess potassium permanganate. Upon addition of H_2O_2, the color of the solution changed to a pale yellow. Then, 80 ml of distilled water was slowly added to the solution. The mixture was stirred for 2 hours at 35°C. The product was washed by centrifugation at 10,000 rpm and resuspension. The pH was 4.5 after washing. The solution was left to settle overnight. The product was

placed in an oven (60 °C) for 15 h. Then, the final product was reduced to a dark powder by grinding in a mortar and pestle.

2.2.4 Fabrication of ssDNA-conjugated graphene oxide on glassy carbon

In this experiment, ssDNA (concentration 5 µM) was first added to the graphene oxide suspended in buffer solution and then DVS was added to the solution. Here, Single-standard DNA (GSurf) was covalently attached to the oxygen functional groups of graphene oxide surface through DVS coupling. The bare GCE electrode was cleaned to a mirror surface with 0.3 mm γ-Al2O3. The clean GCE was sonicated in water for 30 s and then rinsed with deionized water. For immobilization of the oligonucleotide, 5 mg graphene oxide was suspended in a 0.1 M MES buffer (pH 4.5). The mixture was washed with buffer three times. Then, ssDNA (5 µM concentration) was added to the mixture. DVS (10%, V/V) was added to the solution, and the mixture was vortexed for 2 hours at room temperature. The suspension was washed five times by centrifuging at 6000 rpm 10 min and resuspending in 0.1 M MES buffer (pH 4.5). To the DNA-coated GO, 25µL 5% Nafion, 50 µL of ethanol, and 50 µL DI water were added to the surface of GO. The suspension was sonicated for 1 hour. Then, a droplet of 3 µL of the DNA-coated GO in Nafion suspension was applied to the surface of the polished GCE.

2.2.5 Characterization of the electrode response to hybridization

To characterize the difference in biosensor response to GSurf and the dsDNA complex of GSurf and GSurf*, pre-hybridized electrodes were prepared. After conjugation of GO and GSurf DNA as above, we added different concentrations of GSurf* (the reverse complement of the immobilized oligonucleotide) in Tris buffer. The GSurf -coated graphene oxide and

GSurf* were vortexed for 1 hour. The solution was then drop-cast on the surface of the GCE as above.

To test the response of the GSurf-coated electrode to GSurf* in solution, we dipped the drop-cast GSurf-coated GO electrode in a solution of GSurf*. The GSurf-coated electrode was immersed in 10 µL of Tris buffer (25 mM) containing GSurf* at the concentration noted in the text. The GO was loosely attached to the graphite electrode and was handled carefully. As a negative control, the GSurf-coated electrode was immersed in non-complimentary DNA (mut-GSurf*). DNA hybridized for 1 hour at room temperature. shows the conjugation of an ssDNA onto the GO surface and hybridization with a complementary target.

2.2.6 Electrochemical measurement of complementary DNA with CV and EIS

The electrochemical characteristics of the modified glassy carbon electrode were measured using cyclic voltammetry (CV, as shown in figure 9) and electrochemical impedance spectroscopy (EIS). CV measurements were carried out in 25 ml of 10 mM potassium ferricyanide and 1 M potassium chloride (KCl). A conventional 3-electrode system was used as noted above with potential range from -600 to +200 mV and scan rate was 100 mVs^{-1}. EIS was measured in 25 ml of 10 mM potassium ferricyanide in 1 M KCl. The range of frequencies was from 1MHz to 1Hz and the amplitude is 25 mV in a given open circuit voltage.

2.2.7 Detection of complementary DNA with the intercalating dye, MB

DNA (GSurf) was coupled to the GO surface as detailed above. The electrode was dipped into complementary DNA (GSurf*) at an indicated concentration. The electrode was washed by dipping in 0.1 mol L^{-1} PBS: 0.1 mol L^{-1} NaCl + 0.01 mol L^{-1} sodium phosphate buffer (pH 7.45)

buffer. To measure the captured DNA analyte, MB was accumulated onto the surface-hybridized DNA. The hybridized electrode was immersed into 0.1 mol L^{-1} PBS buffer (pH 7.45) containing 25 µM MB for 10 min with gentle stirring. Intercalated MB accumulated by the dsDNA was measured by using DPV with potential between -0.6V and +0.3V (see figure.9). This experiment was repeated with 3 different electrodes for each concentration.

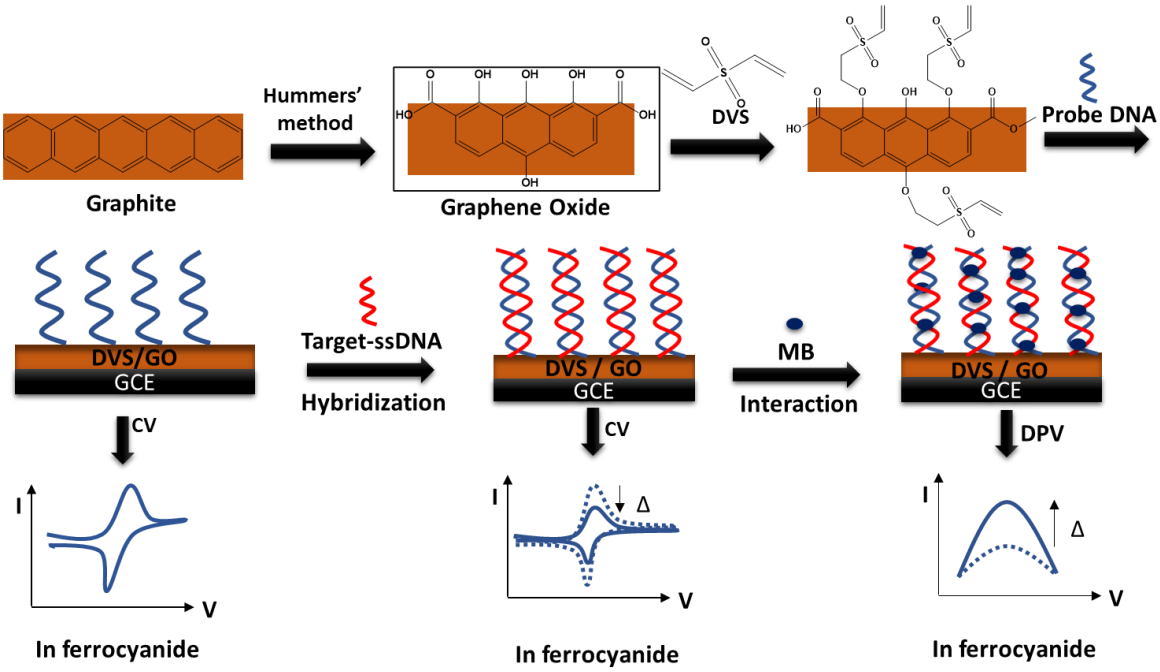

Figure 9. Graphical outline of the conjugation of DNA to the electrode surface and the application to detection of complementary DNA with CV and DPV.

2.2.8 Fluorescent microparticle binding DNA coated GO

GO was coated with GSurf as described above using DVS. To demonstrate the availability of GSurf for hybridization, we applied fluorescent microparticles coated with the reverse complementary DNA, GSurf*. GSurf* was acquired from IDT with an amine modification for attachment. Carboxylate coated, green-fluorescent microparticles (Bangs Labs, Fishers, IN)

were conjugated to the GSurf* DNA with standard EDC chemistry (per Manufacturer Technical Note 205). Briefly, particles were washed and resuspended in 0.1 mol L^{-1} PBS: 0.1 mol L^{-1} NaCl + 0.01 mol L^{-1} sodium phosphate buffer (pH 7.45), followed by adding 0.1 volume of 1 M EDC with stirring, followed by the addition of DNA in water to a final concentration of 20µM. This was allowed to conjugate for 2 hours. The resulting DNA-conjugated microparticles were then washed 3 times in 0.1 mol L^{-1} PBS (pH 7.45) by centrifugation and resuspension. The GSurf*-coated microparticles were applied to GSurf-coated GO and allowed to sit for 45 minutes. The surface was then gently rinsed with 0.1 mol L^{-1} PBS buffer (pH 7.45). The resulting immobilized microparticles were imaged with a fluorescence microscope (Nikon Microphot FX). As a control reaction, the above was repeated with non-complementary DNA (mut-GSurf*) on the microparticle surface.

2.2.9 Reaction of DVS with Thymidine

To how DVS reacts with DNA, we reacted DVS with a mononucleotide and analyzed the result by LC/MS/MS. First, 100µL DVS (10%, V/V) was mixed with thymidine (5 mg) in 5 mL of 50 mM phosphate buffer solutions for 4h at room temperature (pH 7.4). The solution was diluted with ethanol. The progress of the reactions between DVS and thymidine was monitored by LC-MS/MS (Waters Q-Tof Premier Quadrupole-Time of Flight Mass Spectrometer).

2.3 Results and discussion

2.3.1 Conjugation of DNA to Graphene Oxide (GO) electrode

The conjugation of DNA to the surface of GO was investigated through EIS and the capture of fluorescent microparticles. **Figure 10A** shows the Nyquist plots of the faradaic impedance

spectra for the biosensor. The sensor was measured at 5 stages of preparation: 1. The bare electrode before adding GO; 2. The electrode after drop-casting unconjugated GO (which shows reduced resistance to electron capture, typical for GO electrodes); 3. After reaction with DVS on the surface of GO; 4. After immobilization of GSurf on the DVS; 5. After hybridization of DNA complementary to the immobilized probe (Figure S1).

Nyquist plots of the bare electrode and GO drop cast electrode show a characteristic semicircular profile at the 1MHz to 1Hz frequency region. At all stages after the addition of DVS, the Nyquist plots appear as a straight line due to the high molecular weight of DVS and its strong inhibitory effect on charge transfer. The charge transfer resistance (Rct) at the GCE/GO/DVS is higher than that at the GCE/GO, showing the blocking effects of DVS.

When we quantitatively compare the EIS spectra and charge transfer resistance of the DVS electrode after immobilization of DNA (GSurf) and after hybridization (adding GSurf*), we can see a further increase in charge transfer resistance proportional to the blocking effects of the negatively charged DNA. We take this as evidence that GSurf is conjugated and able to capture complementary GSurf*.

We confirmed the results of EIS with fluorescence microscopy. The conjugated DNA on the GO surface should capture fluorescent particles coated with complementary DNA. **Figure 10B** shows a schematic of the strategy by which the surface-immobilized DNA captured fluorescent particles. GSurf*-coated green fluorescent microparticles were captured on the GSurf-conjugated GO surface. Green fluorescent microparticles coated with a non-complementary DNA (negative control) adsorbed nonspecifically at a significantly lower

frequency. This can be seen in the representative micrographs (**Figure 10C**) and a bar graph (**Figure 10D**). The number of fluorescent particles per micrograph (~0.1 mm^2) indicates much higher numbers of captured particles in the case where the DNA is complementary. Error bars are the standard deviation of three representative images.

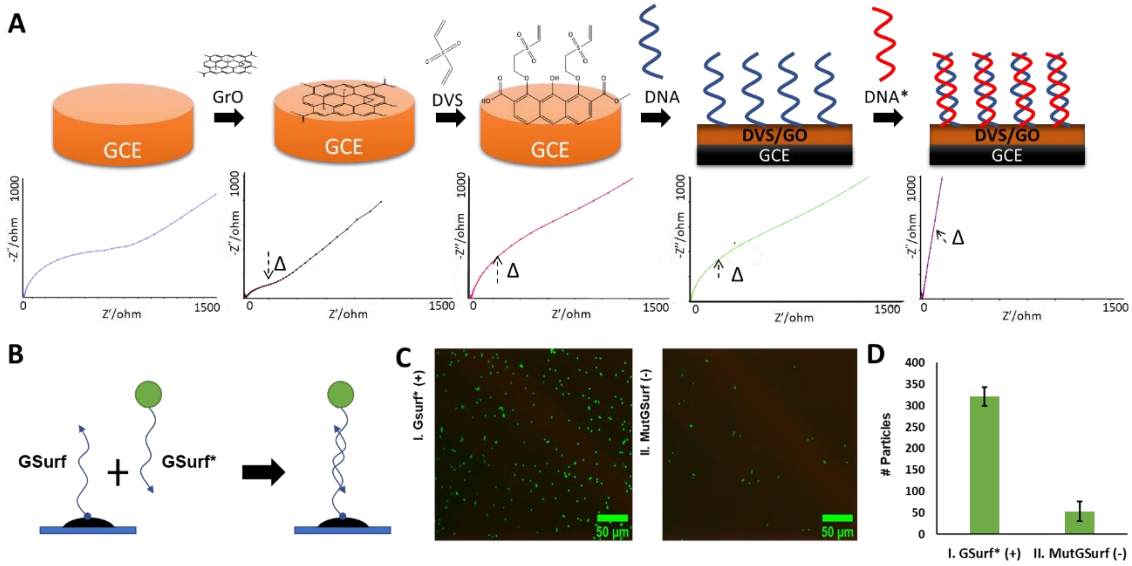

Figure 10. Conjugation and corresponding data confirm DNA attachment. (A) the Nyquist plots of the faradaic impedance spectra at different electrodes: bare GCE, GCE/ GO, GCE/GO/DVS, GO, GCE/GO/DVS/GSurf and GCE/GO/DVS/GSurf/GSurf* (B) Illustration of surface-immobilized DNA captured fluorescent particles. (C) Fluorescence micrographs of DNA-coated electrode exposed to green, fluorescent microparticles with complementary DNA (experimental, left) and non-complementary DNA (negative control, right). (D) Bar graph shows the average number of fluorescent particles per micrograph.

2.3.2 Conjugation of DNA to DVS is by attachment to nitrogenous bases

The products of DNA reacting with DVS were characterized by mass spectrometry (using a quadrupole-time of flight tandem mass spectrometer). Thymidine was used as a model nucleotide to investigate the reaction between DVS and DNA. We originally hypothesized that DVS would react with hydroxyls on the ribose ring. The mass spectrum suggests, rather, that the DVS reacts with the nitrogenous base. **Figure 11A** shows the total ion chromatogram (TIC) of the reaction product mixture. The highest peak with retention times of 4 min shows a mass

spectrum (**Figure 11B**) with the highest abundance ion at m/z 361. We attributed this peak to the DVS-thymidine conjugate. **Figure 11C** is the fragment ion mass spectrum. The most abundant peak has m/z 244.9 and is consistent with a thymine divinyl sulfone link, minus deoxyribose. We interpret this to mean that the fragmentation occurred between ribose and thymine. The peak m/z 152.7 may be DVS hydroxyl products. The smallest peak m/z 118 is consistent with the pentose sugar fragment from the parent ion.

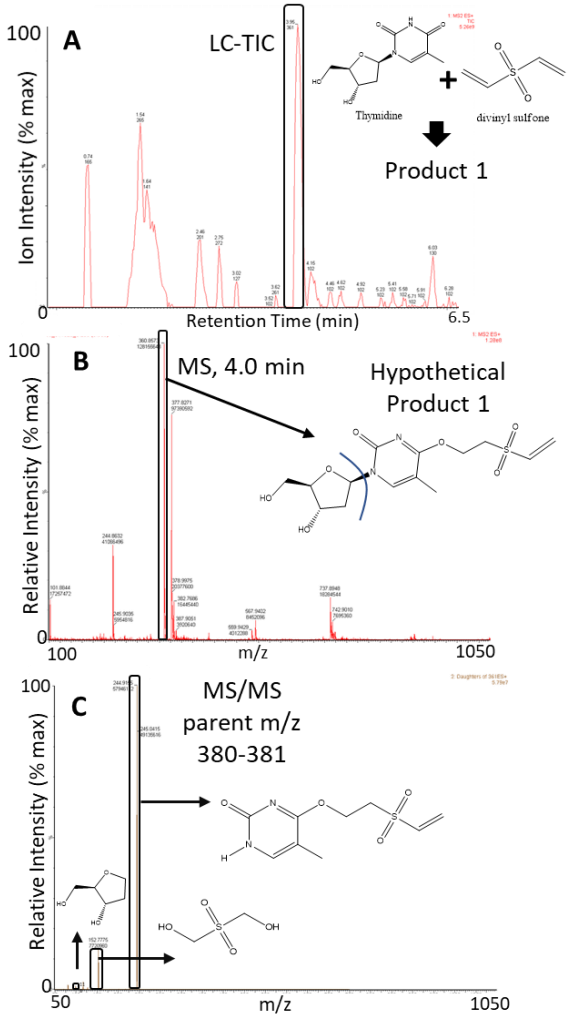

Figure 11: LC-MS data of solution-phase conjugation of DVS to the nucleotide. (A) Total ion chromatogram of the reaction mixture of DVS and Thymidine. (B) Mass spectrum of the peak at 4 minutes, the major product of the reaction. Hypothetical product structure with molecular mass 361 is shown as an inset. (C) The fragment mass spectrum of Product 1 shows fragments and hypothetical structures.

2.3.3 Detection of complementary DNA with CV and iron (II/III) hexacyanide

In this work, cyclic voltammetry (CV) was used to monitor the hybridization of complementary DNA (GSurf*) to the immobilized DNA (GSurf). The hybridization of GSurf* to immobilized DNA adds a significant negative charge to the surface, which we expected to repel ferricyanide ions in solution. This resulted in a reduction in charge transfer efficiency and a decrease in the peak current in the cyclic voltammogram. **Figure 12A** shows a schematic of the experiment for the detection of hybridization by this method. **Figure 12B** shows typical CV data for bare GO, GO/DVS, GO conjugated to GSurf, and the electrode exposed to complementary DNA (experimental) or non-complementary DNA (negative control). Qualitatively, DNA decreases charge transfer (as expected), and non-complementary DNA has no effect.

In more detail, GO/DVS showed a lower CV current than GO alone, just as it showed higher charge transfer resistance in EIS. DNA and its complement also further reduced the redox current of the probe in solution. The peak current change is proportional to the concentration of analyte (complementary) DNA. DNA-coated electrodes (bearing GSurf) were dipped into solutions containing different concentrations of complementary DNA (GSurf*). **Figure 12C** shows typical CV peak currents after the capture of complementary DNA. The oxidative peak currents decrease proportionally to the concentration of GSurf* (see **Figure 12D**). Based on the calibration curve in **Figure 12 B**, we estimate that we have a limit of detection of 6.99 μM.

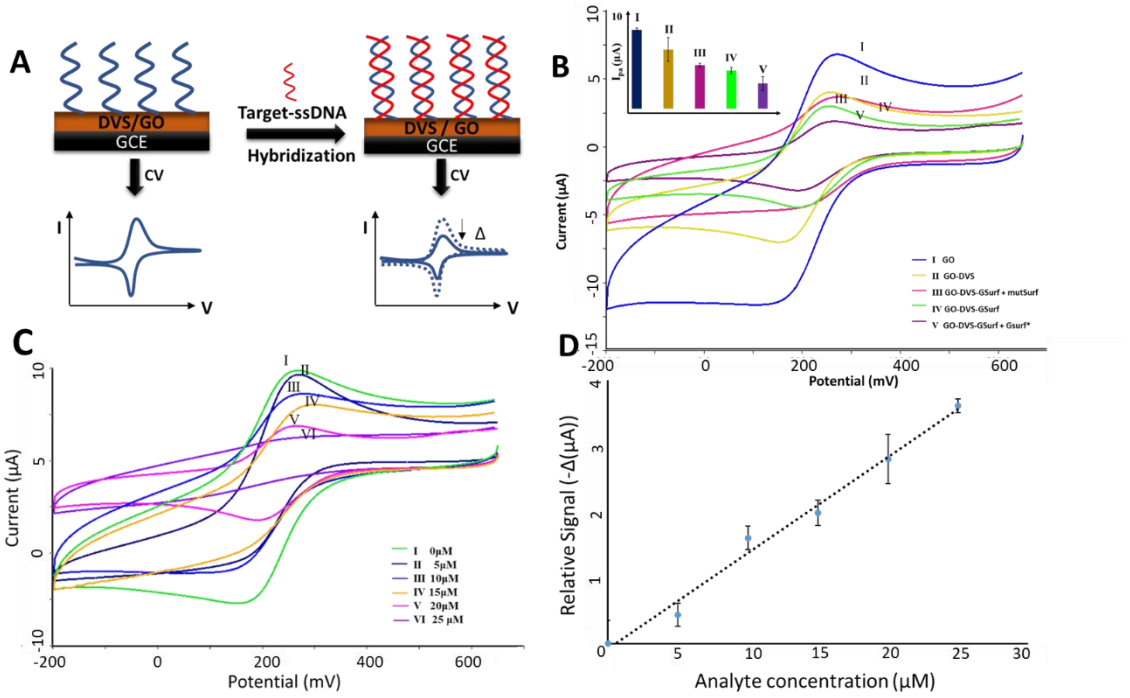

Figure 12: CV to detect complementary DNA. (A) Illustration of hybridization of complementary DNA with immobilized DNA. (B) Cyclic voltammogram of iron ferricyanide using electrodes at each stage of the electrode fabrication. (C) Cyclic voltammogram of the DNA-modified GO electrode with increasing concentrations of complementary DNA from 0 to 25 μM. (D) Calibration curve derived from the cyclic voltammogram peak current as a function of the concentration of analyte DNA.

2.3.4 Detection of complementary DNA with DPV and methylene blue

We set out to detect DNA with DPV by measuring the electrochemical activity of an intercalating dye. When analyte DNA associates with conjugated, complementary DNA, it generates a dsDNA complex. This complex can then associate with the electroactive dye Methylene Blue (Mb) from the solution. Mb associates more strongly with dsDNA than with ssDNA. Hybridization results in more Mb being immobilized near the electrode[37–39]. **Figure 13A** illustrates how the peak current is increased due to more efficient charge transfer in the presence of complementary DNA. **Figure 13B** shows how the peak current increased as a function of the concentration of complementary DNA. We dipped the electrode in

complementary analyte DNA at concentrations ranging from 0 µM to 25 µM under optimal conditions. This was converted to a calibration curve (**Figure 13C**) for quantifying ssDNA in solution. We estimate that we have a limit of detection of 1.44 µM. Our results show a comparable detection limit to other published DNA biosensors based on electrode-conjugated oligonucleotides (Table 1).

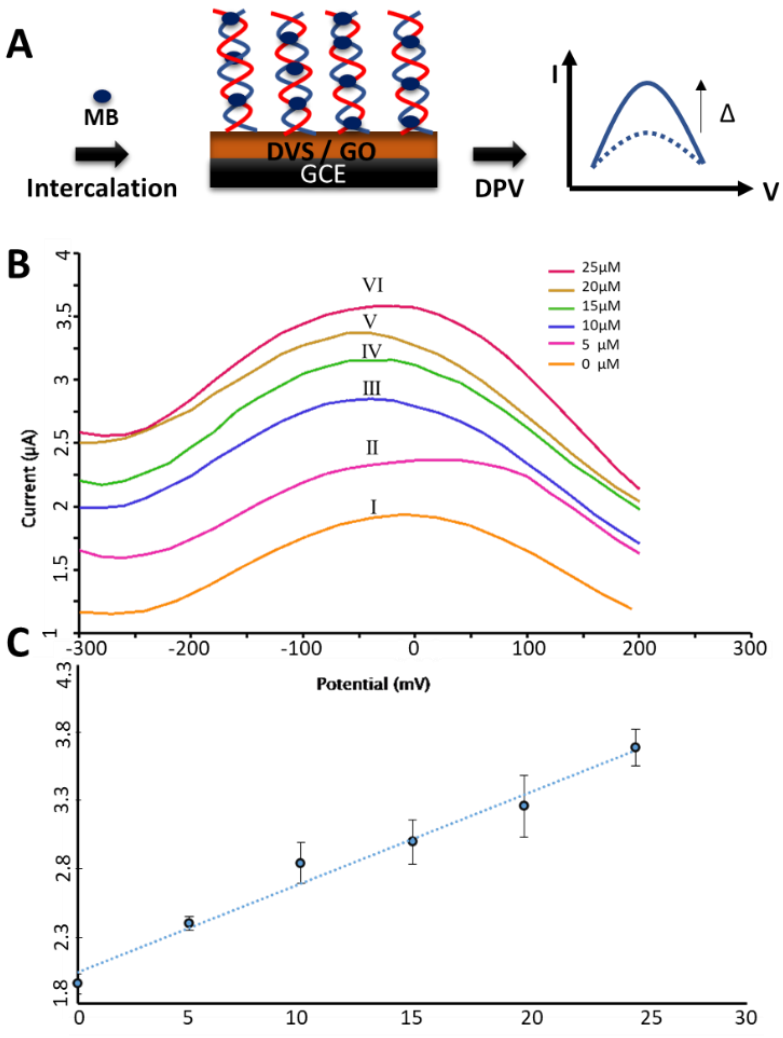

Figure 13: DPV to detect complementary DNA. (A) Illustration of hybridization of complementary DNA with immobilized DNA and electroactive dye Mb. (B) Differential pulse voltammetry current signal response of the modified GCE electrode with increasing concentrations of complementary DNA from 0 to 25 µM. (C) Calibration curve derived from the DPV peak current as a function of the concentration of analyte DNA.

2.3.5 Detection of complementary DNA with EIS and ferricyanide

We also used EIS to measure complementary DNA (GSurf*) using the immobilized DNA (GSurf) electrode. As with CV, DNA reduced the charge transfer to ferricyanide. This resulted in an increase in net resistance, which showed up as a positive signal in EIS. A DNA-coated electrode (bearing GSurf) was dipped into solutions containing different concentrations of complementary DNA (GSurf*). GSurf* represents an analyte nucleic acid in solution. As can be seen in Figure 14A, the EIS spectrum changes as a function of analyte concentration.

Finding a suitable equivalent circuit (e.g., Randles) is the first step to investigate the parameters in our electrochemical system. Given an equivalent circuit, we can calculate uncompensated resistance (i.e., solution or electrolyte resistance), charge transfer resistance, and double-layer capacitance. This circuit consists of a parallel capacitor (with capacitance C) and a charge transfer resistor (modeling the resistance of redox reactions, Rct), both in series with the supporting electrolyte resistor (with resistance Rsol). The fit of the EIS spectral data to the equivalent circuit was acceptable between the experimental data and Randles circuit model via Zview software. We also tried the Warburg impedance model, but it gave a poor fit to the data under most conditions. Randles circuit is best for modeling a free diffusing species. This is very common for simulating analytical electrochemistry experiments where freely diffusing ferricyanide is present. *al*.[40] and Gao *et al*.[41].

Semicircular spectra are common for redox species in solution (like ferricyanide). With the bare electrode (no conjugated DNA), we do get a small semicircle in the spectrum because charge transfer to ferricyanide is efficient (the semicircle diameter is proportional to the

resistance to charge transfer). When the electrode is coated with DNA (and especially when analyte concentrations are high), the ferricyanide species are repelled from the surface. Repulsion of the ferricyanide makes the resistance to charge transfer much higher. The diameter of the semicircle would be very large, and so the line appears straight.

A Randle's circuit with a constant phase element was used in all cases to model the impedance of the DNA-coated electrode. From the model, we derived the resistance to charge transfer, Rct. This was used to compare the impedance response of the modified GCE electrode with DVS at different concentrations of DNA.

In general, intrinsic resistance (Rs) is related to the combined resistance of electrolyte and electrode material, charge transfer resistance (Rct) is originated from the electronic and ionic resistances at the electrode-electrolyte interface, whereas Warburg diffusion resistance (Rw) is related to the resistance of ionic diffusion in an electrolyte, which is frequency-dependent.

The calibration curve in Figure 14B shows the EIS resistance parameters (Rct, resistance to charge transfer) of the electrode as a function of analyte DNA concentration. We estimate that we have a limit of detection of 0.076 μM.

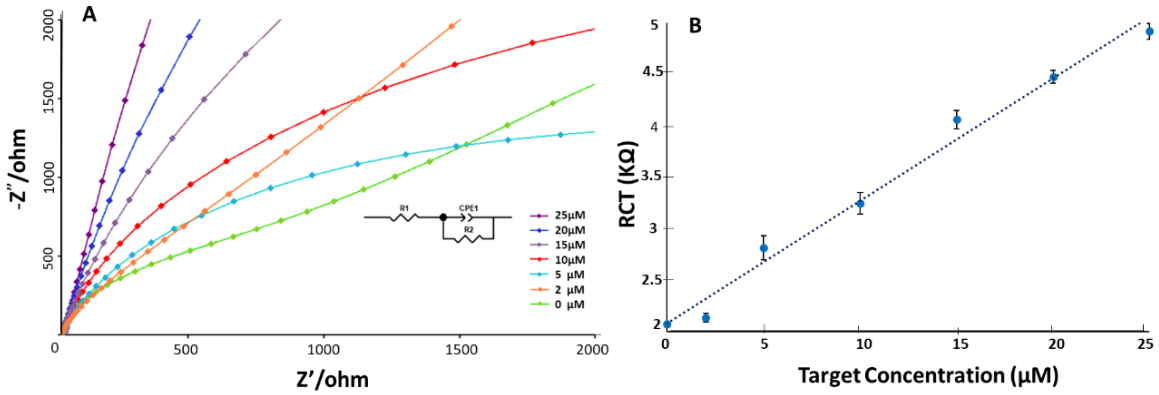

Figure 14: EIS detection of complementary DNA. (A) Nyquist plots of the DNA biosensor of the modified GCE electrode with increasing concentrations of complementary DNA from 0 to 25 µM. Inset: Randles equivalent circuit. (B) Calibration curve derived from the EIS charge transfer resistance (Rct) as a function of the concentration of analyte DNA.

Table 1: Comparison of published conjugation methods for electrochemical DNA sensors

Electrode	Modifier	Detection methods	Target	Dynamic Range	Detection limit	Ref
Glassy carbon	Graphene-COOH	DPV	adenine and guanine.	$5x10^{-4}$- 0.2 µM	0.5µM	[42]
Glassy carbon	Carbon nanotubes	DPV	DNA	$3.1x10^{-4}$-$6.2x10^{-3}$ µM	$0.16x10^{-4}$ µM	[43]
Glassy carbon	SWNTs ,Ethylenediamine	DPV	DNA	$4X10^{-5}$-$11X10^{-5}$ µM	$2x10^{-5}$ µM	[44]
Glassy carbon	Reduced graphene oxide	EIS	DNA (HIV-1 gene)	1×10^{-6}- 1×10^{-3} µM	1.0×10^{-7} µM	[45]
Pencil graphite	Graphene Oxide EDC/NHS	DPV	Hepatitis B virus (HBV) sequences	20 to 160 µg/mL	2.02 µM	[46]
Glassy carbon	Au nanoparticles, toluidine blue–graphene oxide (Au NPs/TB–GO)	DPV	MDR1 gene	1.0 × 10−5 to 1.0 × 10−3 µM	2.95×10^{-6} µM	[47]

Graphene	AuNPs were able to hybridize, following the AuNPs-catalyzed silver deposition	DPV	DNA	2 x 10^{-4} µM to 0.5 µM	72 x 10^{-6} µM	[48]
Graphene	Fe_3O_4 nanoparticles	DPV	DNA	10 $^{-11}$ µM to 10^{-4} µM	2 x 10^{-12} µM	[49]
Graphene	oxide-yttria nanocomposite	Chronoamperometric	DNA	10 $^{-11}$ µM to 10^{-3} µM	5.95 x 10^{-12} µM	[50]
Glassy carbon	Graphene oxide, DVS	EIS	DNA	2.0 × 10^{-3} to 25 × 10^{-3} µM	7.6x10^{-2} µM	This work

2.4 Conclusion

DVS conjugation allowed us to build a label-free electrochemical biosensor for the detection of DNA using one of three detection modes. Iron (II/III) hexacyanide can be used as a probe for CV or EIS. Mb can be used as a probe for DPV. GCE/GO is an effective electrode material due to the large specific surface area for electron transfer and numerous active sites to bind with DVS. EIS and fluorescent microparticles demonstrate that unmodified DNA (GSurf) was successfully conjugated to the surface of GO through DVS. LC-MS data shows that the DVS/DNA bonds likely formed at sites on the nitrogenous bases. The major product of the reaction between a deoxyribo-mononucleotide (deoxythymidine) and DVS shows the appropriate m/z and a fragmentation pattern consistent with reaction at a site on thymine. While our illustrations show the DNA immobilized end-on to the surface of the electrode, DNA molecules were likely captured by diverse reactive sites.

Electrochemical methods can be used as a simple detector of reverse-complimentary nucleic acids. We use the model oligonucleotide GSurf* as a demonstration. The fabrication process

of the electrochemical sensor is relatively rapid, low-cost, and simple (i.e., compared to gold electrodes and thiol bonds). Our method for using DVS as a linker to covalent attachment of DNA to carbon nanomaterials is efficient, robust, and readily commercially available. Although our specific implementation did not reach the extreme limits of detection obtained elsewhere, the simplicity of this technique is significant. Additionally, this method does not require a modified probe nucleic acid; an unmodified oligonucleotide can be conjugated to the GO surface. This method could be used to detect any analyte that can hybridize, such as the amplification products from asymmetric PCR[51], strand displacement amplification[52], or rolling circle amplification[53] to achieve low detection limits.

2.5 Supplementary Information

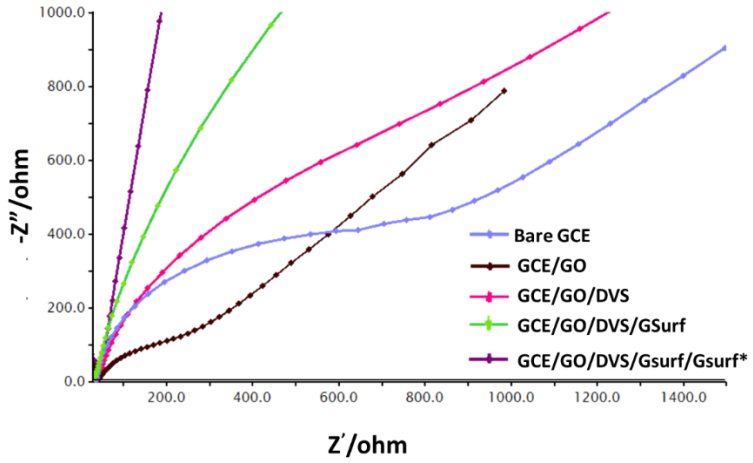

Figure S1. The Nyquist plots of the faradaic impedance spectra at different electrode bare GCE, GCE/ GO, GCE/GO/DVS, GO, GCE/GO/DVS/GSurf and GCE/GO/DVS/GSurf /GSurf*

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Chapter 3: Facile Fabrication of DNA Biosensors Based on Oxidized Carbon Black and Graphite Oxide

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3.1 Introduction

Biosensors can detect diverse analytes. We want to detect nucleic acids from pathogens. If our sensor is sensitive, we can detect contamination in food or drugs. Electrochemical biosensors have been demonstrated by many groups for this purpose. Electrochemical biosensors are inexpensive, sensitive, simple, and rapid [1–3]. Label-free, electrochemical DNA detection is especially attractive as it does not require chemically modified probes [4]. However, DNA probes for electrochemical detection must be conjugated to the electrode surface. This can be accomplished by covalent attachment to carbon or by a gold-thiol link. Most electrochemical methods use gold electrodes and thiol attachment chemistry. We demonstrate an alternative with several advantages [5,6]. We show that DNA can be conjugated to specific forms of carbon by well-known 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) coupling. The disadvantages of gold-thiol chemistry include a narrow

potential window and high cost. Additionally, the surface of bare gold must be pristine for good functionalization. This can lead to irreproducible results. This can be overcome with careful technique but represents a significant barrier to entry.

Carbon electrodes are an attractive alternative. Carbon has a wide potential window, high electron transfer, electrical conductivity, low background current, and is affordable [7]. Graphite oxide and carbon black oxide are widely used in electrochemical biosensors due to their high conductivity at room temperature, high surface area, thermal stability, and low cost [8–11]. These materials can be drop cast onto an existing conductive surface such as a glassy carbon rod. Glassy carbon is used for its corrosion resistance and homogenous surface [12]. Different methods have been studied to anchor DNA strands. Other labs have attached DNA by adsorption, entrapment in a polymer matrix, and electro grafting [13–15]. In this study, we show that DNA can be directly conjugated to conductive carbon particles. The particles can be formed into an electrode by drop-casting in Nafion. We demonstrate the effectiveness of this approach by detecting reverse-complementary DNA using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

3.2 Experimental Process

3.2.1 The Materials and Methods Section

The DNA oligonucleotides used in this paper were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). The sequences of the oligonucleotides were: Probe single-stranded DNA (ssDNA): /5AmMC6/TTGAGGAGGAGGAGGAGAGGCGGGTTGAGG and complementary double-stranded DNA (dsDNA): 5’-TCTCCTCCTCCTCCTCTTTTCTGAATAAGA-

3’. 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), was purchased from Sigma Aldrich Co. All other reagents were of analytical reagent grade. All of the solutions were prepared with Millipore deionized water.

3.2.2 Synthesis of Graphite Oxide and Carbon Black Oxide

Graphite oxide (GrO) and oxidized carbon black (CbO) were prepared from graphite powder and carbon black powder, respectively, by following Hammer’s method. Firstly, 0.5 g of $NaNO_3$ and 3 g of graphite powder were dissolved in 25 mL concentrated 98–99% sulfuric acid. Then the mixture was stirred for 3 h in an ice bath. We slowly added 3 g of $KMnO_4$, as a potent oxidizing agent, to the mixture at room temperature. The mixture was stirred for 12 h. The oxidation reaction was interrupted by the addition of 20 mL 30% H_2O_2 solution. Immediately after the addition of 30% hydrogen peroxide, the color of the mixture changed to pale yellow. Subsequently, 120 mL of purified water was added to the solution. The resultant product was repeatedly centrifuged with H_2O_2 to adjust the pH to four. The final product was placed in a 60°C oven for 12 h. Synthesis of CbO proceeded in the same manner [16,17].

3.2.3 Covalently Immobilization of DNA

Single-stranded DNA was covalently attached to the carboxyl group of CbO and GrO surfaces through EDC coupling. The glassy carbon electrode (GCE) was polished with 0.3 mm γ-Al_2O_3., then sonicated in deionized water for about 30 s and then rinsed with deionized water. For immobilization of the oligonucleotide, 5 mg oxidized graphite and carbon black were suspended in a 1 M MES (pH 4.5) containing 0.1 M EDC. Amine-modified ssDNA was added

to the suspension (1 µM final concentration) and vortexed for 2 h at room temperature. The suspension was then washed with PBS buffer (pH 7.4) by centrifugation and resuspension. Finally, 50 µL of ethanol, 25 µL 5% Nafion, and 50 µL DI water were added. The mixture was sonicated for 1 h. Then, a droplet of 2.5 µL of the Nafion-carbon-DNA suspension was applied to the surface of the GCE. For hybridization, 4 µL of the complementary oligonucleotide was mixed in 200 µL of 20 mM NaCl in PBS buffer solution (containing 137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM Phosphate Buffer pH 7.4) and applied to the ssDNA modified GrO/CbO. After 20 min, the surfaces were extensively washed for 20 min. Finally, the electrode was dried at room temperature for at least 3 h. Figure 15 shows the conjugation of an ssDNA onto the GrO surface and hybridization with a complementary target.

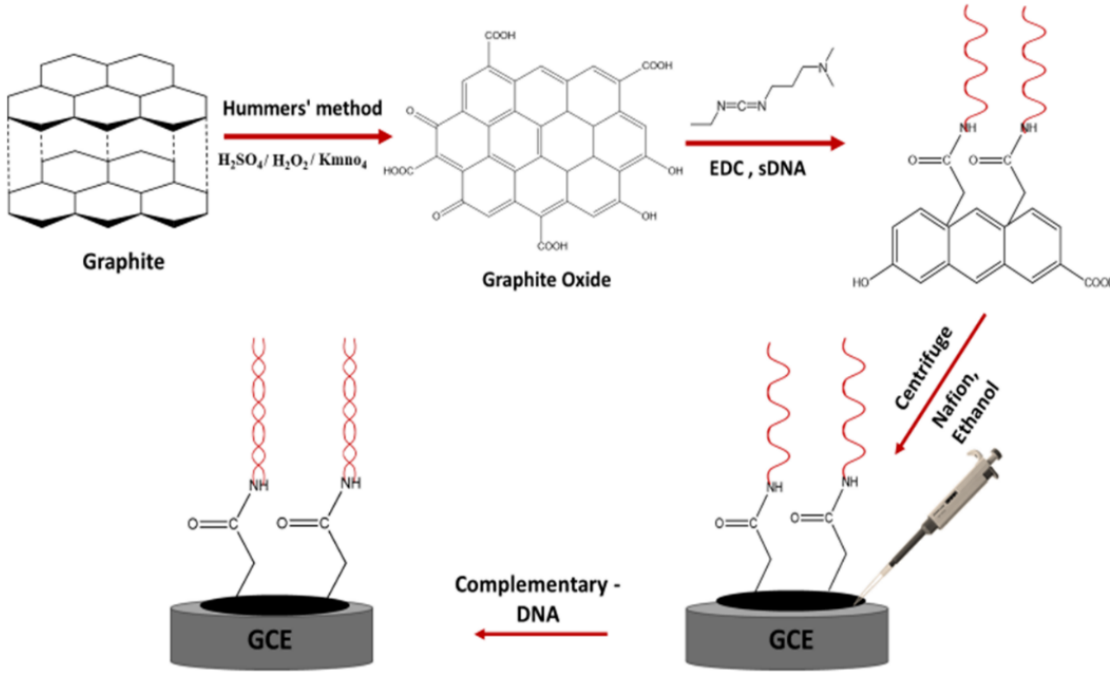

Figure 15. The immobilization of an ssDNA onto the graphite oxide (GrO) surface and hybridization with a complementary target. EDC: 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide; glassy carbon electrode

3.2.4 Electrochemical Detection

A conventional three-electrode system (Pine WaveDriver Galvanostat, Pine instruments, Durham, NC, USA) was used for electrochemical characterization with modified GrO/CbO coated glassy carbon as working electrode, saturated Ag/AgCl (3.5 M KCl) as a reference electrode, and Pt as a counter electrode in a 0.1 M KCl solution containing 6 mM of $K_3Fe(CN)_6$. All measurements were performed at room temperature. Cyclic voltammetry and electrochemical impedance spectroscopy of the redox probe were performed to detect reverse-complimentary DNA. Cyclic voltammetry measurements were carried out between −0.4 V and 0.6 V. The scan rate of these measurements was 100 mV s^{-1}. EIS was performed to monitor the whole procedure in the modification of the electrodes. Impedance measurements were carried out between 1 MHz and 1 Hz applying an AC amplitude of 25 mV. The electrolyte for the impedance measurement was 1 M KCl, containing 6 mM potassium ferricyanide.

3.3 Result and Discussion

Figure 16 presents the performance of the CbO and GrO electrodes conjugated to DNA. Single-stranded DNA (ssDNA) was immobilized on the surface through the formation of covalent amide bonds between the amino groups of the oligonucleotides and carboxyl groups on the GrO and CbO. In Figure 16 B, the anodic and cathodic current at 180 mV was the oxidation and reduction of iron cyanide in solution. The electron transfer (and, therefore, the current) was reduced when the electrode was conjugated to DNA (Figure 16 AII). The current was further reduced when complementary DNA was added (Figure 16 AIII). Figure 16 C shows

the performance of the CbO electrode conjugated to DNA. Conjugation CbO to DNA also caused a decrease in charge transfer and reduced current. We attributed the reductions in current to the electrostatic repulsion between DNA and ferricyanide. This increased after the addition of complementary DNA.

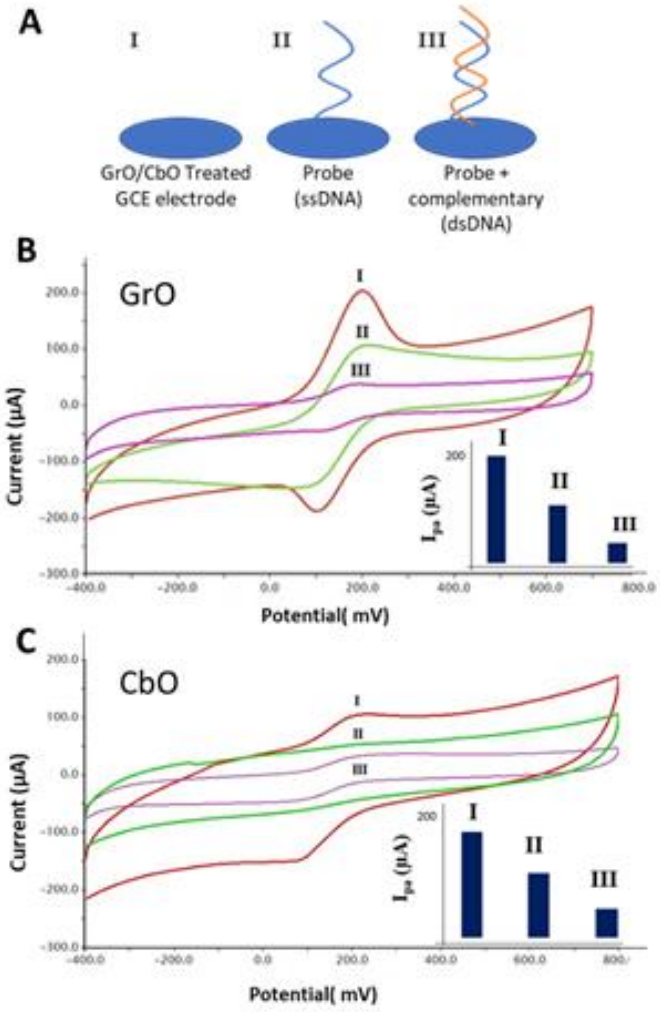

Figure 16. DNA conjugation detected with Cyclic Voltammetry. (**A**) The schematic shows (I) the no-DNA control, (II) single-stranded DNA (ssDNA)-coated electrode, and (III) the double-stranded DNA (dsDNA)-coated electrode. (**B**) Cyclic voltammograms of the GrO-modified electrodes recorded in 10 mM potassium ferricyanide [$K_3Fe(CN)_6$] in 1 M potassium nitrate at a scan rate of 0.1 Vs^{-1}, in the potential range between −0.4 to +0.7 V A. for the (I) GrO electrode, (II) ssDNA-GrO electrode, and (III) dsDNA-GrO electrode. (**C**) Equivalent cyclic voltammograms for (I) CbO electrode, (II) ssDNA-CbO electrode, and (III) dsDNA-CbO electrode. Insets show the baseline-corrected peak anodic current for each sample.

We interpreted the greater reduction in current on GrO (as compared to CbO) to two causes: lower resistance and more efficient functionalization. GrO showed higher efficiency charge transfer from GrO to ferricyanide. The current for the unmodified GrO was much higher than the unmodified CbO. High performance of charge transfer on GrO was due to the regular structure of graphite (which reduces resistance). GrO also showed better functionalization. GrO consists of graphene oxide sheets and displays oxygen-containing functional groups on the surface (alcohols, quinones, and carboxylic acids). As a consequence, GrO has a high potential to create a covalent bond between oxygen and amines [18–20].

Nyquist plots of the EIS spectra of CbO/GrO electrodes in a 1M KCl aqueous solution containing 6 mM [$K_3Fe(CN)_6$] are shown in Figure 17. The bare GrO/CbO electrodes had a small AC impedance, indicating that the electron transfer between the electrode and electrolyte was fast (Figure 17 B,C). The AC impedance increased after the GrO/CbO were conjugated to the DNA. This suggested more resistance to electron transfer between the electrode and the ferricyanide. The resistance was further increased when complementary DNA is added. EIS corroborated the CV results and indicated that the GrO had a lower resistance to electron transfer compared to the CbO electrode.

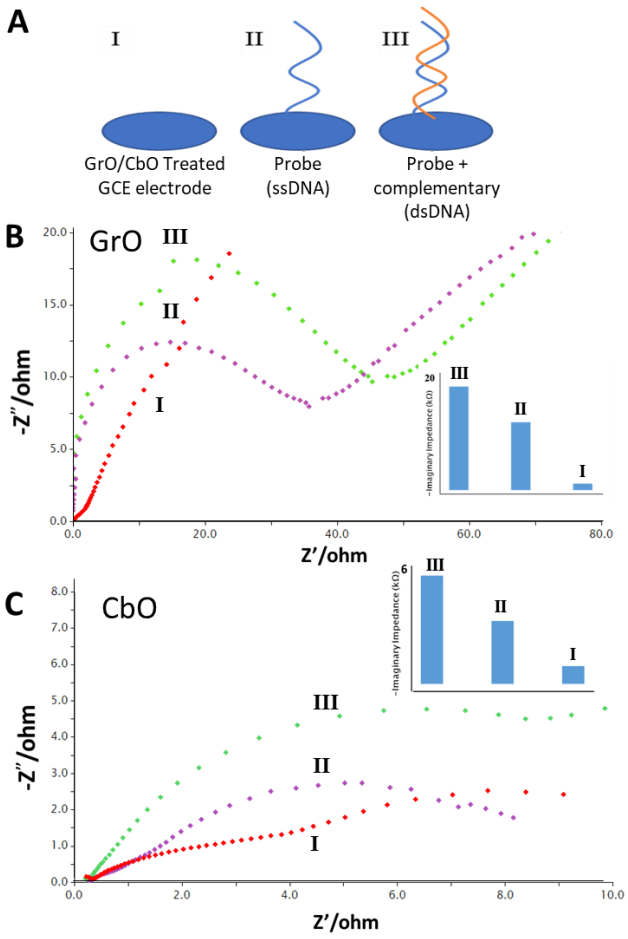

Figure 17. DNA conjugation detected with Electrochemical Impedance Spectroscopy (EIS). (**A**) A schematic shows (I) the no-DNA control, (II) ssDNA-coated electrode, and (III) dsDNA-coated electrode. (**B**) Nyquist plots of the EIS spectra recorded in 10 mM potassium ferricyanide [$K_3Fe(CN)_6$] in 1 M potassium nitrate at a frequency between 1 MHz and 1 Hz and an AC amplitude is 25 Mv for (I) GrO electrode, (II) ssDNA-GrO electrode, and (III) dsDNA-GrO electrode. (**C**) Equivalent Nyquist plots for the EIS spectrum of (I) CbO electrode, (II) ssDNA-CbO electrode, and (III) dsDNA-CbO electrode.

3.4 Conclusions

This study has introduced a method for conjugating amine-modified DNA to carbon black oxide and graphene oxide. We showed that graphene oxide and carbon black oxide bind DNA, and this prevents charge transfer to ferricyanide. This phenomenon allows for detecting reverse-complementary DNA. Reduced peak current was attributed to the complementary DNA. This is a label-free biosensor; it requires no indicators or labels on the DNA. We show

that graphite oxide is superior in performance to carbon black oxide. We attribute the higher performance to the ordered structure and lower resistance in graphite oxide compared to the more disordered, paracrystalline structure of carbon black [21]. This is a simple and rapid conjugation method for generating DNA sensors.

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Chapter 4. Conclusion

4.1 Conclusion

In our study first, we introduced an electrochemical method used as a simple detector of reverse-complimentary nucleic acids. We highlighted the properties of carbon nanomaterials as immobilization platforms. The fabrication process of the electrochemical sensor based on carbon structure such as graphene oxide is relatively simple, low-cost, and rapid (i.e., compared to gold electrodes and thiol bonds) [1–3]. We showed that the Immobilization of DNA through covalent bonds is the encouraging immobilization method for electrochemical sensor electrodes [4]. Because the covalent bonds between attachment groups on DNA (i.e., modified DNA) and different functional groups on electrode surfaces are strong.

In chapter 2 we discussed the design of an electrochemical biosensor for the hybridization of DNA. We immobilized unmodified, single-stranded (ss) DNA oligonucleotides on graphene oxide (GO) using divinyl sulfone (DVS). In this study, DVS used as a linker to covalent attachment of DNA to the surface of carbon materials. Reverse-complimentary DNA was detected by using multiple electrochemical methods. The results show that DNA can be successfully conjugated to conductive carbon for electrochemical sensors. Reverse-complimentary DNA was detected by using multiple electrochemical methods. The results show that DNA can be successfully conjugated to conductive carbon for electrochemical sensors. In chapter 3 we demonstrated the detection of reverse-complimentary DNA by using graphene oxide and carbon black oxide as a platform. Amine-modified DNA was immobilized on the surface of the modified electrode through 1-ethyl-3-(3-dimethyl aminopropyl)

carbodiimide (EDC) coupling. We detected reverse-complimentary DNA using Electrochemical Impedance Spectroscopy (EIS) and Cyclic Voltammetry (CV) in a ferricyanide solution. The change in current or impedance was measured. We showed the carbon structure can significantly enhance the sensitivity of the biosensor for the reverse-complementary DNA[5, 6].

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