Design, synthesis, and application of electroactive reporters for nucleic acid biosensing

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**DESIGN, SYNTHESIS, AND APPLICATION
OF ELECTROACTIVE REPORTERS
FOR NUCLEIC ACID BIOSENSING**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2008

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ELECTROACTIVE REPORTERS
FOR NUCLEIC ACID BIOSENSING**

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2008

Abstract

This thesis discusses the design, synthesis, and application of electroactive reporters for nucleic acid biosensing. The reporters are able to specifically recognize and sensitively report the hybridization of target nucleic acids to capture probes in an electrochemical biosensor. Each molecule contains a DNA intercalating moiety, 1,4,5,8-naphthalenetetracarboxylic diimide, as the recognition unit that binds to nucleic acid duplexes. Four classes of compounds were studied: symmetrical mono- and bis-intercalators linked to either Os or Ru metal complex, symmetrical mono-intercalators linked to EDOT, and an asymmetrical mono-intercalator linked to both Os complex and EDOT. A range of redox potentials was obtained by modifying the ligands of the metal complexes, while binding properties was enhanced by positive charge.

Three compounds were used in different detection schemes. In each scheme, direct detection was achieved by measuring the electroactivity of Os or Ru complex. Lower detection limits were obtained through various amplification strategies. The Os complex catalytically oxidizes ascorbic acid, leading to an amplified current signal in amperometry and a detection limit of 60 fM for oligonucleotide samples. The Ru complex catalytically oxidizes guanine bases, leading to an increase of peak current in cyclic voltammetry and a detection limit of 1.5 pM for TP53 cDNA. The EDOT moiety acts as ‘seeds’ that facilitates selective polymerization of poly(EDOT). Under controlled conditions, the amount of polymer deposited was positively related to target DNA concentrations as low as 20 pM.

In conclusion, the advantageous electrochemical and binding properties of the newly synthesized reporters qualify them for application in electrochemical detection platforms that can potentially be incorporated into point-of-care nucleic acid biosensors.

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List of Publications

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List of Abbreviations

AA	ascorbic acid
CV	cyclic voltammetry
CP	capture probe
DNA	deoxyribonucleic acid
ds-DNA	double-stranded DNA
ss-DNA	single-stranded DNA
$E_{1/2}$	half wave potential
EB	ethidium bromide
EDOT	3,4'-ethylenedioxythiophene
MLCT	metal to ligand charge transfer
NTCDI	1,4,5,8-naphthalenetetracarboxylic diimide
NOESY	nuclear overhauser enhancement spectroscopy
PIND	propyl imidazole NTCDI
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPy	polypyridine
SWV	Square wave voltammetry
SNP	Single nucleotide polymorphism
TE	Tris EDTA buffer
UV-Vis	Ultra violet-visible

1 Introduction

1.1 Background and motivation

Since Watson and Crick proposed the double helical structure of deoxyribonucleic acid (DNA) in 1953,¹⁻⁴ nucleic acid research has been an essential part of life science. The completion of the genome project in 2003 and the successful genetic sequencing of life-threatening viruses have provided an unparalleled opportunity to further our understanding of the role of genetic factors in human health. The challenge now lies in applying this genome-based knowledge to the prevention, diagnosis, and treatment of diseases.^{5, 6} Areas of specific interest are the accurate molecular classification of diseases, prediction and pre-symptomatic detection of these diseases, and prediction of drug response.⁷

In this regard, nucleic acid biosensors are among the most promising tools for molecular diagnostics today.⁸⁻¹¹ A biosensor is defined as a device that utilizes biochemical mechanisms to transform chemical information, i.e. the concentration of a specific analyte, into an analytically useful signal.¹² Since the development of the first biosensor by Clark,^{13, 14} this device has evolved and played an important part in healthcare. Aside from application in the diagnosis of genetic or infectious diseases,^{15, 16} nucleic acid biosensors are also useful in genetic profiling, environmental monitoring,¹⁷ detection of biowarfare agent,^{18, 19} and drug screening.²⁰

As for all sensors, an ideal nucleic acid biosensor should be sensitive, selective, and accurate. Given the increasing concern that fast-mutating viruses can cause infectious diseases that will lead into pandemics, there is a clear and immediate need for point-of-care detection that can be applied for large numbers of subjects. Diagnostic systems that

are low-cost, easy-to-use, portable, and miniaturizable—while maintaining the level of accuracy and sensitivity of laboratory diagnostics—are therefore highly desirable. The creation of such diagnostic systems is a direct motivation for this project.

Over the past decade, many important technological advances have provided us with the tools needed to achieve this goal. We have identified the electrochemical detection method as the most promising, and further focused our efforts on a series of electroactive molecules as reporters in electrochemical nucleic acid biosensors.

1.2 Objectives

The objective of this thesis was to design, synthesize, and use electroactive reporters for nucleic acid biosensing, with the focus on sensitive and selective detection schemes toward point of care diagnosis of diseases. This goal can be realized through four concrete steps. First, design electroactive reporters that are capable of recognizing the presence of target nucleic acid sequences and generating an amplified electrochemical signal. Second, synthesize, characterize, and study the relevant properties of these reporter molecules. Third, develop suitable detection strategies utilizing the reporters, taking into account the needs for point of care molecular diagnosis. And fourth, apply these detection platforms in actual electrochemical detection of target nucleic acids.

1.3 Scope

The term ‘electroactive reporters’ refers to a series of newly designed and synthesized molecules that comprise a DNA binding unit and one or more electroactive moieties. The characterization of these molecules focuses on the electrochemical and DNA binding properties, which are relevant for their application in nucleic acid biosensing.

The term ‘nucleic acid biosensors’ refers to electrochemical biosensors based on the use of DNA or its analogs as recognition elements and electroanalytical techniques for sequence-specific detection of nucleic acids. Most of the targets used are DNA oligonucleotides; however the detection of genomic DNA and messenger ribonucleic acid (mRNA) has also been achieved. The discussion in this thesis focuses on the ‘detection’ functionality of a biosensor, i.e. the generation and amplification of signal in the presence of target molecules. Sample preparation, microfluidics and miniaturization, multiplexing, and the incorporation of this detection platform into a working diagnostic device are beyond the scope of this thesis.

1.4 Organization of the thesis

Chapter one provides a brief overview of the motivation and objectives of the project. Chapter two surveys the literature and summarizes the state of the art of nucleic acid biosensing, especially through electrochemical transduction. A brief history and relevant findings about electroactive reporters and conducting polymers are presented to identify possible knowledge gaps to support the formulation of the problem.

Chapter three focuses on a series of novel electroactive compounds for use as reporters in a nucleic acid biosensor. Their molecular design, synthesis, and characterization are discussed and compared. Chapter four discusses the application of transition-metal linked reporter molecules for direct detection of nucleic acids. Chapter five highlights another redox reporter, which has a hybrid structure containing an Os complex and an 3,4-ethylenedioxythiophene (EDOT) monomer, and its use to amplify electrochemical signal through polymer growth. Each chapter consists of a brief introduction, result and discussion, as well as conclusions and experimental details.

2 Literature Survey

2.1 Nucleic acid biosensors

Nucleic acids are important detection targets for several reasons. The identification of infectious organisms, the quantification of gene expression, and genomic sequencing rely on the detection of nucleic acids. Nucleic acids can potentially yield more genetic information than classical serological markers, for example to distinguish between a virulent and an avirulent organism and between the vaccine and wild-type strains based on the pathogen's genotype.

Unlike enzymes and antibodies, nucleic acid probes are readily synthesized in high yield. And since they are more stable than protein markers, nucleic acid samples allow a longer time frame for bioanalysis. Short, synthetic oligonucleotides are ideal for the construction of a biosensor recognition layer because base-pairing interactions between complementary sequences are both specific (sequence selective) and robust. The recognition event, hybridization, is reversible since no covalent bonds are formed. The oligonucleotide layer can thus be regenerated by alternate hybridization and denaturation, opening up possibilities for multiple-use sensors. In short, nucleic acid tests provide an advantageous alternative diagnostic approach when immunochemical tests are ineffective or not practical.

2.1.1 DNA microarrays

Prior to the introduction of biosensors, nucleic acid detection was carried out in membrane blots and gel electrophoresis. The DNA sequencing technique, developed in 1977,^{21, 22} demonstrated how hybridization of complementary oligonucleotide strands

could be used as a basis for nucleic acid biosensing. The invention of the polymerase chain reaction (PCR) allowed amplification and detection of very small quantities of DNA. Hybridization was initially observed on autoradiographic images with radioisotope labels, until Smith and coworkers introduced an optical detection technique using fluorescent tags in 1986.²³

Coupling the advances in biochemistry and semiconductor technologies, DNA microarrays (also called gene chips or biochips) were introduced in the 1990s. It has since become the gold standard of DNA analysis.²⁴ A microarray is an array of individual sensors, each representing a single gene and comprising a ‘receptor’—where the actual sensing chemistry takes place, and a ‘transducer’—where signals are processed into user-readable information.

Schena and coworkers first demonstrated the use of a DNA microarray for the simultaneous measurement of differential gene expression.²⁵ Others have since used microarrays to track the up- and down-regulation of genes under various environmental conditions, under regulator control, or in specific tissue samples. Microarrays were also used to study the genetic diversity of pathogens in different stages of their life cycle.^{26, 27}

Routine clinical DNA analyses today rely on DNA microarrays for identification of single nucleotide polymorphisms (SNPs) that lead to an increased susceptibility to genetically linked diseases. The first commercial microarrays was introduced by Affymetrix and has been used to identify SNPs in genes such as p53 (a tumor suppressor) and BRCA1 and BRCA2 (related to breast cancer).²⁸ Due to the relatively short sequences detected in each spot, multiple replicates of each gene are needed to draw a reliable conclusion in genetic profiling, amounting to a requirement of extremely high array density.

Figure 2-1: Procedure for nucleic acid analysis in a microarray.²⁹

The general procedure of microarray analysis is as follows: templates for genes of interest are amplified by PCR and isolated in the form of single-stranded DNA (ss-DNA). Following purification and quality control, aliquots (~5 nL) are printed on coated glass microscope slides. Target DNA are isolated from the sample, fluorescently labeled with either Cy3 or Cy5 fluorophores, and then allowed to hybridize—under stringent conditions—to the probes on the array. The target is usually a product of reverse transcription of total RNA samples that has been amplified through PCR.

2.1.2 State of the art

Regardless of the sensor density, the molecular basis for most of the current nucleic acid assays is the detection of specific sequences by Watson–Crick hybridization. Such biosensors are based on the same basic principle: a single-stranded “probe” DNA

is immobilized onto a surface to form the recognition layer, whereupon the base-pairing interactions recruit the complementary “target” DNA from a sample solution to the surface. This configuration offers convenient formats for detection of nucleic acid complexes since unreacted molecules can be easily washed away after hybridization, leaving only those which are specifically bound.

The duplex formation can be detected following the association of an appropriate hybridization indicator or through other changes arising from the binding event. Various strategies are used to transduce this hybridization event into a measurable signal. Two of the most popular and effective methods are: labeling the target with reporter molecules and exploiting the differential binding of these reporters to double-stranded DNA (ds-DNA) relative to ss-DNA, or using label-free techniques that rely on the intrinsic change of properties in the presence of target DNA. The state of the art for DNA chips has been reviewed.⁸

Numerous types of labels/ markers/ tags and their corresponding transduction and amplification modes have been proposed. Two main groups of labels have garnered the most attention: optically- or electrochemically-active organic compounds^{10, 30} and nanoparticles or other nanostructures.³¹⁻³⁴ DNA binders and Au and semiconductor nanoparticles^{35, 36} in particular have received immense attention due to their potential for amplification and compatibility with multiple transduction and amplification methods.

Nucleic acid biosensors based on surface plasmon resonance,³⁷⁻³⁹ quartz crystal microbalance,⁴⁰ and field effect transistor^{41, 42} have been extensively studied primarily for their simplicity. Other less established label-free techniques based on capacitive microfabricated cantilever⁴³ and magnetoresistive⁴⁴ are also gaining momentum.

To improve the sensitivity of existing transduction methods, numerous groups are developing novel approaches to nucleic acid probe and target preparation, probe labeling,

and signal read-out. Examples of such studies are the use of silicon nanowires and carbon nanotubes as electrodes,^{45, 46} PNA as capture probes,⁴⁶⁻⁴⁸ magnetic beads for sample separation and pre-concentration,⁴⁹⁻⁵¹ and fiber optics for optical signal transmission.^{52, 53}

The variety of transduction methods, labels, and amplification techniques create a vast number of systems for nucleic acid detection. Among all the possibilities, an improvement in sensitivity and selectivity often comes with the drawback of increasing complexity of the assay. To illustrate this point, two very different detection schemes are compared as illustrated in Figure 2-2.

Figure 2-2: Comparison between (A) a simple but insensitive visual detection based on light scattering⁵⁴ and (B) a very sensitive yet complicated detection using nanobarcodes.⁵⁵ Reprinted with permission from Chem. Rev. 2005, 105, 1547-1562. Copyright 2005 American Chemical Society.

One of the simplest optical detection methods is based on the light scattering properties upon aggregation of Au nanoparticles as a result of hybridization.⁵⁶ It allows visual detection of target DNA in the form of changing colors; however a large

concentration of DNA is needed. On the other extreme, a PCR-like sensitivity has been achieved using nanoscale ‘barcode’ as labels.⁵⁵ To achieve the detection limit in the zeptomolar range, numerous nucleic acid strands, micro- and nanoparticles are used in an assay that involves multiple steps of washing, hybridization, and separation.

An emerging field for DNA analysis is the infectious disease detection¹⁶ targeting a specific fragment of a gene. For these applications, a much lower array density is needed and a myriad of possible sensing and transduction mechanisms can be applied to this format. The ‘lab-on-a-chip’ concept is also an active research area. This configuration integrates multiple processes, including sample collection and pretreatment with the DNA extraction, amplification, hybridization and detection on a microfluidic platform. Such miniaturization of analytical instrumentation is truly needed to enable point-of-care detection. However, the description of these methods is beyond the scope of this thesis.

2.2 Electrochemical nucleic acid biosensors

Optical biosensors are arguably the most widely used type of biosensors in clinical setting today,⁵⁷ where detection of specific nucleic acid sequences are based on labeling the sample nucleic acids with a fluorophore. While fluorescence-based detection technologies have shown tremendous utility, they suffer from the drawbacks of labor-intensive sample preparation, high cost, and complex and bulky fluorescence detection instrumentation.

Electrochemical techniques, on the other hand, are gaining momentum as the method of choice for nucleic acid detection. Since electrochemical reactions directly generate electronic signal, there is no need for expensive signal transduction equipment. The electrode fabrication process is highly compatible with advanced microfabrication technology, enabling miniaturization and fabrication of high density sensor arrays. In

addition, signal amplification through electrocatalysis can potentially increase the sensitivity of the biosensor to extraordinarily high levels. Compared to the current modes of detection, electrochemical detection offers simplicity, miniaturization, and lower cost toward point-of-care application.

The downsides associated with this transduction method are the possible electromagnetic interference at very low signal range, and the limitation of in-vivo application due to the need for electrical perturbations to generate signal.

A vast number of strategies for electrochemical nucleic acid detection have been reported and reviewed.^{10, 58-62} As with other nucleic acid detection schemes, the presence of target nucleic acids is signified by its hybridization to the capture probes. These hybridization detection strategies can be broadly classified into label-free detection and detection based on the use of electroactive reporters. In label-free detection, the signal originates from the redox activities of the nucleic acid itself or from the intrinsic change of the electrical properties of the recognition layer, e.g. impedance, conductivity, and capacitance. Alternatively, electrochemical detection is based on the differences in the electrochemical behavior of the redox reporters following the hybridization event.

2.2.1 Brief history

The concept of electrochemical analysis of nucleic acids was first reported in 1957.^{63, 64} Adenine, guanine, and cytosine are reducible on mercury electrodes while guanine and adenine are oxidizable on graphite electrodes.⁶⁵ Initial studies on nucleic acid electrochemistry were focused on utilizing the changes in electrochemical signals to study DNA structural transition and local conformational changes. Cathodic signals from the

reduction of adenine and cytosine,⁶⁶ as well as anodic signals from oxidation of guanine⁶⁷,⁶⁸ observed through polarography have been used for this purpose.

Compared to ss-DNA, ds-DNA has a significantly lower electrochemical activity. Due to its rigidity, ds-DNA has lower adsorbability on the electrode surface, thus reducing susceptibility to oxidation and reduction. As a result, anodic current of guanine is lowered⁶⁷ and oxidation potential is higher⁶⁸ in ds-DNA. Cathodic currents of adenine and cytosine are also significantly lowered due to the involvement of reductions sites in hydrogen bonding.⁶⁶ Os tetroxide was used as an electroactive probe to study the DNA conformation due to its reducibility in polarography and its selective binding to ss-DNA.⁶⁹ Sensitive differentiation of ss-DNA from a solution of ds-DNA was achieved,⁷⁰ with even lower detection limit detectable with adsorptive stripping analysis combined with a.c. or cyclic voltammetry.

2.2.2 Label-free detection

In label-free detection, the signal is derived from the intrinsic redox activities of the nucleic acids or from the changes in the electrochemical properties of the interface, e.g. impedance, conductivity, and capacitance. Label-free detection strategies are attractive because they minimize the need for added reagents and potentially produce a reusable sensor that can be applied to continuous monitoring devices.⁷¹

Based on the intrinsic redox signal of nucleic acids

Sequence specific detection of nucleic acids utilizes the intrinsic oxidation signal of guanine base in the target.⁷¹⁻⁷⁶ To eliminate interferences, guanine bases in the capture probes were substituted with inosine, an electrochemically inactive base that is still

capable of binding to cytosine. Wang and coworkers successfully detected a 29-mer *Cryptosporidium parvum* DNA by observing guanine oxidation peak through chronopotentiometry on carbon paste electrode with detection limit around 120 ng/mL (~ 6.2 nM).⁷²

Figure 2-3: Label-free hybridization detection scheme based on the intrinsic redox signal of guanine oxidation mediated by an electrocatalytic transition metal complex.⁷⁷

Two avenues have been successfully examined for enhancing the guanine signal, and hence hybridization response. Thorp's group pioneered DNA detection based on the electrocatalytic effect of $\text{Ru}(\text{bpy})_3^{2+}$ to amplify the intrinsic signal of guanine oxidation, as illustrated in Figure 2-3.⁷⁷ This biosensor was used for the determination of PCR products of genomic DNAs and RNAs from viruses as well as genetic abnormalities that cause diseases.^{78, 79} Detection limits as low as 40 amol (400 fM) were achieved using this strategy.^{80, 81}

However, since the compounds are unable to interact selectively with ds-DNA, the assay suffered from high background signal and lack of sensitivity. The analytical signal is superimposed onto an inherently large background current due to direct oxidation of the catalyst and the catalytic oxidation between oligonucleotide CP and the catalyst. Most of the catalytic oxidation current can be eliminated by using peptide nucleic acids as capture probes, but little can be done to reduce the direct oxidation of the

catalyst. A similar strategy has been further explored by other groups,^{82, 83} using different mediators, e.g. methylene blue⁸⁴ and alkylating agent 4, 4 dihydroxy chalcone.⁸⁵ Higher sensitivity was achieved mainly due to the adoption of the solid state approach, allowing removal of the labels after a brief incubation period.

Another successful amplification method entails the adsorptive-accumulation and separation of the hybrid using magnetic beads, followed by potentiometric stripping detection.⁸⁶ A lower detection limit of 40 fmol (250 pg) was achieved with this method through adsorptive chronopotentiometric stripping measurements of the free purine nucleobases in the presence of copper ions.⁴⁹ The tradeoff for the simplicity of label-free detection is the limited sensitivity due to dependence on the amount of guanine in the target. Substituting inosine in the probe is expensive and may reduce the stability of the duplex and the specificity of the hybridization.

Based on the properties of the functional monolayer on electrode surface

Nucleic acid hybridization can also be detected using electrochemical impedance spectroscopy to measure the change of capacitance and electron transfer properties at the electrode/electrolyte interface upon hybridization.^{87,88} A notable example is the application of impedance spectroscopy with enzyme amplification. Hybridization of target DNA recruits alkaline phosphatase enzyme to the electrode surface, initiating a cascade that eventually results in the precipitation of an insoluble indigo derivative on the electrode surface. Using the Faraday impedance spectroscopy to monitor the change of capacitance and electron transfer resistance of the electrode, a single-base mutant of Tay-Sachs disorder could be detected with lower detection limit of 10 pM.⁸⁹

Other detection methods

In recent years several novel avenues of label-free detection have been explored. Mirsky and coworkers exploited the effect of electrode surface modification on electron transfer between electrodes and electroactive species in solution to detect hybridization.⁹⁰ A ferri/ferrocyanide system displays electrochemical activity even at low ionic strength on an electrode coated with thiolated ss-DNA. In contrast, the electrocatalytic signal is significantly attenuated on an electrode coated with duplex DNA that has been hybridized in solution prior to deposition, as observed in cyclic differential pulse voltammetry. This method benefits from its simplicity and the ease of solution-phase hybridization; however the current detection limit of 1 μM needs to be significantly improved.

Another label-free detection method exploits the change in rigidity of oligonucleotide upon hybridization, as depicted in Figure 2-4.^{91, 92} Prior to hybridization, CP molecules are surface confined and ‘shield’ the electrode surface from any ionic activity in the bulk solution. This shielding effect is lost upon hybridization, allowing detection through the voltammetric signal from the ionic activity in the same solution.

Figure 2-4: Label-free hybridization detection scheme based on the change of the nucleic acids' rigidity.⁹²

DNA-mediated charge transfer electrochemistry

Ever since Elley and coworkers proposed the possibility of electrical conduction through DNA,⁹³ this topic has been intensely debated. Early theoretical models attributed the long-range charge transfer to tunneling or electron hopping along the coupled π orbitals of the base pairs. A significant amount of effort was dedicated toward nucleic acid detection using DNA as a mediator of long-range charge transfer. The detection of DNA mismatch or damaged DNA was proposed based on the sensitivity of charge transfer to stacking.⁹⁴⁻⁹⁶

Despite extensive efforts, however, the experimental results were contradictory with the measured resistivity of DNA molecules ranging from 10^{-4} to $10^8 \Omega\text{cm}$, covering the behavior of insulators, wide band-gap semiconductors, and even ohmic to metallic conductors. The emerging consensus is that DNA in itself is an insulator.⁹⁷ Although the molecule can transport electrons over a length of a few base pairs, allowing it to deflect oxidative damage away from important sections,⁹⁸ it fails to conduct over longer distances.

2.2.3 Detection using redox indicators

There are two main approaches based on the use of redox indicators: one involving a pre-labeling step to covalently attach a label to the target DNA or to a second oligonucleotide strand; and another involving a direct binding of reporter molecules to hybridized DNA through specific interactions such as groove binding and intercalation. Both linear-scan or square-wave voltammetric modes⁹⁹ and constant-current chronopotentiometry¹⁰⁰ can be used to detect the association of these redox indicators with the nucleic acid duplex.

Detection with pre-labeling of target DNA

Aside from being detectable at very low concentrations using a simple analytical procedure and simple instrumentation, nucleic acid hybridization labels should ideally have the following properties:

- Easy to attach to nucleic acids using a simple and reproducible labeling procedure
- Stable under nucleic acid hybridization conditions, i.e. high temperature
- Nonobstructive on the nucleic acid hybridization, in solution and in solid phase
- Stable during storage, providing longer shelf-life for commercial hybridization assay kits

In the pre-labeling step, labels are covalently attached either to the target DNA or to a second oligonucleotide strand called the reporter probe, as illustrated in Figure 2-5. When a reporter probe is used, a ‘sandwich’ of CP-target-reporter probe is formed hence the name of sandwich assay.¹⁰¹

Figure 2-5: Comparison between hybridization detection schemes with (A) labeled target and (B) labeled reporter probe (sandwich assay). — CP, — target, and — reporter probe.

Enzymes such as horseradish peroxidase and alkaline phosphatases,¹⁰² glucose oxidase¹⁰³ have been used to label target DNA. Heller and coworkers added a redox

polymer as a mediator to amplify the signal from enzyme labels.¹⁰⁴ Gao and coworkers proposed a different configuration where the electroactive polymer mediator is selectively recruited to the electrode surface through electrostatic interactions with the enzyme labels, as depicted in Figure 2-6. As a result, the background current and detection limits were successfully lowered.¹⁰⁵

Figure 2-6: Hybridization detection scheme using enzyme-labeled reporter probe and electroactive polymer as mediator.¹⁰⁵ Reprinted with permission from *Anal. Chem.* 2004, 76, 1611-1617. Copyright 2004 American Chemical Society.

Figure 2-7: Sandwich assay using nanoparticle labels for simultaneous electrochemical detection of multiple DNA sequences.¹⁰⁶

For Au nanoparticle labels, Wang and coworkers proposed an amplification strategy through catalytic deposition of silver on Au-nanoparticle labels followed by electrochemical stripping of the resulting silver.¹⁰⁷ The same group also demonstrated a

simultaneous detection of multiple target sequences through the use of semiconductor nanoparticles with different oxidation potentials, as depicted in Figure 2-7.^{106, 108}

Figure 2-8: Hybridization detection scheme based on the conformational change of a pre-labeled capture probe.¹⁰⁹

Heeger and coworkers utilized a pre-labeling strategy similar to the one used for optical molecular beacons.¹⁰⁹ This strategy involves ferrocene-labeled, stem-loop structured DNA strands that originally form a surface-confined self assembled structure on the electrode surface. Hybridization induces a large conformational change that significantly increases the electron-transfer tunneling distance between the electrode and the ferrocene label, causing a decrease in redox signal. This change is measured by cyclic voltammetry, resulting in a detection limit as low as 10 pM.

Ihara and coworkers proposed a sandwich assay with ferrocene label on the reporter probe.^{110, 111} An extension to the sandwich assay has been applied in a commercial biosensor called eSensor™. A reporter probe was synthesized, incorporating a ferrocenyl derivative in the oligonucleotide strand. In combination with the alternating current voltammetry, the detection of a single-base mismatch was achieved.¹¹²

A major drawback for the pre-labeling method is inconvenience and cost of the pre-labeling step and the reduced electrochemical activity of the labels attached at the end

of a long DNA segment. For sandwich assay, there is an added complication of finding two probe sequences for each target.

Direct detection without pre-labeling

The redox indicators in direct detection schemes are able to differentiate ss- and ds-DNA through specific interactions such as intercalation and groove binding; hence negating the pre-labeling step. The detection of hybridization is based on the electrochemical signal of the reporters recruited to the electrode surface in the presence of complementary target DNA. These reporters should ideally possess a well-defined, low-potential, voltammetric response.

Although the idea of DNA binding has been around since the 1940s,¹¹³ the binding event has been mostly studied optically.¹¹⁴ Bard and coworkers first demonstrated that the interaction DNA and binders can be studied electrochemically,¹¹⁵ inspiring the use of electroactive DNA binders for sequence-specific detection of nucleic acids.

Figure 2-9: Direct detection of nucleic acids using electroactive reporters (without the pre-labeling step). — CP, — target, — intercalating reporter, and — groove binding reporter.

Milan and Mikkelsen first proposed the idea of using a electroactive indicator—tris(1,10-phenanthroline)cobalt(III) perchlorate or $\text{Co}(\text{phen})_3^{3+}$ —to signify hybridization.⁹⁹ The concentration of target DNA was correlated to the characteristic redox signal of the cobalt complex. Upon hybridization of the target, the modified electrode was immersed in a solution $\text{Co}(\text{phen})_3^{3+}$ to allow binding. The voltammetric analysis was subsequently carried out in the same solution. This is possible because voltammetric peak currents depend on diffusion coefficients and the DNA-bound reporters diffuse much more slowly than free reporters in solution.¹¹⁵

Since then, nucleic acid biosensors based on voltammetric detection of electroactive organic^{96,116-124} or inorganic^{96,99,100, 125-131} indicators interacting preferentially with ds-DNA have been previously reported and reviewed,¹³² with selected examples summarized in Table 2-1.

The organic compounds used as reporters in electrochemical DNA detection bind to ds-DNA either through groove binding or intercalation. These heteroaromatic compounds have been widely used as DNA stains since the 1940s, and still form an important group of cancer drugs today. Their binding properties have been well studied using multiple methods including X-ray crystallography.

The inorganic compounds are mainly made up of transition metal complexes, with $\text{Co}(\text{bpy})_3^{3+}$ being the first electroactive reporter used for sequence specific nucleic acid detection. The cobalt complex, along with several others used in early DNA assays binds through electrostatic interaction. In recent years, metallointercalators—metal complexes that bind through intercalation to ds-DNA—have garnered attention due to their more selective binding.

Table 2-1: Electroactive reporters used in direct detection of nucleic acids

Reporter	DNA binding mode	Electrode	Analysis technique*	Target	Detect ion limit	Ref
Co(bpy) ₃ ³⁺	Electro-static binding	Carbon paste	CV	cystic fibrosis Delta F508 sequence	1.8 fmol	127
Co(phen) ₃ ³⁺		Carbon paste	CP	HIV virus	4 nM	100
Ferrocenyl complex		Gold	ACV	SNP		112
Methylene blue	Inter-calation	Carbon paste	DPV, CV	Hepatitis B virus		129
Hoechst 33258	Minor groove binding	Gold	DPV	oncogene v-myc in pVM623	0.1 pg/mL	116
Dauno-mycin	Inter-calation	Screen printed	LV	cDNA of yAL(3) gene	30 pg/mL	120
Disulfonic acid anthrax-quinone	Inter-calation	Gold	CV			133
Ferrocenyl naphthalene diimide	Inter-calation	Gold	DPV	yeast chlonie transport gene	Sub-pico mole	134

* CV = cyclic voltammetry, DPV = differential pulse voltammetry, LV = linear voltammetry, ACV = alternating current voltammetry.

Although initially studied for their cationic and chemiluminescence properties,¹³⁵ it is the catalytic nature of metallointercalators that makes them ideal candidates as

electrochemical reporters. These compounds exhibit multi-electron transfers that are the sum of the oxidation state changes in the metal center plus redox changes in the ligand. In addition, the enhanced redox capacity allows the complexes to act as "electron reservoirs" and facilitate multi-electron redox catalysis to amplify the detection signal and hence increase sensitivity.

Figure 2-10: Examples of metallobintercalators: (A) $[\text{Pt}(\text{tpy})(\text{SCH}_2\text{CH}_2\text{OH})]^+$, the first known metallobintercalator,¹³⁶ (B) $[\text{Rh}(\text{phi})(\text{Me}_2\text{trien})]^{3+}$, the only metallobintercalator with known DNA-bound X-ray crystal structure, and (C) $[\text{Rh}(\text{phi})(\text{bpy})_2]^{3+}$ developed by Barton,¹³¹ (bpy= bipyridine, tpy= terpyridine, phi=phenanthrene quinone diimine).

One of the earliest metal complexes known to bind to DNA by intercalation is the square planar Pt(II) complex containing terpyridine unit.¹³⁶ Barton and coworkers have designed and studied other metallobintercalators made of Rh, Ru, and Os complexes.¹³¹ The octagonal coordination of metal, as depicted in Figure 2-10, means their ligands are most likely to be bidentate, large, and planar. Certain ligands may then be able to insert in between DNA base pairs (intercalate), with the metal center positioned at either grooves of the DNA backbone. As the ligand size increases beyond the suitable limit for intercalation, however, the use of metal complexes is directed toward detection of DNA mismatch by binding at defective sites with larger gaps (Figure 2-10 C).

Unlike organic DNA binders, the binding of metallointercalators to ds-DNA is harder to predict, with $[\text{Rh}(\text{phi})(\text{Me}_2\text{trien})]^{3+}$ (Figure 2-10 B) being the only compound for which the DNA binding has been structurally characterized by X-ray crystallography.¹³⁷ Combining the more ideal binding properties of organic intercalators and the catalytic and cationic properties of a transition metal complex would thus result in a superior electroactive reporter.

Takenaka and coworkers reported an electrochemical detection scheme using a redox reporter that comprises a ferrocene-labeled naphthalene diimide intercalating unit.¹³⁴ The high binding constant of the intercalating unit allows the reporter to form a more stable complex with ds-DNA, while the electrocatalytic nature of ferrocene enabled signal amplification for sensitive detection.

This design and application of redox reporters, combining a well-known aromatic intercalating unit with electrocatalytic transition metal complex, will be further explored in Chapter 4.

2.2.4 Construction

Electrode preparation

Carbon and Au electrodes have been most widely used for electrochemical nucleic acid biosensors.¹³⁸ Carbon-based electrodes, used in various forms including paste,¹⁰⁰ pencil lead,¹³⁹ carbon fibers, and screen printed electrodes,¹⁴⁰ are relatively easy to prepare and can be regenerated.¹³⁹

Gold electrodes are usually in the form of a disk sealed in a plastic support or in disposable sheets and arrayed patterns for commercial products. The pre-treatment of the electrode surface before derivatization and immobilization of the recognition layer is

highly crucial. The surface preparation starts with mechanical polishing with alumina slurry, followed by treatment in a highly oxidative or basic condition, e.g. oxygen plasma, piranha solution, or other strong oxidants. Before use, the electrode surface is regenerated by potential cycling in dilute sulfuric acid until the desired electrochemical profile is achieved.¹¹⁶

Probe immobilization

The recognition layer on an electrochemical nucleic acid biosensor is most commonly formed by an array of oligonucleotide strands matching the sequence of interest. Aside from the commonly used oligonucleotides, DNA dendrimers¹⁴¹ and PNA were explored as capture probes. PNA is a DNA analog with peptide backbone that can form Watson Crick duplex with DNA.¹⁴²⁻¹⁴⁵ It offers certain advantages as a CP, mainly attributed to the neutrality of its backbone, leading to a more stable and specific binding.¹⁴⁶ Furthermore, a mismatch in PNA/DNA duplexes is much more destabilizing than one in DNA/DNA duplexes (with a lowering of the T_m by 15 °C versus 11 °C, respectively). Such mismatch discrimination is of particular importance in the detection of disease-related mutations.

For carbon electrodes, one of the simplest immobilization techniques is the adsorptive accumulation, where the negatively charged capture probes are recruited to the anodically pretreated electrodes.^{147, 148} Immobilization through the attachment of biotinylated probes to avidin-coated surfaces,¹⁴⁰ carbodiimide covalent binding to activated surfaces,¹²⁷ and attachment to polymer-coated surfaces¹⁰⁴ have also been achieved.

The quality of the recognition layer, i.e. the reactivity, orientation, accessibility, and stability of the surface-confined probes, determines its efficiency in capturing the

target. Probe immobilization through random covalent binding of chemically modified oligonucleotides on to the electrode surface is therefore not recommended.¹⁴⁹

Figure 2-11: Two self-assembly strategies for CP immobilization: (A) self assembly of functionalized SAM followed by covalent attachment of CP via end group reactions, and (B) direct self assembly of thiol-functionalized CP on the electrode surface. ● thiol, ● hydroxyl, amino, or carboxy group, and ● amide

With the successful characterization of self assembly of sulfur-containing molecules on a variety of metals,^{150, 151} self assembly has been preferred as an elegant method for immobilizing small molecules on Au.¹⁵² Two immobilization strategies are possible, as depicted in Figure 2-11. In the indirect method, hydroxyl-, amino-, and carboxy-terminated thiol can be coupled to oligonucleotide through covalent carbodiimide coupling.^{120, 153} Alternatively, the recognition layer can be directly formed from self assembly of alkanethiol- or phosphorothioester-functionalized capture probes.^{89, 111} Quantization of immobilized DNA can be achieved either electrochemically¹²⁵ or through quartz crystal microbalance.¹⁵⁴

A second monolayer of thiol is often added to control the CP orientation, as depicted in Figure 2-12, and at the same time protect the electrode surface from

nonspecific adsorption of target or labels.¹⁵⁵⁻¹⁵⁷ Obviously, this surface passivation layer has to be stable under the electrochemical conditions required for the assay.¹⁵⁸

Figure 2-12: Surface passivation by self-assembly of thiol monolayer to improve the quality and robustness of the recognition layer.⁵⁹

Hybridization and washing step

The hybridization between CP and target at the transducer-solution interface is the heart of all nucleic acid detection schemes. The hybridization process is controlled by numerous experimental variables, including salt concentration, temperature, viscosity, the presence of accelerating agents, contacting time, base composition (G + C, %), and length of probe sequence.¹⁵⁹ Careful control of the hybridization event is thus required.

While crucial for identification of many genetic diseases, many nucleic acid biosensors are not capable of selectively detecting a point mutation. Considering that hybridized strands with mismatched bases have reduced stability (depending on the number and location of mismatches), they can be discriminated by controlling the stringency of hybridization, e.g. by using elevated temperatures. Control of the hybridization time can be used for tuning the linear dynamic range, with shorter time offering an extended range at the cost of lower sensitivity.¹⁶⁰ Detection limits ranging from the nanomolar to the picomolar concentration range can thus be achieved in connection to 5 and 60 minutes hybridization times, respectively.

2.2.5 Electroanalytical techniques

Table 2-2: Nomenclature of the methodology of electrochemistry

Method	Controlled variable	Measured variable
Potentiometry	$i = 0$	E
Controlled potential		
Polarography (DME)	E	i
Voltammetry	E	i
Linear sweep	$E(t)$	i
Cyclic sweep	$E(t)$	i
Rotating disk	E, ω	i
Pulse	$\Delta E(t)$	Δi
Chronoamperometry	E -step	$i(t)$
Chronocoulometry	E -step	$\int i dt$
Controlled current		
Chronopotentiometry	i	$E(t)$
Galvanostatic	i	$E(t = 0)$
Coulometric titrations	i	$(it) / F$
Conductivity	V (AC)	i (AC)

Amperometry measures the current resulting from the electrochemical oxidation or reduction of an electroactive species at a constant potential. Pt, Au or C- based working electrodes are well suited for this technique, with a reference electrode that may also serve as the auxiliary electrode if currents are low (from 10^{-9} to 10^{-6} A). The

amperometric current is directly correlated to the bulk concentration of the electroactive species or its production or consumption rate within the adjacent biocatalytic layer. As biocatalytic reaction rates are often chosen to be first order dependent on the bulk analyte concentration, steady-state amperometric currents are usually proportional to the bulk analyte concentration. Amperometric electrodes measure current as a function of concentration. The minimum slope (response) of the minimum graph is 100 nA/decade of concentration.

Electrochemical stripping analyses involve a preconcentration of the analyte (or its salt or derivative) onto the working electrode, prior to its direct or indirect detection. The combination of an effective accumulation step with an advanced measurement procedure results in a very low detection limit, and makes stripping analysis one of the most important techniques in trace analysis.

Anodic stripping voltammetry (ASV) was first used for the cathodic accumulation of metals as an amalgam followed by their anodic determination, while cathodic stripping voltammetry (CSV) was first used for the indirect determination of organics as mercury salts, involving anodic oxidation of mercury and subsequently cathodic reduction of the mercury ion. Adsorptive transfer stripping voltammetry (AdTSV), in which the electrode is transferred to a different electrolyte after accumulation and before determination, is usually applicable for studies of large biomolecules such as nucleic acids.

2.3 DNA binders

2.3.1 Classification of DNA binders

Table 2-3: Types and mechanism of interaction between DNA and small molecules

Type of interaction	Binding mechanism	Examples
External association	Association in the atmosphere of ions of the DNA polyelectrolyte due to electrostatic forces with the negatively charged DNA phosphate sugar backbone	$\text{Ru}(\text{bpy})_3^{2+}$ $\text{Co}(\text{bpy})_3^{3+}$
Groove binding	van der Waals contact, hydrophobic, and/or hydrogen bonding, as governed by geometric and steric factors	Netropsin Hoechst 33258 $[\text{Ru}(\text{tmp})_3]^{2+}$
Intercalation	Insertion of a ligand of the complex in the DNA base pairs stack, leading to significant π -electron overlap	Ethidium bromide $[\text{Ru}(\text{dppz})_3]^{2+}$ $[\text{Ru}(\text{phehat})_3]^{2+}$

* tmp= tetramethyl phenanthroline; dppz=dipyridophenazine; phehat=phenanthroline hexaazatriphenylene

Figure 2-13: Comparison between two DNA binding mechanisms: (A) minor groove binding of DAPI and (B) intercalation of doxorubicin.¹⁶¹ [Chem. Soc. Rev., 2007, 36, 280-295] – Reproduced by permission of The Royal Society of Chemistry.

As can be seen in Figure 2-13, the structure of an organic compound influences its mode of binding to ds-DNA. Extended flexible chains tend to wrap around the DNA minor groove, while the more rigid polyaromatic structures are inserted in between base pairs. Intercalators can be further classified based on their position with respect to DNA grooves. Classical intercalators have one or two side groups at either major or minor groove only. Examples: acridines (proflavin), anthracyclines (adriamycin), and ethidium bromide. Threading intercalators have side groups (usually bulky substituents) residing at both minor and major grooves. Examples: nogalomycin, threading anthracyclines (daunomycin with carbohydrate ligand and actinomycin with circular peptide substituting phenoxazone).

Figure 2-14: Comparison of the molecular structures of (A) classical intercalator ethidium bromide and (B) threading intercalator $[\Delta,\Delta-\mu-(bidppz)-(phen)_4Ru_2]^{4+}$.

In order for the polyaromatic ring of a threading intercalator to be fully intercalated, one of the bulky side groups has to penetrate the stack of base pairs. The electrostatic and hydrophobic interactions between these side groups with DNA backbone provide additional contributions to the DNA binding energies in addition to the energetics of the intercalating unit itself.¹⁶² This leads to kinetic inertness or stabilization of the complexes formed from threading intercalator and ds-DNA as compared to those formed from classical intercalators, though its verification is a matter of further research. On the

other hand, threading intercalators exert little, if any, stabilizing effect on ss-DNA. They should then be able to discriminate between ds- and ss-DNA with a large margin, and even confer sequence selectivity in some cases. The unique properties of threading intercalators will be further discussed and exploited in this project.

2.3.2 Studies of DNA binding mechanism

The most convenient method to determine the DNA binding mechanism of a binder is optical methods based on the UV-Visible (UV-Vis) absorbance and fluorescence.¹⁶³ Monitoring of DNA-drug interactions using spectrophotometric methods relies on the fact that the fluorescence and electronic absorption spectra of the free ligands are altered upon binding.¹⁶⁴ Circular dichroism spectroscopy, proposed by Rodger and Norden,^{165, 166} were based on the changes in the circular dichroism of DNA solution in the presence of binders.¹⁶⁷

For example, the fluorescence of ethidium bromide is enhanced,¹⁶⁸ while those of aminoacridines and anthracyclines are quenched.¹⁶⁹⁻¹⁷² In contrast, the UV-Vis absorption spectrum of the DNA-bound drug is simultaneously shifted to longer wavelength (bathochromic shift) and the molar extinction coefficient $\epsilon_{\max}$ at the $\lambda_{\max}$ value is depressed (hypochromic effect). These findings were further substantiated with hydrodynamic and structural studies such as viscometry, sedimentation, gel electrophoretic techniques, and X-ray diffraction.¹⁷³

In viscometry, the increase of the viscosity of a DNA solution provides direct evidence of intercalation. Cohen and coworkers observed an increase of intrinsic viscosity and decrease of sedimentation constant for sonicated, rod-like calf thymus DNA with increasing amounts of proflavine.^{174, 175} This phenomena, ascribed to the increment of

hydrodynamic volume owing to increased stiffness of DNA upon intercalation, is consistent with the intercalation hypothesis proposed by Lerman.¹⁷⁶ Subsequent viscometric studies correlated the reduced-viscosity values to the contour length of DNA. A linear increase of around 2-3 Å was found due to the helix extension of DNA fragments for every bound molecule of ethidium, proflavine,¹⁷⁷ and various Pt-based metallointercalators.¹⁷⁸⁻¹⁸⁰

The crystallographic study by Sobell, Neidle, Rich, and their colleagues provided the first truly atomic description of intercalation complexes, and unequivocally proved that the DNA duplex could indeed stretch to accommodate acridine and phenanthridine chromophores between two base pairs.

Today, techniques that are employed most frequently are X-ray crystallography and two dimensional Nuclear Overhauser Enhancement Spectroscopy (NOESY) NMR spectroscopy. Combined with quantum chemistry, molecular mechanics, and molecular dynamics, a complete molecular description of DNA-ligand interaction at the atomic level has been achieved. Unfortunately, the application of these techniques are limited to complexes that can crystallize and diffract X-ray radiation for crystallography, and are sufficiently stable to allow build-up of NOE signals before the complex dissociates.

The binding mechanism of NTCDI to ds-DNA has been studied since 1982.¹⁸¹ Substituents next to the ring were found to greatly influence binding. A series of NTCDI-based compounds was thus studied, each containing different substituents that range from a small methyl group to a bulky adamantyl amide of lysine. Viscometric titrations, circular dichroism, and UV-Vis spectrophotometry results indicated that all of these compounds bind ds-DNA through intercalation. Unlike classical intercalators, the bulky substituents of NTCDI reside at opposite (minor and major) grooves of DNA. This

signifies a threading intercalation mode, which is possible when the bulky substituents are able to pass through the openings in the double helix caused by DNA ‘breathing’ motions.

The same group further demonstrated the threading intercalation mode of NTCDI compounds through kinetic and equilibrium analyses.¹⁸² Spectrophotometric binding studies indicate strong binding of the diimide to DNA, with little base pair preferences. Stopped-flow kinetics experiments demonstrate that the diimide both associates and dissociates from DNA more slowly than classical intercalators with similar binding constants. During association, NTCDI forms two ion pairs in its complex with DNA as expected for a simple dication. During dissociation, however, only one ion pair is broken. The free side chain can then slide between base pairs to put both diimide side chains in the same groove, followed by rapid full dissociation of the diimide. These dissociation characteristics are similar to those of monocationic intercalating ligands. When combined, the results of kinetics studies clearly distinguish NTCDI compounds as threading intercalators.

More recently, multiple NTCDI units were incorporated into poly-intercalators.^{183, 184} The structure of the resulting DNA-NTCDI complexes were studied using a combination of two-dimensional NOESY NMR data and molecular modeling.¹⁸⁵⁻¹⁸⁷ Although the linker groups can reside in either minor or major grooves, NTCDI units are always positioned parallel to the DNA base pairs, further confirming the intercalative binding.

2.3.3 DNA binding constant

Peacocke and Skerrett demonstrated for the first time a truly quantitative measurement of a reversible drug-DNA binding.¹⁶⁴ Among the numerous quantitative

techniques, optical absorbance and fluorescence techniques have found universal application in quantifying the DNA binding of various DNA binders.

Equilibrium binding titration

The equilibrium binding titration involves monitoring the progressive absorbance or fluorescence change of a drug solution upon serial addition of DNA or oligonucleotide aliquots. Addition of DNA solution to the drug solution results in a drop in absorbance and an increase or decrease of fluorescence depending on the optical behavior of the drug being examined. The absorbance A measured at any wavelength reflects both the free and DNA-bound drug species:

$$A = A_f + A_b = \varepsilon_f \cdot C_f + \varepsilon_b \cdot C_b$$

This direct method is applicable to DNA or oligonucleotide samples at a range of conditions (pH, ionic strength, temperature). However, its use is restricted to fluorescent binders or DNA-binder complexes. Similarly, UV-Vis becomes difficult if the drug only weakly absorbs at wavelengths beyond those associated with DNA titrant (e.g. 260 nm).

Fluorescent intercalator displacement (competitive binding) assay

Morgan and coworkers¹⁸⁸ proposed an indirect method to determine the apparent binding constant (K_{app}) from a fluorescence-based competition assay. DNA sample were first saturated with excess amount of ethidium reporter intercalator. Quantitative titrations were then carried out to determine the C_{50} value, i.e. the concentration of compound of interest that is needed for 50% displacement of initially bound ethidium reporter. Since free ethidium reporters are $4000 \times$ less fluorescent than those that are DNA-bound, the

displacement event can be easily quantified through the reduction of fluorescence signal. The C_{50} value of a compound is inversely proportional to the binding constant.

Subsequent works^{189, 190} also demonstrated an excellent correlation between K_{app} and the intrinsic binding constant K_a obtained from spectrophotometric and equilibrium dialysis studies. As a whole, this relatively simple procedure is appealing because it offers a quick, flexible, and reliable indicator of relative binding affinity of one or a series of compounds.

Building on the previous work by Bruice,^{191, 192} Boger and co-workers proposed a quantitative method based on Scatchard analysis for cases where the binding stoichiometry is 1:1.^{193, 194} The stoichiometry was determined using a titration curve, i.e. the change in fluorescence vs. the amount of intercalator added as depicted in Figure 2-15 (A). The mathematical intersection of the pre- and post-saturation portions of the curve provides ΔF_{sat} . Saturation was achieved at DNA:ligand ratio of 1:1, justifying the use of Scatchard analysis.

Equation 1 was then used to establish the free ligand concentration [*free ligand*] that is needed to generate a Scatchard plot.

$$\text{fraction of DNA-ligand complex} = \left(\frac{\Delta F}{\Delta F_{sat}} \right) \frac{1}{X}$$

$$\text{fraction of free ligand} = \left[1 - \left(\frac{\Delta F}{\Delta F_{sat}} \right) \frac{1}{X} \right]$$

$$\text{concentration of free ligand} = [\text{free ligand}] = [\text{total DNA}] \left[X - \frac{\Delta F}{\Delta F_{sat}} \right] \quad (\text{Equation 1})$$

Where X = molar equivalent of ligand vs. DNA, ΔF_x = change in fluorescence, and ΔF_{sat} = maximum change in fluorescence when DNA is saturated with ligand.

Starting from the definition of binding constant K_a :

$$K_a = \frac{[DNA - ligand]}{[free DNA][free ligand]}$$

$$[DNA - ligand] = K_a [free DNA][free ligand]$$

$$[DNA - ligand] = K_a [[total DNA] - [DNA - ligand]][free ligand]$$

$$\frac{[DNA - ligand]}{[free ligand]} = K_a [total DNA] - K_a [DNA - ligand] \quad (\text{Equation 2})$$

Since $[DNA - ligand]$ is proportional to ΔF and $[total DNA]$ is constant for a given assay,

equation 2 can be re-written as $\frac{\Delta F}{[free ligand]} = C - K_a \Delta F$.

The plot of $\frac{\Delta F}{[free ligand]}$ vs. ΔF therefore yields a linear portion of the

Scatchard plot and provides $-K_a$ as the slope of this portion of the curve as illustrated in Figure 2.15 (B).

Figure 2-15: (A) Change of fluorescence signal upon titration of ligand under study, and (B) Scatchard plot for determination of binding constant.¹⁹³ Reprinted with permission from J. Am. Chem. Soc. 2001, 123, 5878-5891. Copyright 2001 American Chemical Society.

2.3.4 DNA intercalators

Since it was first proposed by Lerman in 1961,¹⁷⁶ the intercalation hypothesis has been extensively studied due to its important application in cancer drugs, labels in nucleic acid biosensing, and many other diagnostic schemes.

Energetic of intercalation

ΔG_{obs} measures the tendency of complex formation between the intercalator and ds-DNA, and can be derived using the equation:

$$\Delta G_{obs} = \Delta G_{conf} + \Delta G_{l+r} + \Delta G_{hyd} + \Delta G_{pe} + \Delta G_{mol}^{195}$$

The driving force for intercalation is the linear combination of free energies associated with various processes. They follow as:

- ΔG_{conf} : conformational changes in the both the DNA and intercalator
- ΔG_{l+r} : reduction of the rotational and translational degrees of freedom
- ΔG_{hyd} : hydrophobic interactions
- ΔG_{pe} : the polyelectrolyte contribution
- ΔG_{mol} : noncovalent molecular interactions between intercalator and DNA bases

A major driving force comes from the *polyelectrolyte effect*, in which the release of counter ions upon addition of cationic intercalators decreases the free energy of the system. Initially, there was a strong repulsive interaction among the closely spaced charge groups in the polyion. The binding of an intercalator increases the space between the DNA phosphate groups, hence increasing the charge spacing in the polyion. The release of the territorially bound counter ions also reduces the local concentration gradient relative to the bulk of solution. As a result, the stresses imposed on the system are

reduced and the formation of a DNA-intercalator complex is favored. Increasing ionic strength (e.g. Na^+ concentration) thus reduces the driving force for complex formation due to the more prominent screening effect among the phosphate groups and a reduced concentration gradient of counter ions. Intercalation is also a favorable *hydrophobic transfer* process. The removal of a nonpolar intercalator from aqueous solution into the hydrophobic intercalation site within the DNA duplex causes a disruption in the water shell surrounding it, resulting in favorable entropy contributions.

The equilibrium binding constant ΔG^0_{obs} is related to the equilibrium binding constant K_a by the Gibbs equation:

$$\Delta G^0_{obs} = -RT \ln K_a$$

where R is the gas constant and T is the absolute temperature (K).

The intercalation process

The intercalation process can be broken down into three main stages. First is the conformational transition of DNA to form the intercalation site. This step is characterized by localized unwinding of the contiguous base pairs at the intercalation site and an increase in the distance between the phosphate groups on the sugar phosphate backbone on both strands. The DNA base pairs separate by 3.4 Å, effectively forming a cavity for the incoming compound to reside. This is followed by the transfer of the intercalator from aqueous solution to the intercalation site. The planar, nonpolar polycyclic aromatic ring is taken from the aqueous solution and buried within the DNA. The final step involves the optimization of the energy associated with the complex. Once the intercalator has inserted between adjacent base pairs, the energy of the complex is optimized through noncovalent interactions between the intercalator and the base pairs at the binding site. This is

achieved mainly by the stacking π -bond interactions between the electron-deficient polyaromatic rings and the electron-rich bases.

Poly- intercalators

Bis-intercalation was discovered in 1974,¹⁹⁶ and was proven to have potential advantages of tighter binding and sequence-selectivity.¹⁷⁰ Threading polyintercalators that occupy both DNA grooves are expected to display high DNA binding affinity and/or specificity, slow rates of DNA dissociation, and potent inhibition of protein-DNA interactions

Poly- intercalators have been reported to have binding constants around one order of magnitude higher than their single intercalator counterparts. Poly-intercalators made of symmetrical homometallic dinuclear complexes with two Ru complexes linked by a flexible linker has a reported dissociation constant in the nM range (corresponding to $K_a \sim 10^9 M^{-1}$).¹⁹⁷ The highest binding constant reported so far ($K_{app} = 10^{14} M^{-1}$) was obtained from a polyamine-linked triacridine synthesized by Laugaa and coworkers.¹⁹⁸

2.4 Conducting polymers in nucleic acid biosensing

Since their inception in the 1970s,¹⁹⁹⁻²⁰¹ conducting polymers have been used in applications ranging from anti-static coating to electrostatic capacitors and magnetic storage media. Leclerc and coworkers first suggested the use of conjugated polymers for biomolecular detection.²⁰²⁻²⁰⁴ Detection is accomplished mainly by monitoring the alterations of the electrical and optical properties due to the conformational change of the polymer backbone upon binding of the sample.²⁰⁵ The advantage of these sensors is the potential of conducting polymers to exhibit collective properties that are sensitive to very

minor perturbations, providing an opportunity for amplification through changes in transport properties, electrical conductivity or rate of energy migration.²⁰⁶ Therefore, assays based on local properties such as the electrochemical oxidation and reduction potentials of conducting polymers will not fully utilize the collective properties of the system.²⁰⁶

In most of nucleic acid detection assays reported thus far, conducting polymer takes on the role of the transduction element. Sequence specificity comes from the incorporation of nucleic acid probes into the conducting polymer through various manners: entrapment within polymers during their electrochemical growth, simple adsorption onto electropolymerized films, chemical coupling or affinity interactions between bioreceptors and electropolymerized films, or direct electrochemical polymerization of the bioreceptor itself.^{207, 208}

2.4.1 Optical detection

In optical nucleic acid biosensors, conducting polymers transduce the oligonucleotide hybridization into colorimetric or fluorometric output.²⁰⁹ Leclerc and coworkers synthesized a series of water-soluble cationic polythiophene derivatives for this purpose. These are direct assays that involve no chemical reactions and rely solely on the difference of electrostatic interactions and conformational structures between electroactive and photoactive cationic polythiophene derivative with ss- or ds-DNA.

In a departure from the usual solid-state detection, Bazan and coworkers proposed the use of conducting polymer in a homogenous assay.²¹⁰ Förster Resonance Energy Transfer (FRET) principle was applied to amplify the fluorescence signal of a polythiophene derivative. An extension of this method was successfully applied to

achieve a very low detection limit of 3 zM, enabling the detection of single nucleotide polymorphisms (SNPs) from clinical samples without the need for PCR.²¹¹

2.4.2 Electrochemical detection

Electrodes modified with conducting polymers and oligonucleotide capture probes monitor changes in the oxidation potential, electroactivity, as well as conductivity of the conducting polymer.²¹²⁻²²⁰ Since most of these assays rely on the intrinsic electrical and electrochemical properties of the conducting polymer films upon interaction with sample nucleic acids, they have the advantage of being simple, direct, and label-free.

Touele and coworkers synthesized an oligonucleotide-functionalized pyrrole monomer, which was then electropolymerized onto a microelectrode.²²¹ Garnier and coworkers used this immobilization method, and further detected the hybridization through the increase in the polypyrrole (PPy)'s oxidation potential and reduced current response in CV.^{212, 214} Such changes in the electronic properties of PPy have been attributed to the higher rigidity of the polymer backbone following the hybridization. A similar observation was reported for poly(EDOT).²²⁰ A successful differentiation of one-base mismatched was obtained through a similar method using a modified polythiophene.²¹⁶

In recent years, several groups have reported the electrochemical detection of nucleic acids using conducting polymer in conjunction with impedance spectroscopy,²²² electrostatic modulation of ion-exchange kinetics,²¹⁷ and ferrocene labels.²²³

2.4.3 poly(EDOT) for biosensor

Interest in poly(EDOT) is highlighted by its high conductivity, small band gap, optical transparency, and ease of processing. Poly(EDOT) stands out from other conducting polymers such as polyanilines and polypyrroles due to its stability at its oxidized conducting state and the low toxicity of the monomers.²²⁴ In its pure form or as a complex with polystyrene sulfonate, poly(EDOT) has found widespread use as antistatic coatings and as conducting layer in electrochemiluminescent devices, organic light emitting devices (OLED), and organic field effect transistors (OFET).²²⁵

As with other conducting polymers, poly(EDOT) is extensively conjugated and possesses a spatially delocalized band-like electronic structure that stem from the splitting of interacting molecular orbitals of the constituent monomer units.²²⁶ The cyclic voltammetry of electronically conducting polymers is characterized by broad non-Nernstian waves.²²⁷

The use of poly(EDOT) and its derivatives in biosensing has been summarized.²²⁸ Poly(EDOT) is an ideal candidate for immobilization matrix-cum-physicochemical transducer because it is highly stable, can be synthesized and deployed at physiological pH and its conductivity does not change significantly with changes in counter ions.²²⁵

Despite the extensive effort in using conducting polymers in solid state nucleic acid detection, there has only been limited efforts in making use of the dynamic growth of conducting polymers to amplify the analytical signal.^{229, 230} It could in principle provide a highly efficient signal amplification route with controllable amplification power, where hundreds to millions of repeat units are connected to form long conducting polymer chains. This method will be further exploited in Chapter five.

3 Electroactive DNA Intercalators

3.1 Introduction

Numerous organic and inorganic DNA intercalators have been synthesized and used as electrochemical reporters in DNA detection.^{96, 99, 100, 116-131} Organic DNA intercalators such as daunomycin¹²⁰ and ethidium bromide²³¹ bind with satisfactory specificity to double-stranded DNA (ds-DNA) but provide relatively low redox signal with limited possibility for amplification. And while inorganic metallointercalators generate strong electrocatalytic redox signal, their usage for DNA detection is limited by the lack of specific binding to ds-DNA. There is therefore a need for reporter molecules that possess optimum binding properties to ds-DNA as well as high, easy-to-amplify electrochemical signal. We plan to achieve this through molecular design, i.e. by selecting discrete moieties that each possesses a desired property and combining them through flexible synthetic routes.

This chapter discusses the design, synthesis, and characterization of new electroactive intercalators based on 1,4,5,8-naphthalenetetracarboxylic diimide (NTCDI), a known DNA intercalating agent. Their electrochemical activity is derived from incorporation of transition metal complexes (**3** and **4**), 3,4-ethylenedioxythiophene (EDOT) moieties (**8**), or a combination of both (**9**). The molecular structure of these electroactive intercalators is presented in Figure 3-1.

Figure 3-1: Molecular structures of the electroactive intercalators being studied

3.2 Molecular design

3.2.1 Objectives of molecular design

The aim of molecular design is to create novel, multi-functional nucleic acid hybridization reporters that combine the advantageous properties of known DNA labels, i.e. the ability to bind specifically to ds-DNA, and to generate an amplified redox signal at the desired potential range. Amenability to chemical modification and ease of synthesis are additional design considerations.

Constructing the intercalators from discrete building blocks introduces flexibility to the design process. Unlike most other electroactive intercalators, the intercalating moiety and the electroactive component in these reporter molecules are not covalently bonded to each other; rather they are connected through a modifiable linker. Tuning the length and flexibility of these linker groups can possibly improve the binding ability of the intercalators while maintaining their redox activities. The effect of variation in linker groups on the binding properties of the molecules will be discussed further in this chapter.

3.2.2 Components

All the electroactive reporters we designed share a common duplex-recognition moiety: NTCDI, an electron deficient polyaromatic that was first proposed as a DNA intercalator in 1982.¹⁸¹ This moiety was shown to bind to ds-DNA through threading intercalation,¹⁸² an observation that was further verified through viscometric titrations with DNA, circular dichroism spectroscopy, UV-Vis spectrophotometry, and a combination of NMR spectroscopy and molecular modeling. The value of its binding constant averages around 10^5 M^{-1} depending on experimental technique and parameters, making it one of the strongest DNA intercalators. In addition, this diimide can be

synthesized through nucleophilic substitution of the commercially available naphthalene-tetracarboxylic dianhydride. The proven binding capacity and ease of synthesis make NTCDI an ideal choice for an intercalating moiety. Each reporter molecule contains one NTCDI intercalating unit (mono-intercalators), except for compounds **4a–b** that contain two (bis-intercalators). Neutral poly-intercalators^{183-187, 232} containing multiple NTCDI chromophores have been previously synthesized with binding constants higher than that of mono-intercalators. It is therefore interesting to investigate the binding properties of poly-intercalators containing positively charged metal complexes.

The reporters discussed in this thesis can be classified based on their electroactive components. The first group (series **3** and **4**) incorporate electroactive moieties in the form of Os or Ru complex. Os and Ru coordination complexes with bulky polyaromatic ligands such as phenanthroline (phen) and dipyrrophenazine (dppz) have been previously studied for their use in biosensing. These complexes have also received much attention for their ideal catalytic behavior. The M(II), M(III), and M(IV) oxidation states are substitution inert, and the fast switching among these oxidation states provides many reaction pathways with lower kinetic energy barrier. Os and Ru coordination complexes are therefore capable of generating amplified electrocatalytic signals. Furthermore, the pyridine ligands are easily substituted, providing simple synthetic routes for tethering these metal complexes to other constructs. These features make Os and Ru pyridine complexes ideal candidates for electroactive component in our reporters.

In series **3** and **4**, the metal complexes are linked to the intercalating unit through 1-(3-aminopropyl)-imidazole. The linker's amine group reacts with the anhydride to form imide group on the intercalating unit while its imidazole ligand replaces one of the chloride ligands on the metal complex, effectively connecting the two components. Several structural modifications were also designed to study the effects of these variations

on the properties of the intercalators. Bipyridine ligands with methyl (—CH_3) and methoxy (—OCH_3) substitutions were used in bis-intercalators **4a-CH₃**, **4a-OCH₃**, **4b-CH₃**, and **4b-OCH₃**.

For the second group (**8a-c**), EDOT monomers serve as the electroactive moiety. Although the usage of EDOT in various schemes of DNA detection has been previously reported,²²⁵ the incorporation of EDOT with an intercalating moiety has not been reported. The redox potential of EDOT monomer is too high to be of use in direct electrochemical detection; however its capability of polymer growth presents an intriguing possibility for signal amplification.

For symmetrical systems with NTCDI as intercalating unit and EDOT as electroactive unit, their properties can be tuned by modifying the linker groups. Methyl-, hexyl-, and tetraethylene glycol linkers, providing various lengths, flexibility, and polarity, were successively studied. As they are essentially functionalized EDOT monomers, compounds **8a-c** can be polymerized. The resulting conducting polymers possess novel properties mostly brought about by the unique combination of n-type and p-type semiconducting behavior in the same molecule. The electrochemical and optical properties of these polymers will be briefly discussed, but their use in other applications is outside the scope of this thesis.

Asymmetric tailoring of NTCDI with one Os complex and one EDOT unit into a hybrid structure (**9**) results in a unique molecule with potential for multi-functional detection of DNA. Each component performs a specific role in enhancing the selectivity and sensitivity in application of this intercalator for DNA sensing.

3.3 Organic syntheses

3.3.1 Synthesis of transition-metal linked intercalators

The synthesis of propylimidazole NTCDI (PIND, **1**) is depicted in Scheme 3-1. This nucleophilic substitution reaction is based on previously reported procedures.^{233, 234} Dianhydrides of the starting material were converted to diimides upon nucleophilic attack by the amine group of the linker chain. An intermediate containing acid and amide groups were formed, followed by elimination of water at elevated temperature to form the imide.

Scheme 3-1: Synthesis of intercalating unit PIND

Os and Ru metal complexes (**2a** and **2b**) were synthesized from their corresponding salts based on literature procedures. The oxidation state of the Os center was reduced from 4+ to 3+, and Ru from 3+ to 2+ during formation of the organometallic complexes. These reactions were therefore carried out in an inert environment to prevent re-oxidation of the product.

Scheme 3-2: Synthesis of transition metal complexes series **2a** and **2b**

a) K₂OsCl₆, DMF, reflux, 2 h, 96%. b) RuCl₃, DMF, reflux, 2 h, 92%

Electroactive mono-intercalators **3a–b** and bis-intercalators **4a–b** were formed from intercalating unit **1** and the corresponding metal complexes **2a** or **2b** through ligand exchange between one of the chloride ligands on the metal complex with the imidazole ligand of the linker. Ligand exchange occurs readily in coordination complexes and is a commonly used method to modify the structure and properties of these complexes. Syntheses of mono- and bis-intercalators differ only in terms of the ratio of starting materials used.

Scheme 3-3: Synthesis of transition-metal linked mono-intercalators **3a** and **3b**

The redox potential of Ru and Os metal complexes changes upon ligand exchange. The shift of redox potentials, observed through cyclic voltammetry, therefore provides a convenient method to monitor the formation of **3** and **4**. As shown in Figure 3-2 (A), only one pair of redox waves at $E_{1/2} = 0.0$ V was observed at the initial stage of **3a** synthesis, corresponding to the redox process of the starting material **2a**. Upon addition of **1**, a new pair of voltammetric peaks appeared at 0.16 V, indicating the ligand exchange on the Os metal center and the formation of **3a**. Both electron transfer processes are clearly resolved. The slightly larger peak-to-peak potential separations are mainly due to the lower conductivity of the reaction medium. Voltammetric peak intensity of the product increased with longer reaction time, and the peaks of starting material diminished gradually. Both pairs of redox peaks reached a steady state after

30–40 minutes of refluxing. After purification, cyclic voltammograms of the purified **3a** displayed only one pair of redox waves, confirming the purity of the product. A similar process was observed during synthesis of **3b**, as shown in Figure 3-2 (B).

Figure 3-2: Normalized cyclic voltammograms of (·····) starting materials (A) **2a** and (B) $\text{Ru}(\text{bpy})_2\text{Cl}_2$ at 0 minutes, (---) reaction mixture after 30 minutes of refluxing with 0.5 equivalent of **1** in ethylene glycol, and (—) purified products (A) **3a** and (B) **3b**. Voltammograms recorded in PBS at a scan rate of 100 mV/s.

Scheme 3-4: Synthesis of transition-metal linked bis-intercalators series **4a** and **4b**

a) **2a** series, ethylene glycol, reflux, 2h, 45–55%. b) **2b** series, ethylene glycol, reflux, 2 h, 55–65%.

Bis-intercalators series **4a** and **4b** are also produced through ligand exchange (Scheme 3-4), similar to formation of mono-intercalators **3a–b**. Six variants of metal

complexes were used to form six corresponding bis-intercalators in series **4a** and **4b**. The syntheses were also monitored through cyclic voltammetry as shown in Figure 3-3.

Figure 3-3: Normalized cyclic voltammograms of (·····) starting materials (A) **2a** and (B) $\text{Ru}(\text{bpy})_2\text{Cl}_2$ at 0 minutes, (---) reaction mixture after 30 minutes of refluxing with 2 equivalents of **1** in ethylene glycol, and (—) purified products (A) **4a** and (B) **4b**. Voltammograms recorded in PBS at a scan rate of 100 mV/s.

During the ligand exchange reaction, two new pairs of redox peaks were observed in addition to the initial starting material peak. These peaks represent the two consecutive ligand exchanges during the formation of bis-intercalators. The formation of bis-intercalator generally takes longer than mono-intercalator. The redox potential of the Os metal complex increases from $E_{1/2} = 0.0$ V to 0.16 V and 0.43 V, and that of Ru increases from $E_{1/2} = 0.40$ V to 0.65 V and 0.81 V.

3.3.2 Synthesis of EDOT-linked intercalators

The starting material for syntheses of all functionalized EDOTs is EDOT-OH, which was synthesized according to a previously described method.^{235, 236} Amine-

functionalized EDOTs (**7a–c**) were synthesized through intermediates **5a–c**. Nucleophilic substitution yielded the corresponding azide-functionalized EDOTs (**6a–c**), and subsequently Staudinger reduction of azides led to formation of the desired **7a–c**.²³⁷⁻²³⁹

Scheme 3-5: Synthesis of amine-modified EDOT with various linkers (**7a–c**).

a) for **5a**: MsCl , Et_3N , CH_2Cl_2 . for **5b-Cl**: NaH , $\text{Br}(\text{CH}_2)_6\text{Cl}$, 18-crown-6, THF, reflux, 18 h. for **5c-OTs**: NaH , tetraethylene-glycol ditosylate, 18-crown-6, THF, reflux, 18 h. b) NaI , acetone, reflux, 18 h. c) NaN_3 , $\text{DMF}/\text{H}_2\text{O}$, 100°C , 18 h. d) 1. PPh_3 , 50°C , 1 h. 2. NaOH , $\text{THF}/\text{H}_2\text{O}$, 50°C , 2 h.

Amine functionalized EDOTs were subsequently tethered to the intercalating moiety as depicted in Scheme 3-6. Similar to the formation of **1**, **8a–c** were formed through nucleophilic substitution between dianhydride and amine groups. Zinc acetate was employed as an acid catalyst in these reactions.

Scheme 3-6: Synthesis of symmetrical EDOT-linked intercalators (**8a–c**).

3.3.3 Synthesis of asymmetrical hybrid intercalator

Scheme 3-7: Synthesis of asymmetrical intercalator **9**

a) 1. NaN_3 , cat. NaI, H_2O , $80^\circ C$, 1 h. 2. TsCl, cat. DMAP, Et_3N , CH_2Cl_2 , RT, 18 h.
 b) NaH, cat. NaI, DMF, RT, 18 h. c) 1. PPh_3 , THF, $50^\circ C$, 1 h. 2. NaOH, THF/ H_2O , $50^\circ C$, 2 h. d) 1. 1-(3-aminopropyl)imidazole, DMA, $125^\circ C$, 18 h. 2. $SOCl_2$, DMF, $55^\circ C$ 2 h. e) $Zn(OAc)_2$, pyridine, $120^\circ C$. f) **2a**, ethyleneglycol, $180^\circ C$, 6 h.

Two synthetic routes were attempted for the synthesis of **8d**, both based on nucleophilic substitution.^{240, 241} We first tried a one-step approach by mixing the aminopropyl imidazole and EDOT monomer simultaneously with the dianhydride precursor. The product was then purified with column chromatography. However, this method allowed a maximum yield of 50%. Since the amine-functionalized EDOT **8c** was synthesized through four steps, a more efficient two-step reaction was attempted. The

aminopropyl imidazole was first linked to one side of the dianhydride and the crude intermediate was extracted prior to reaction with **8c** to form the diimide, as depicted in **Scheme 3-7**. Again, nucleophilic substitution took place during formation of **8d**. In this case, thionyl chloride was used to dehydrate the acid in order to form the imide bond.²⁴²

The hybrid compound **9** was obtained through a ligand exchange between **8d** and **2a**, similar to that used for synthesis of **3** and **4**. The limited solubility of **9** in water, due to the more hydrophobic EDOT tethering group, required a modified purification method. Extraction in chloroform separated the product from the highly water soluble starting materials. Similar to other compounds containing a metal complex, the formation of **9** was monitored through cyclic voltammetry as depicted in Figure 3-4.

Figure 3-4: Normalized cyclic voltammograms of (·····) starting material **2a** at 0 minutes, (---) reaction mixture after 30 minutes of refluxing with 1 equivalent of **8d** in ethylene glycol, and (—) the purified product **9**. Voltammograms recorded in PBS at a scan rate of 100 mV/s.

3.4 Characterization

3.4.1 Electrochemical properties

Cyclic voltammetry was applied to study the electrochemical properties of the intercalators because it offers a rapid identification of redox potentials and convenient evaluation of the effect of media upon the redox processes. All potentials are referenced to Ag/AgCl unless otherwise stated.

Figure 3-5: Normalized cyclic voltammograms of (A) mono-intercalator **3a** (—) and bis-intercalator **4a** (---) with Os metal complex, and (B) mono-intercalator **3b** (—) and bis-intercalator **4b** (---) with Ru metal complex in PBS at a scan rate of 100 mV/s.

The cyclic voltammograms of intercalators containing metal complexes are shown in Figure 3-5. The intercalators behaved exactly as expected for a highly reversible redox couple in solution phase. They exhibit good stability, as evidenced by the negligible difference in electrochemical signal upon numerous potential cycling. At the scan rate of 100 mV/s, a one-electron exchange system exhibiting an ideal Nernstian behavior was displayed. The peak current is proportional to the square root of the potential scan rate, confirming a standard diffusion-controlled electrochemical kinetics. The peak-to-peak

potential separation is very close to 59 mV and is independent of the scan rate. These results indicate high electron transfer rate and the accessibility of electrode surface to the metal redox centers, allowing a reversible heterogeneous electron transfer to take place.

Unlike the classical Os or Ru complexes with six coordinating N atoms such as $\text{Ru}(\text{bpy})_3^{2+}$, the coordination polyhedron in mono-intercalators **3a–b** includes an electron donating chloride ligand. As a result, the 2+/3+ oxidation of mono-intercalators **3a** and **3b** occurs at lower potential ($E_{1/2} = 0.16$ V and 0.65 V, respectively). Redox potentials of bis-intercalators **4a** and **4b** are higher than those of mono-intercalators **3a** and **3b**. This is caused by the replacement of another chloride ligand with imidazole, leading to a lower electron density of the metal complex that translates to a higher redox potentials. The stability, reversibility, and electron transfer rate of the **4a** and **4b** are comparable to those of **3a** and **3b**.

Table 3-1: Redox potentials of transition-metal intercalators (V, vs. Ag/AgCl, ± 0.01 V)

	$E_{1/2}$		$E_{1/2}$
3a	0.16	3b	0.65
4a	0.43	4b	0.81
4a-CH₃	0.31	4b-CH₃	0.75
4a-OCH₃	0.19	4b-OCH₃	0.56

The redox potentials of the mono- and bis-intercalators containing transition metal complexes are summarized in Table 3-1. Substitutions on the bipyridine ligands change the ligands' stability and influence the overall redox potential of the complexes. Methyl and methoxy are both electron donating groups that increase electron density and

facilitate oxidation of the redox species they are attached to, resulting in lower oxidation potentials. The methoxy group, with a free lone pair electron adjacent to the π system, exerts a stronger resonance effect than the inductive effect of the methyl group. As a result, compounds with methoxy substitution (**4a-OCH₃** and **4b-OCH₃**) exhibit lower redox potentials compared to those with methyl groups (**4a-CH₃** and **4b-CH₃**). The range of redox potential values of these intercalators allow them to act as mediators in various redox systems, leading to a possible use in a variety of detection schemes. Detailed discussion on these schemes will be presented in Chapter 4.

Cyclic voltammetry study of EDOT-linked intercalators **8a**, **8b**, and **8c** were carried out in acetonitrile, allowing for a wide potential range and measurement of the redox activities of NTCDI as shown in Figure 3-6.

Figure 3-6: Cyclic voltammograms of symmetrical EDOT-intercalators (A) **8a**, (B) **8b**, and (C) **8c** in 0.1 M $n\text{Bu}_4\text{PF}_6/\text{CH}_3\text{CN}$ at a scan rate of 100 mV/s.

The two pairs of redox waves observed at negative potentials (typical $E_{1/2}$ value around -1.10 and -0.70 V) signify the two-stage reversible redox reactions of NTCDI, while an anodic peak at oxidative potentials (typical $E_{1/2}$ value around 1.60 V) represents

the oxidation of EDOT. These redox peaks confirm the presence of each component and indicate that individual redox activity is preserved in the hybrid molecule. The redox potentials of these components are listed in Table 3-2.

Table 3-2: Redox potentials of EDOT-linked intercalators (V, vs. Ag/AgCl, ± 0.01 V)

	NTCDI 1			NTCDI 2			EDOT
	$E_{pa,1}$	$E_{pc,1}$	$E_{1/2}$	$E_{pa,2}$	$E_{pc,2}$	$E_{1/2}$	E_{pa}
8a	-0.91	-1.23	-1.07	-0.46	-0.69	-0.57	1.59
8b	-1.04	-1.18	-1.11	-0.61	-0.78	-0.69	1.55
8c	-0.9	-1.4	-1.16	-0.52	-1.01	-0.76	1.61

The oxidation potentials of EDOT moiety in **8a**, **8b**, and **8c** are roughly independent of the linker group and are comparable to the value for unsubstituted EDOT monomers. The redox potential of the NTCDI unit, on the other hand, decreases slightly with increasing linker length and are comparable to values previously reported for similar compounds.²⁴³ For all three compounds, the relatively large peak separation ($\Delta E_p = 82$ to 495 mV, average 252 mV) indicate poor reversibility of NTCDI redox reaction.

Cyclic voltammogram of asymmetrical intercalator **9** displays two pairs of redox peaks for NTCDI ($E_{1/2} = -1.04$ V and -0.63 V) and one pair for Os complex ($E_{1/2} = 0.17$ V). These redox peaks are well resolved and can be clearly detected, again indicating the preservation of redox activity of the discrete components in this hybrid structure.

Figure 3-7: Cyclic voltammogram of asymmetrical intercalator **9** in 0.1 M $n\text{Bu}_4\text{PF}_6/\text{CH}_3\text{CN}$ at a scan rate of 100 mV/s.

3.4.2 Spectrophotometric properties

Typical UV-Vis absorbance spectra of representative intercalator molecules are presented in Figure 3-8. These spectra are superimposed with those of the relevant starting materials, so they serve to confirm the formation of the desired compounds.

Figure 3-8: UV-Vis absorption spectra of (A) symmetrical transition-metal linked intercalator **3a** (—) superimposed with its starting materials **2a** (---) and **1** (····), (B) symmetrical EDOT-linked intercalator **8c** (—) with **7c** (---) and naphthalene dianhydride (····), and (C) asymmetrical intercalator **9** (—) with **2a** (---) and **8d** (····).

UV-Vis spectrum of **3a** in Figure 3-8 (A) exhibits an intense band at 297 nm from the intraligand (IL) $\pi \rightarrow \pi^*$ bipyridine transitions in bipyridine and a broad band in the visible region (~ 532 nm) generated by spin-allowed metal-to-ligand charge-transfer (MLCT) transitions.²⁴⁴ Compared to the absorption of the starting material **2a**, the MLCT peak is red-shifted from 412 nm, possibly due to ligand exchange reaction that creates another type of MLCT in addition to the existing $\text{Os}(\text{d}\pi) \rightarrow \text{bpy}(\pi^*)$ transition. In the new $\text{Os}(\text{d}\pi) \rightarrow \text{imidazole}^*$ transition, the conjugated imidazole groups of PIND have a lower π^* level relative to the original chloride ligand. This leads to a lower energy and hence a longer wavelength being absorbed in UV-Vis spectroscopy. The $\pi \rightarrow \pi^*$ transition in PIND is represented by absorption peaks at 361 and 380 nm, also containing some contribution from the underlying MLCT absorbance. The UV-Vis spectrum of the final compound thus reveal signatures of both its starting materials, and are consistent with prior reports on a similar compound.^{181, 245} This result confirms the formation of **3a**.

For **8c**, as seen in Figure 3-8 (B), NTCDI exhibits a $\pi \rightarrow \pi^*$ transition as indicated by absorption peaks at 360 and 380 nm. Compared to the absorption peak of the starting material, red shifts occur upon conversion to NTCDI when the oxygen atom of dianhydride is replaced with nitrogen of the imide group. The presence of EDOT in the compound was indirectly confirmed by the formation of imide group. **8a** and **8b** showed similar absorption spectra, indicating that spectral activities of NTCDI moieties in these compounds are independent of the linker groups.

Figure 3-8 (C) depicts the absorption spectrum of **9** compared to its starting materials: **8d** and **2a**. The hybrid structure exhibited $\pi \rightarrow \pi^*$ transitions of bipyridine and NTCDI, as well as the $\text{Os} \rightarrow \text{imidazole}^*$ MLCT. Similar to **3a**, exchanging chloride ligand with imidazole results in a lower energy absorption. EDOT absorption is not observable

at this optical range; however their presence is indicated by the formation of imide. This result again reinforces that the NTCDI and Os complex retain their activity and individual properties in the hybrid structure.

Absorption peaks characterizing the $\pi \rightarrow \pi^*$ transitions of NTCDI were found at the same wavelengths ($\lambda_{\text{max}} = 361$ and 380 nm) for mono- and bis-intercalators with Os and Ru metal centers. The intraligand and MLCT transitions, on the other hand, are dependent on the metal center and ligand substitution. As a result, absorption peaks were observed at slightly different wavelengths as listed in Table 3-3. Besides the position of λ_{max} , the relative intensities of the absorption peaks are also proportional to the ratio between NTCDI and metal complex contained in the compounds, as shown in Figure 3-9.

Figure 3-10 shows a comparison of UV-Vis spectra among bis-intercalators with various substitutions on their bipyridine ligands. Methyl and methoxy substitutions on the bipyridine ligand reduce the intensity of its intraligand $\pi \rightarrow \pi^*$ transition, with methoxy group exerting a more pronounced effect that completely eliminate the absorption peak at 297 nm.

Table 3-3: λ_{max} of UV-Vis absorption of transition-metal linked intercalators (± 0.5 nm)

	IL (bpy)	MLCT		IL (bpy)	MLCT
3a	297	532	3b	291	485
4a	294	520	4b	292	488
4a-CH₃	294	530	4b-CH₃	292	500
4a-OCH₃	-	-	4b-OCH₃	-	500

Figure 3-9: UV-Vis absorption spectra of (A) mono-intercalator **3a** (—) and bis-intercalator **4a** (---) with Os complex, and (B) mono-intercalator **3b** (—) and bis-intercalator **4b** (---) with Ru complex.

Figure 3-10: UV-Vis absorption spectra of bis-intercalators in series **4** showing the effect of ligand substitutions. (A) Bis-intercalators containing Os complexes without substitution (—), with methyl (---), and with methoxy (····) substitutions. (B) The corresponding spectra for bis-intercalators containing Ru complexes. For clarity, the spectra of substituted intercalators are shifted by 1.0 and 2.0 units, respectively.

3.5 Polymer of EDOT-intercalator

3.5.1 Electrochemical properties

Symmetrical EDOT-intercalators **8a–c** can be polymerized to form conducting polymer films that incorporate intercalating NTCDI moieties. As shown in Figure 3-11, polymerization was achieved by potentiodynamic cycling between -0.05 and 1.55 V. The maximum applied potential was set just above the onset oxidation potential of the EDOT monomers to reduce the risk of decomposition of monomer or polymer from overoxidation. EDOT oxidation peak shifted to a lower potential and new, broad redox waves grew in subsequent scans, indicating polymer growth on the electrode surface.

Figure 3-11: Electropolymerization of 10 mM of symmetrical EDOT-linked intercalators (A) **8a**, (B) **8b**, and (C) **8c** in 0.1 M $n\text{Bu}_4\text{NPF}_6/\text{CH}_2\text{Cl}_2$ at a scan rate of 100 mV/s.

The resulting polymers **poly8a**, **poly8b**, and **poly8c** adhered strongly to the working electrode and appeared homogeneous. Under identical polymerization conditions, **8b** and **8c** formed increasingly thicker polymer films than **8a**. Their polymerization was facilitated by the flexible linkers that provide better separation between EDOT and the rigid polyaromatic ring, allowing EDOT moieties to behave more like unsubstituted monomers and polymerize more easily.

Electroanalytical study of the polymer films is presented in Figure 3-12. The broad redox peak of the poly(EDOT) backbone was detected at ~ 0.2 V, which is ± 0.9 V more positive compared to the value for methyl-functionalized EDOT.²⁴⁶ In the negative potential range, reduction peaks of NTCDI unit were detected at around -0.5 V. Compared to the monomer form, these peaks were positively shifted and are no longer resolved. This indicates a less efficient electron transfer from the poly(EDOT) backbone to the side groups, an effect that becomes more prominent with increasing linker length.

Figure 3-12: Cyclic voltammograms of polymers (A) **poly8a**, (B) **poly8b**, and (C) **poly8c** in 0.1 M $n\text{Bu}_4\text{PF}_6/\text{CH}_3\text{CN}$ at scan rates of 200, 150, 100, 50, and 10 mV/s.

The presence of distinct redox characteristics of EDOT and NTCDI in these polymers indicates the absence of internal electrochemical communications between the two moieties. Peak current for NTCDI decreases while that of poly(EDOT) simultaneously increases in the order of **poly8a**, **poly8b**, and **poly8c**, i.e. the conductivity of poly(EDOT) films are higher when the monomers are distant from the rigid NTCDI ring. This indicates a difference in electron transfer mechanism between these two components: polaronic and bipolaronic delocalization on the conjugated chain leads to a band-like, p-type conductivity of poly(EDOT) backbone, while electron hopping drives

the n-type conductivity of NTCDI.²⁴⁷ Furthermore, the linear correlation between scan rate and current intensity confirms that the electrochemistry of these films is surface-controlled.

Figure 3-13: Conductivity plots of intercalator-grafted polymers. (A) **poly8b** and (B) **poly8c** in 0.1 M solution of *n*Bu₄PF₆ in CH₃CN (—) and in CH₂Cl₂ (---). (C) **poly8b** and (D) **poly8c** in 0.1 M solution of LiClO₄ in water.

Conductivity tests were carried out on polymer films electropolymerized on interdigitated microelectrodes with 5 μ m gaps. As shown in Figure 3-13, both **poly8b** and **poly8c** were conductive in aqueous and organic media. The conductivity of **poly8a** cannot be measured using this method, indicating a very low conductivity. **Poly8c** exhibited a higher conductivity because of the longer linker between EDOT and NTCDI

units. Contrary to common observations on poly(EDOT) films, **poly8b** exhibited a conductivity curve with a larger hysteresis in aqueous medium than in organic electrolyte solutions. This is most likely due to the influence of NTCDI stacking in this polymer.

3.5.2 Spectroelectrochemistry

Spectroelectrochemical study was done by first electropolymerizing the films on ITO electrodes. Various potentials were applied to these films and the change of their UV-Vis absorption spectra was monitored, as presented in Figure 3-14. All three polymers exhibit similar spectroelectrochemical characteristics. At a fully reduced state, a broad peak was observed at $\sim 450\text{--}700\text{ nm}$. This peak subsequently decreased when the films were oxidized, and completely disappeared when potentials above 0 V were applied. Various absorption bands were observed at longer wavelengths. Absorption from the polaron state at $\sim 1000\text{ nm}$ first appeared, followed by the absorption band from the bipolaron states. This corresponds to the observation of color change, from dark blue at reduced states to almost colorless at oxidized states.

Figure 3-14: Spectroelectrochemical response of polymers (A) **poly8a**, (B) **poly8b**, and (C) **poly8c** immersed in 0.1 M $n\text{Bu}_4\text{PF}_6/\text{CH}_3\text{CN}$ at -0.9 V (—), -0.5 V (—), 0.0 V (—), $+0.5\text{ V}$ (—), and $+0.9\text{ V}$ (—).

Spectroelectrochemistry was also utilized to characterize the co-polymers of **8a**, **8b**, and **8c** with various amounts of EDOT. At a sufficiently negative potential, the broad absorption peak of poly(EDOT) can be observed at $\lambda_{\text{max}} = 600$ nm. By comparing this peak to that of NTCDI's signature peaks at 360 and 380 nm, the ratio of unmodified EDOT to EDOT-linked intercalators (**8a–c**) can be estimated. The UV-Vis spectra of three such copolymers are presented in Figure 3-15. Indeed, the ratio of NTCDI peak to that of EDOT was positively related to the amounts of EDOT and **8a–c** used in the copolymer systems. The relative spectrophotometric activity of the NTCDI unit with respect to the poly(EDOT) backbone is higher in **8a** compared to **8b** and **8c**. This suggests a dependence of electronic transitions, and hence molecular conformation, on the length of the linker group between NTCDI and EDOT moieties.

Figure 3-15: Normalized spectroelectrochemical spectra of copolymers of EDOT with (A) **8a**, (B) **8b**, and (C) **8c** at NTCDI:EDOT ratios of 1:10 (—), 1:5 (—), and 1:1 (—). Measurements were taken at an applied potential of -0.8 V in 0.1 M $n\text{Bu}_4\text{PF}_6/\text{CH}_3\text{CN}$.

3.6 DNA binding study

Given existing studies that have adequately demonstrated the intercalative binding of NTCDI moieties to ds-DNA through multiple binding studies,¹⁸¹⁻¹⁸⁷ efforts in this area

were geared toward confirming rather than establishing the binding mechanism. Two different methods were used to quantify the binding constants of the molecules, especially in comparison to widely used intercalators. Even though ds-DNA was used in the binding studies, the results and discussions also apply to other hybridized nucleic acids, including the duplex of DNA-mRNA and PNA-DNA.

3.6.1 UV-Vis spectrophotometry

DNA binding can be observed spectrophotometrically by examining the UV-Vis absorption of the molecules in the presence of ds-DNA. The UV-Vis absorption spectra of symmetrical Os-intercalator **3a**, symmetrical EDOT-intercalator **8c**, and asymmetrical hybrid intercalator **9** are presented in Figure 3-16, along with the changes observed upon addition of increasing amounts of salmon sperm DNA.

Figure 3-16: UV-Vis absorption spectra of 25 μM of (A) **3a**, (B) **8c**, and (C) **9** in PBS containing 0, 50, 100, 150 and 200 μM (base pair) of salmon sperm DNA

As shown in Figure 3-16 (A), the addition of ds-DNA to **3a** resulted in gradual decrease (hypochromism) and red shift (bathochromism) of NTCDI's absorbance peaks. These are signatures of intercalative binding,²⁴⁸ where the fused planar aromatic ring of

an intercalator inserts itself between the base pairs of ds-DNA. Hypochromism arises from the interaction between the π systems of NTCDI unit and that of DNA base pairs when they are positioned relatively parallel upon intercalation. The gradual change of spectral activity indicates that the molecules bind to specific sites on the ds-DNA instead of saturating all the available sites. At a DNA base pair:**3a** ratio of 4:0, NTCDI absorbance decreased by 35% and the peaks shifted by ~ 3 nm to 363 and 383 nm. Similar phenomena were also previously observed with NTCDI having aliphatic tertiary amine side chains.^{181, 245} Subsequent addition of DNA (DNA base pair/**3a** ratio >4.0) did not produce further change. This indicates that for **3a**, the average loading of intercalator is one for every 4 base pairs. The same spectrophotometric effects and binding stoichiometry were obtained for **3b**.

The UV-Vis spectrum of **8c** showed little change upon addition of ds-DNA, as depicted in Figure 3-16 (B). Negligible hypochromism and no red shift were detected, implying a very limited binding of this compound to ds-DNA. The same observations were also obtained from other symmetrical EDOT intercalators **8a** and **8b**. This limited binding may be caused by two factors. First, the limited solubility of these compounds in aqueous solutions makes them more prone to precipitation, hence reducing their availability and binding capacity to ds-DNA. Second, the absence of positive charge means that there is no electrostatic interaction between these compounds and DNA. The chance of collision between **8a–c** and ds-DNA is solely controlled by diffusion and therefore reduced.

As shown in Figure 3-16 (C), intercalator **9** showed significant hypochromism and red shift in the presence of ds-DNA, with binding stoichiometry of 1 molecule per 4 DNA base pairs. The binding capacity of this hybrid molecule is higher than that of **8c**.

Introduction of the positively charged Os complex helps increase the water solubility of this molecule despite its large hydrophobic group, and most importantly it brings the molecules to the vicinity of DNA and increases the chance of binding.

To investigate the difference between binding properties of mono- and bis-intercalators, the result of UV-Vis binding study on **3a** and **4a** is presented in Figure 3-17. Hypochromism and bathochromism were also observed for the bis-intercalator, indicating the same binding mechanism through intercalation of NTCDI. However, the saturation of hypochromism was only achieved at one **4a** molecule per 6 base pairs. This is due to the fact that each molecule of bis-intercalator occupies a larger binding site.

Figure 3-17: UV-Vis absorption spectra of 25 μM of (A) mono-intercalator **3a** and (B) bis-intercalator **4a** in the presence of increasing amounts of ds-DNA (25 μM increments).

3.6.2 Circular dichroism spectroscopy

Circular dichroism (CD) studies reveal intercalative binding by demonstrating the change in DNA conformation due to insertion of molecules in between its base pairs. The CD spectra of DNA in the presence of series **4** bis-intercalators are presented in Figure 3-18. All four intercalators induced similar types of spectral change in DNA, albeit at

varying degrees. The spectral changes at ~ 260 and 290 nm are consistent with the changes observed on DNA conformation at local binding sites of ethidium bromide.^{249, 250} This result reinforces the conclusion from UV-Vis study in confirming the intercalative binding. Ru-based intercalators appear to cause more pronounced spectral changes. **4a-OCH₃** and **4b-OCH₃** cause more distortion to ds-DNA conformation. This suggests that, aside from intercalation of NTCDI, the interaction between transition metal complexes with DNA backbone also create a conformational change in DNA.

Figure 3-18: Circular dichroism spectra of 200 μM (base pairs) salmon sperm DNA before (—) and after the addition of 400 μM of **4a** (—), **4b** (—), **4a-OCH₃** (---), and **4b-OCH₃** (---) in PBS buffer.

3.6.3 Viscometry

The insertion of intercalating unit increases the length of DNA strand and the viscosity of a DNA solution.¹⁷⁴ The result of viscometry study is summarized in Table 3-4. All of the compounds tested increased the DNA viscosity, further confirming their intercalative binding. With two intercalating units per molecule, bis-intercalators (series **4**)

cause more elongation to DNA and thus a more significant increase of viscosity compared to mono-intercalators (series **3** and **9**).

Table 3-4: Viscosity of 500 μM solution of sonicated salmon sperm DNA in $0.1 \times \text{PBS}$ after the addition of 100 μM intercalators (η) relative to the original viscosity (η^0).

	η/η^0		η/η^0		η/η^0
3a	1.069	4a	1.076	4b	1.126
3b	1.070	4a-CH₃	1.084	4b-CH₃	1.068
9	1.044	4a-OCH₃	1.118	4b-OCH₃	1.080

3.6.4 Gel electrophoresis

A fluorescent intercalator displacement (FID) assay was conducted to confirm the intercalators' binding mechanism and determine their binding constant to ds-DNA. In FID assay, a fluorescent intercalator first saturates the ds-DNA. A non-fluorescent intercalator, in this case the molecule being studied, was then introduced with gradual increase in concentration. We hypothesized that the fluorescent and non-fluorescent intercalators would bind to similar sites in the ds-DNA, creating a competition for a fixed number of binding sites. Changes in fluorescence intensity are thus expected as the ds-DNA-bound fluorescent intercalators are displaced by the non-fluorescent intercalators. The fluorescent intercalator used in this assay must display sufficient contrast of fluorescence signal between its DNA-bound and free (displaced) states to provide the required sensitivity. It also has to possess fast intercalation kinetics to minimize the time needed to reach equilibrium. Ethidium bromide (EB), one of the most widely-used DNA intercalators, fulfill these criteria²⁵¹ and thus will be used in this study.

The FID assay was carried out in combination with gel electrophoresis. Figure 3-19 shows representative gel electrophoretic data obtained from EB-saturated DNA (EB:hairpin oligonucleotide ratio = 3:2) treated with increasing **3a** concentrations, whereby lanes 1 to 5 correspond to different **3a**:EB ratios. DNA-bound EB (negative overall charge) moved toward the positive pole and while free EB (positively charged) moved toward the negative pole. We observed decreasing fluorescence intensity when **3a**:EB ratio was increased. This showed that more **3a** molecules were bound to the ds-DNA and larger amounts of EB molecules were displaced. For example, at a **3a**:EB molecular ratio as low as 1:4 (lane 3), more than 60% of the bound EB was replaced. This is shown by the diminished fluorescence intensity of bound EB and the increased fluorescence intensity of free EB.

Figure 3-19: Gel electrophoretic results of the competitive binding study of mono-intercalator **3a**. Each sample contains 5 μ M hairpin oligonucleotides and 7.5 μ M EB in PBS buffer. **3a**:EB ratio from lanes 1 to 5 are 0:8, 1:8, 1:4, 3:8, and 1:2.

A closer examination of the gel image showed that there was a systematic change in DNA mobility accompanying the weakening of fluorescence intensity. With increasing ratio of **3a**:EB, the fluorescent bands appeared closer to their initial position in the wells, signifying a lower DNA mobility. This is due to the high molecular weight and dicationic nature of **3a** that collectively lower the mobility of DNA molecules they

are bound to. These results indicate that **3a** bind to ds-DNA *via* the same mechanism as EB, i.e. intercalation, and that it is a stronger DNA intercalator than EB.

In a more quantitative treatment, this gel electrophoretic study was also used to estimate the binding constant K_a using a modified version of the method proposed by Boger.¹⁹³ As discussed in Chapter 2, the fluorescence titration data was first used to determine stoichiometry of the binding. Figure 3-20 (A) shows that the intersection point of pre- and post-saturation portions of the titration curve is at ~0.97. This indicates a 1:1 binding stoichiometry of **3a** to DNA, which is reasonable considering that there are only 5 base pairs in the hairpin DNA. This simple stoichiometry allows the use of Scatchard plot to estimate binding constant.

The next step was to determine the free intercalator concentration at each point:

$$[\text{free intercalator}] = [DNA]_T \left[X - \frac{\Delta F_x}{\Delta F_{sat}} \right]$$

These values were then used in the Scatchard plot (Figure 3-20 B). Finally, the binding constant can be estimated from the slope of the plot, giving a K_a value of $1.94 \times 10^7 M^{-1}$. This value corresponds to an approximately 40-fold enhancement of binding constant over NTCDI.

This considerable enhancement may be brought about by the electrostatic attraction. After intercalation of NTCDI group with ds-DNA, the two cationic side groups of **3a** form ion-pairs with phosphates on each side of the ds-DNA, effectively locking the intercalating group in between the base pairs of ds-DNA. This excellent binding property prompted subsequent use of this intercalator in DNA detection, which will be discussed in Chapter 4.

Figure 3-20: (A) Normalized change of ethidium bromide fluorescence signal in the presence of increasing amounts of **3a** in the gel electrophoresis samples. (B) Scatchard plot for the titration of hairpin oligonucleotides/EB mixtures with **3a**.

3.6.5 Fluorometry

Another FID assay was also conducted through fluorometry to study the binding of the intercalators. The same criteria were used to select the suitable fluorescent intercalators, keeping in mind that binding stoichiometry dictates the use of bis-intercalators to compete with **4**. YOYO-3²⁵² and thiazole orange (TO)²⁵³⁻²⁵⁶ was chosen for this purpose. Both intercalators have been shown to have a greatly enhanced fluorescence upon binding to ds-DNA and are among the most efficient DNA intercalators in the market.

The change of fluorescence signal of initially-bound YOYO-3 during titration of bis-intercalators series **4a** is depicted in Figure 3-21. The decrease of fluorescence signal of the initially bound YOYO-3 was due to its displacement by the gradually added non-fluorescent intercalators. The molecules being studied occupy the same binding sites as YOYO-3, which is a well characterized intercalator. This finding further validated intercalation as the binding mode to ds-DNA. A much smaller amount of **4a-OCH₃** was

needed to displace the initially bound YOYO-3 compared to other molecules in the same series, indicating a significant variation of binding strengths.

Figure 3-21: Fluorescence intensities of a mixture of 25 μM YOYO-3 in 75 μM salmon sperm DNA during titration of various amounts of (A) **4a**, (B) **4a-CH₃**, and (C) **4a-OCH₃**

In a more quantitative approach,^{163, 188, 190} the same data set was used to estimate the apparent binding constant of the newly synthesized molecules:

$$K_{app} = K_{FI} \times \frac{C_{FI}}{C_{50}}$$

where K_{app} is the apparent binding constant of the molecule being studied, K_{FI} is the binding constant of the fluorescent intercalator, C_{FI} is its initial concentration, and C_{50} is the concentration of the molecule of interest that is needed to replace 50% of the bound fluorescent intercalator.

The fluorescence intensity of YOYO-3 in the presence of series **4** bis-intercalators is summarized in Figure 3-22. Using $K_{YOYO-3} = 1.5 \times 10^8 \text{ M}^{-1}$, the apparent binding constants of bis-intercalators in this series were calculated and summarized in Table 3-5. Substitution of methoxy group on the metal complex increases binding constant significantly. Bis-intercalators bind more strongly to ds-DNA than mono-intercalators due

to the chelating effect (binding at multiple sites). This binding may be further enhanced by the electrostatic attraction between the cationic metal complex and the anionic DNA backbone. Verification of this phenomenon requires single crystal crystallography, which is beyond the scope of this thesis.

Figure 3-22: Normalized YOYO-3 fluorescence signal at various amounts of bis-intercalators **4a** (●), **4b** (▲), **4a-CH₃** (●), **4b-CH₃** (▲), **4a-OCH₃** (●), and **4b-OCH₃** (▲).

Table 3-5: Apparent binding constants of transition-metal linked bis-intercalators (M^{-1})*

	$\frac{C_{YOYO-3}}{C_{50}}$	K_{app}		$\frac{C_{YOYO-3}}{C_{50}}$	K_{app}
4a	2	3×10^8	4b	2	3×10^8
4a-CH₃	2	3×10^8	4b-CH₃	2	3×10^8
4a-OCH₃	5	7.5×10^8	4b-OCH₃	3	4.5×10^8

* The uncertainty of the values is within the same order of magnitude.

An identical study was also carried out for mono-intercalators **3a-b**, **8a-c**, and **9** as presented in Figure 3-23. The addition of compounds **3a-b** and **9** caused significant

decrease of fluorescence intensity due to the displacement of TO molecules. However, compounds **8a–c** did not cause any significant effect on the fluorescence signal (Figure 3-23 B), indicating a much lower binding constant than that of TO. The binding capacity of **8a–c** was further limited by their low solubility in aqueous solutions. As a result the concentration of molecules **8a–c** in solution was not high enough to compete with and displace TO.

Figure 3-23: Fluorescence intensities of mixture of 25 μM TO in 75 μM salmon sperm DNA during titration of various amounts of (A) **3a**, (B) **8b**, and (C) **9**

Table 3-6: Apparent binding constant of transition-metal linked mono-intercalators*

	$\frac{C_{TO}}{C_{50}}$	K_{app}		$\frac{C_{TO}}{C_{50}}$	K_{app}
3a	17.5	$5.8 \times 10^7 M^{-1}$	9	0.25	$7.5 \times 10^5 M^{-1}$
3b	12.5	$4.1 \times 10^7 M^{-1}$			

* The uncertainty of the values is within the same order of magnitude.

The apparent binding constants were calculated using a published binding data¹²⁷ $K_{TO} = 3.3 \times 10^6 M^{-1}$, as summarized in Table 3-6. K_{app} value of $6 \times 10^7 M^{-1}$ for **3a**

is very close to the K_a value found earlier from the Scatchard analysis, hence verifying the validity of this method. While symmetrical EDOT intercalator have very limited binding, asymmetrical intercalator **9** with its hybrid structure demonstrates a binding constant that is comparable to that of TO. This observation reinforces an earlier hypothesis that the presence of a cationic group, i.e. the metal complex, enhances DNA binding by increasing the chance of collision and locking the intercalating unit in place upon intercalation. The binding constant of **9** is high enough to justify its potential use in DNA detection, as will be discussed in Chapter 5.

3.7 Conclusions

A series of redox-active DNA intercalators were designed for use as reporters in nucleic acid detection. Each compound contains NTCDI as the intercalating unit and either Os or Ru complexes, EDOT, or a combination of these as the electroactive component. These redox reporters provide selective binding to ds-DNA through intercalation as well as signal amplification through electrocatalysis. NTCDI was linked to the metal complexes through ligand exchange reactions, while EDOT was covalently linked to NTCDI through linkers of various lengths and hydrophilicity. Spectroscopic and spectrophotometric characterization confirmed the formation of these compounds.

The electrochemical properties of these compounds were studied using cyclic voltammetry. For compounds containing Os or Ru complexes, the redox potential is dependent on the intervalence coupling between the ligands and the metal center. Substitutions of methyl and methoxy group on the ligands led to a range of redox potential values. An intercalator with a hybrid structure displayed the electrochemical activities of NTCDI, Os complex, as well as that of EDOT.

Building on the existing reports of the intercalative nature of NTCDI, the binding mechanism of these compounds to ds-DNA was confirmed through UV-Vis spectrophotometry, viscometry, and circular dichroism studies. Hypochromism and red shift of UV-Vis absorption spectra, increase of viscosity, and induced circular dichroism confirmed the intercalative binding of these molecules. Competitive binding studies, observed through fluorometry and gel electrophoresis, were carried out to determine the binding constants. Compounds containing metal complexes were shown to bind more strongly due to the additional electrostatic interaction between the cationic side groups with anionic DNA backbone. Three compounds were selected for subsequent use in nucleic acid detection, as will be discussed in subsequent chapters.

3.8 Experimental

3.8.1 Organic syntheses

Materials

1,4,5,8-naphthalene tetracarboxylic dianhydride ($\geq 95\%$), 1-(3-aminopropyl)-imidazole (98%), 4,4'-dimethyl-2,2'-dipyridyl (99.5%), 4,4'-dimethoxyl-2,2'-dipyridyl (97%), Ru trichloride, and cis-bis(2,2'-bipyridine)dichloro Ru(II) hydrate (99%), bromochlorohexane (97%), tetraethylene glycol ditosylate (97%), 18-crown-6 (99%), sodium iodide (98%), sodium azide (99.5%), sodium hydroxide, triphenylphosphine (99%), and zinc acetate dihydrate ($\geq 98\%$) were purchased from Aldrich (St. Louis, MO). Potassium hexachloroosmate (99%) was purchased from Strem Chemicals (Newbury Port, MA), while 2,2'-bipyridine (99%) was purchased from Avocado Research Chemicals (Lancaster, UK). Anhydrous sodium hydride (95%) was purchased from Sigma-Aldrich, and kept and used inside a glovebox. All chemicals were of reagent grade and used as

received. Anhydrous solvents such as pyridine, tetrahydrofuran (THF), dimethylacetate (DMA), dimethylformamide (DMF), dichloromethane (DCM), and acetonitrile (ACN) was purchased from Sigma-Aldrich in a sure-seal bottle and back-filled with N₂. All other solvents were purchased from J.T. Baker (Phillipsburg, NJ).

General methods

NMR spectroscopic study was done on a Bruker 400 MHz system from Bruker Biospin GmbH (Karlsruhe, Germany). MS was carried out on a Finnigan/MAT LCQ Mass Spectrometer from ThermoFinnigan (San Jose, CA) fitted with an ESI probe. UV-Vis spectrophotometry was run on an Agilent 8453 diode array spectrophotometer. Single crystal X-ray crystallography data was obtained from a Bruker AXS SMART APEX CCD X-Ray Diffractometer from Bruker AXS GmbH (Karlsruhe, Germany).

Synthesis procedure

N,N'-bis[1(3-propyl)-imidazole]-1,4,5,8d-naphthalene diimide (PIND, 1). 1

was prepared following a general procedure previously reported.^{240, 241} In brief, 1,4,5,8-naphthalene tetracarboxylic dianhydride (0.30 g, 1.12 mmol) was added to a magnetically stirred mixture of 1-(3-aminopropyl)-imidazole (0.3 mL, 2.47 mmol, 10% excess) and tetrahydrofuran (3.0 mL). The rate of addition was controlled to minimize clumps. The reaction mixture was refluxed overnight and then cooled to room temperature. Next, it was dispersed in 10 mL of acetone:water (3:1) mixture and poured into rapidly stirred anhydrous ether to precipitate the compound. The precipitate was

collected by suction filtration through a fine fritted funnel and washed briefly with ethanol. Purification was performed by crystallization from chloroform:ethanol (1:1 by volume) and dried under vacuum at 40°C overnight to give **1** as yellow crystals (0.46 g, 85% yield). ^1H NMR (300 MHz CDCl_3) δ 8.76 (4H), 7.54 (2H), 7.26 (2H), 4.27 (4H), 4.12(4H), 2.31 (4H) and 1.83 10(2H). $[\text{PIND}+\text{H}^+] = 483.3$ and $[\text{M}+2\text{H}^+]/2 = 242.3$. HR-MS (FAB): calcd. for $\text{C}_6\text{H}_{22}\text{N}_6\text{O}_4+\text{H}^+$ 483.1781 $[\text{M}+\text{H}^+]$; found 483.1770.

cis-bis(2,2'-bipyridine)dichloroosmium(III) chloride (2a) , **cis-bis(4,4'-dimethyl-2,2'-bipyridine)dichloroosmium(III) chloride (2a-CH₃)**, and **cis-bis(4,4'-dimethoxy-2,2'-bipyridine)dichloroosmium(III) chloride (2a-OCH₃)**. Synthesis of this transition metal complex is based on a published procedure.²⁵⁷ 2,2'-bipyridine (0.65 g, 4.2 mmol) and potassium hexachloroosmate (0.95 g, 2.0 mmol) were magnetically stirred in 20 mL of DMF and refluxed for 1 hour. Upon removal of potassium chloride precipitates by filtration, the reaction mixture was vigorously stirred in diethyl ether to remove DMF. The product **2a**, in the form of brick-colored powder (1.09 g, 96% yield) was obtained upon vacuum drying. **2a-CH₃** and **2a-OCH₃** were synthesized by analogous treatment of potassium hexachloroosmate with the suitable substituted 2,2'-bipyridine compounds.

cis-bis(4,4'-dimethyl-2,2'-bipyridine)dichlororuthenium(II) (2b-CH₃) and **cis-bis(4,4'-dimethoxy-2,2'-bipyridine)dichlororuthenium(II) (2b-OCH₃)**. A mixture of

Ru(III) trichloride hydrate (1 g, mmol, 3.8 mmol, 20% excess) of 4,4'-dimethyl-2,2'-bipyridine (1.2 g, 6.5 mmol), and lithium chloride (1.1 g, 26 mmol) in 60 mL of DMF was stirred under reflux for 8 hours. The solvent was removed by vigorous stirring in diethyl ether. The crude product was then dissolved in chloroform and washed with water. Upon drying, the product **2b** was obtained in the form of black powder (1.72 g, 70% yield). **2b-OCH₃** was synthesized following an identical procedure using 4,4'-dimethoxy-2,2'-bipyridine

N,N'-bis[1(3-propyl)-imidazole]-1,4,5,8d-naphthalene diimide linked to Os(bpy)₂Cl₂ (3a) and Ru(bpy)₂Cl₂ (3b). **3a** was synthesized in a single-step ligand-exchange reaction. Small portions of **1** (0.12 g, 0.25 mmol) was added to a solution of **2a** (0.32 g, 0.52 mmol, 10% excess) in 8.0 mL of fresh-distilled ethylene glycol and was refluxed for 30–40 minutes. The progress of the ligand-exchange reaction, indicated by the disappearance of redox peak of starting materials and formation of those of the products, was monitored by cyclic voltammetry. The product **3a** was precipitated twice from KCl saturated ethanol and was further purified by crystallization from ethanol, yielding the product as dark brown flakes (0.20 g, 52% yield). The product showed a single pair of reversible redox waves with an $E_{1/2}$ of 0.16 V in PBS. By substituting the Os metal complex **2a** with the corresponding Ru complex **2b**, the corresponding **3b** was

obtained with similar appearance and slightly lower yield (0.21 g, 60% yield). HR-MS (ESI): calcd. for $C_{66}H_{54}N_{14}O_4Os_2Cl_3^+$ 1595.2748 [M^+]; found 1595.2744 and calcd. for $C_{66}H_{54}N_{14}O_4Ru_2Cl_2^{2+}$ 690.0953 [M^{2+}]; found 690.0976

Bis- intercalators with Os complexes (4a, 4a-CH₃, and 4a-OCH₃) and Ru complexes (4b, 4b-CH₃, and 4b-OCH₃). A mixture of **1a** (0.72 g, 1.5 mmol, 50% excess) and **2a** (0.3 g, 0.5 mmol) was heated to 150 °C in 10 mL of anhydrous ethylene glycol. The reaction progress was similarly monitored using cyclic voltammetry. Reaction was stopped after two hours and the solvent removed with diethyl ether. The product was extracted into water and washed with chloroform, giving **4a** as dark brown powder (0.35 g, 48% yield).

A series of similar bis-intercalators (with one PIND moiety linked to each side of a Os(bpy)₂ complex) were synthesized using identical procedures and substituting the Os complexes **2a**, **2a-CH₃**, and **2a-OCH₃** with the appropriate Ru complexes **2b**, **2b-CH₃**, and **2b-OCH₃**, respectively. Reaction yields vary in the range of 45% to 65%, with Os-containing intercalators generally having lower yields. HR-MS (ESI): calcd. for $C_{72}H_{60}N_{16}O_8OsCl^+$ 1503.4088 [M^+]; found 1503.4088 and calcd. for $C_{72}H_{60}N_{16}O_8Ru^{2+}$ 689.1906 [M^+]; found 689.1888.

Hydroxymethyl EDOT was synthesized according to a previously described procedure.^{235, 236} Syntheses of **5b-Cl**, **5b-I**, **5c-OTS**, and **5c-I** were carried out using literature methods.²⁵⁸

2-methanesulfonyl-2,3-dihydrothieno[3,4-b][1,4]dioxine (EDOT-C₁-OMs, 5a-OMs). In a 100-mL round-bottom flask with a stir bar, EDOT-OH (924 mg, 5.4 mmole) was loaded and the flask was backfilled with argon three times. Dry CH₂Cl₂ (15 mL) and triethylamine (0.94 mL, 0.68 g, 6.7 mmole) were introduced, and the reaction mixture was cooled in an ice bath. Methanesulfonyl chloride (0.50 mL, 0.79 g, 6.5 mmole) was added dropwise. The ice bath was removed and the reaction mixture was stirred for 18 hours. Water was added to the mixture, the layers were separated, and the aqueous layer was extracted twice with CH₂Cl₂. The combined organic layers were washed with 5% aqueous H₂SO₄, aqueous saturated NaHCO₃ solution and brine, dried with MgSO₄, and evaporated. The crude product was obtained as yellow oil, and used directly for the next step.

2-azido-2,3-dihydrothieno[3,4-b][1,4]dioxine (EDOT-C₁-N₃, 6a). In a 100-mL round-bottom flask, crude product **5a-OMs** from the previous step (5.4 mmole) was dissolved in THF (5 mL) and EtOH (10 mL), and freshly prepared aqueous NaN₃ solution (2.80 g, 43.2 mmole NaN₃ in 10 mL H₂O) was added. The mixture was stirred under

reflux condenser at 80 °C for 48 hours. The majority of the organic solvents were removed by a rotary evaporator, then the aqueous layer was extracted 3 times with ethyl acetate. The combined organic layers were dried with MgSO₄ and evaporated. The product was purified on silica gel flash column (hexane:ethyl acetate = 20:1). The product **6a** (985 mg, 93%) was obtained as a thick colorless oil. ¹H NMR (400 MHz, CDCl₃): δ 6.38 (dd, 2H, *J* = 11.2, 3.6 Hz), 4.33 (ddd, 1H, *J* = 12.0, 6.8, 2.4 Hz), 4.21 (dd, 1H, *J* = 12.0, 2.4 Hz), 4.07 (dd, 1H, *J* = 12.0, 6.8 Hz), 3.59 (dd, 1H, *J* = 13.2, 6.0 Hz), 3.50 (dd, 1H, *J* = 13.2, 6.0 Hz).

2-amino-2,3-dihydrothieno[3,4-b][1,4]dioxine (EDOT-C₁-NH₂, 7a). In a 100-mL round-bottom flask with a stir bar, the azide **6a** (1.43 g, 7.2 mmole) was dissolved in THF (25 mL), and triphenylphosphine (PPh₃; 2.08 g, 7.10 mmole) were added as a solid. Vigorous evolution of nitrogen was observed. The reaction was heated at 50 °C for 1 hour, whereupon freshly prepared NaOH solution (2 M, 25 mL) was added, and the mixture was heated with vigorous stirring for another 2 hours. The majority of THF was removed by a rotary evaporator after acidification with concentrated HCl (pH < 3). The aqueous layer was extracted 3 times with CH₂Cl₂, and the combined organic layers were discarded. NaOH was then added to the aqueous layer, and the resulting solution (pH > 8) was extracted three times with CH₂Cl₂. The combined organic layers were dried with Na₂SO₄ and evaporated. Product **7a** (1.23 g, 100%) was obtained as a colorless viscous liquid. ¹H NMR (400 MHz, CDCl₃): δ 6.33 (dd, 2H, *J* = 8.8, 4.8 Hz), 4.21 (dd, 1H, *J* = 11.2, 2.0 Hz), 4.13 (ddd, 1H, *J* = 11.2, 7.6, 2.0 Hz), 4.07 (dd, 1H, *J* = 12.0, 7.6 Hz), 2.97 (m, 2H),

1.43 (broad s, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 141.7, 141.6, 100.0, 99.6, 75.2, 66.6, 42.3.

***N,N'*-bis[11-(2,3-dihydrothieno[3,4-b][1,4]dioxin-3-yl)methyl]-1,4,5,8d-naphthalene-tetracarboxydiimide. (Bis-EDOT-ND, **8a**).** Naphthalene dianhydride (35.6 mg, 0.133 mmole), EDOT-NH₂ **7a** (50.0 mg, 0.292 mmole), and zinc acetate (20.4 mg, 0.093 mmole) were mixed together in pyridine (10 mL) and refluxed overnight. The reaction mixture was filtered through a short column of silica gel with CH_2Cl_2 as eluent. The organic solution was then washed with HCl (1 N) and deionized water, dried with MgSO_4 , and the solvent was removed by a rotary evaporator. The crude product was further purified by flash chromatography (hexane:ethyl acetate = 5:1) to yield **8a** as an orange solid (64.0 mg, 0.111 mmole, 78%). ^1H NMR (400 MHz, CDCl_3): δ 8.81 (t, 4H, $J = 6.4$), 6.35 (d, 2H, $J = 3.6$ Hz), 6.29 (d, 2H, $J = 3.6$ Hz), 4.75 (dd, 2H, $J = 13.6, 7.6$ Hz), 4.35 (dd, 2H, $J = 13.6, 4.8$ Hz), 4.26 (dd, 2H, $J = 12.4, 2.4$ Hz), 4.12 (dd, 2H, $J = 11.6, 6.4$ Hz). ^{13}C NMR (100 MHz, CDCl_3): δ 163.0, 141.4, 141.1, 131.6, 127.1, 126.7, 100.4, 100.2, 100.1, 71.4, 66.7. HR-MS (FAB): calcd. for $\text{C}_{28}\text{H}_{18}\text{N}_2\text{O}_8\text{S}_2 + \text{H}^+$ 575.0577 $[\text{M} + \text{H}^+]$; found 574.0575.

2-((6-chloro-hexyloxy)methyl)-2,3-dihydrothieno[3,4-b][1,4]dioxine (EDOT-C₆-Cl, 5b-Cl). A solution of EDOT-OH (688 mg, 4.00 mmole) and 18-crown-6 (52.8 mg, 0.200 mmole) in anhydrous THF (10 mL) was added dropwise at 0°C to another air-free flask containing a suspension of sodium hydride (95%, 505 mg, 20.0 mmole) in anhydrous THF (60 mL). The mixture was then introduced to bromochlorohexane (1.60 g, 8.00 mmole) and was refluxed under N₂ overnight. After quenching the excess sodium hydride with water, THF was removed *in vacuo*. The reaction mixture was then washed with brine, and extracted three times with ethyl acetate. The combined organic phase was dried with MgSO₄, and purified by flash chromatography (hexane:ethyl acetate = 10:1) to yield **5b-Cl** (680 mg, 2.34 mmole, 59%) as colorless crystals. ¹H NMR (400 MHz, CDCl₃): δ 6.34 (d, 1H, *J* = 4 Hz), 6.32 (d, 1H, *J* = 3.6 Hz), 4.33–4.27 (m, 1H), 4.24 (dd, 1H, *J* = 11.6, 2.4 Hz), 4.06 (dd, 1H, *J* = 11.6, 7.6 Hz), 3.68 (dd, 1H, *J* = 10.4, 5.2 Hz), 3.60 (dd, 1H, *J* = 10.4, 5.6 Hz), 3.53 (t, 2H, 6.8 Hz), 3.50 (t, 2H, *J* = 6.8 Hz), 1.82–1.73 (m, 2H), 1.64–1.55 (m, 2H), 1.50–1.31 (m, 4H).

2-((6-iodo-hexyloxy)methyl)-2,3-dihydrothieno[3,4-b][1,4]dioxine (EDOT-C₆-I, 5b-I). A solution of **5b-Cl** (680 mg, 2.34 mmole) and sodium iodide (1.72 g, 11.5 mmole) was refluxed in acetone (20 mL) for 18 hours. The reaction mixture was then filtered to remove precipitates, and acetone was removed *in vacuo*. It was then dissolved in ethyl acetate, and washed with saturated Na₂S₂O_{3(aq)}. After purification by flash chromatography (hexane:ethyl acetate = 10:1), the product was obtained as a viscous, light yellow liquid (647 mg, 1.69 mmole, 74%). ¹H NMR (400 MHz, CDCl₃): δ 6.34 (d,

1H, $J = 3.6$ Hz), 6.32 (d, 1H, $J = 4$ Hz), 4.34–4.27 (m, 1H), 4.24 (dd, 1H, $J = 11.6$, 2 Hz), 4.06 (dd, 1H, $J = 11.6$, 7.6 Hz), 3.68 (dd, 1H, $J = 10.4$, 5.2 Hz), 3.60 (dd, 1H, $J = 10.4$, 5.6 Hz), 3.50 (t, 2H, 6.4 Hz), 3.20 (t, 2H, $J = 7.2$ Hz), 1.80–1.75 (m, 2H), 1.65–1.50 (m, 2H), 1.50–1.31 (m, 4H).

2-((6-azido-hexyloxy)methyl)-2,3-dihydrothieno[3,4-b][1,4]dioxine(EDOT-C₆-N₃, **6b).** To a solution of **5b-I** (647 mg, 1.69 mmole) in DMF (4 mL), an aqueous solution (4 mL) of sodium azide (439 mg, 6.76 mmole) was added. After 18 hours of refluxing, DMF was removed by washing with saturated NH₄Cl_(aq). The reaction mixture was extracted in ethyl acetate, the organic layer was washed with water and dried with MgSO₄, and the solvent was removed by a rotary evaporator. After purification by flash chromatography (hexane:ethyl acetate = 19:1), **6b** was obtained as a viscous light yellow liquid (420 mg, 1.41 mmole, 84%). ¹H NMR (400 MHz, CDCl₃): δ 6.34 (d, 1H, $J = 3.6$ Hz), 6.32 (d, 1H, $J = 4$ Hz), 4.33–4.26 (m, 1H), 4.23 (dd, 1H, $J = 11.6$, 2.4 Hz), 4.05 (dd, 1H, $J = 11.6$, 7.2 Hz), 3.68 (dd, 1H, $J = 10.4$, 4.8 Hz), 3.59 (dd, 1H, $J = 10.4$, 6.4 Hz), 3.49 (t, 2H, 6.4 Hz), 3.26 (t, 2H, $J = 7.2$ Hz), 1.67–1.50 (m, 4H), 1.45–1.30 (m, 4H). ¹³C NMR (100 MHz, CDCl₃): δ 141.7, 141.7, 99.9, 99.8, 77.4, 72.8, 72.0, 69.3, 66.4, 51.5, 29.6, 29.0, 26.7, 25.8. HR-MS (FAB): calcd. for C₁₃H₁₉N₃O₃S+H⁺ 298.1225 [M+H⁺]; found 298.1209.

2-((6-amino-hexyloxy)methyl)-2,3-dihydrothieno[3,4-b][1,4]dioxine (EDOT-C₆-NH₂, 7b). A solution of **6b** (297 mg, 1.00 mmole) in THF (5 mL) was mixed with triphenylphosphine (288 mg, 1.10 mmole), and heated to 50 °C for 1 hour. 5 mL of NaOH solution (2 M) were subsequently added, and the reaction was continued for another 2 hours. THF was removed by rotary evaporator, and the aqueous reaction mixture was acidified to pH < 3. The aqueous phase was washed with CH₂Cl₂. NaOH was then added, and the resulting solution (pH > 10) was extracted with CH₂Cl₂. The organic layer was dried with Na₂SO₄, and the solvent was removed *in vacuo*. The purified product **7b** was distilled with Kugelrohr apparatus (170 °C at 20 mTorr) as a viscous yellow liquid (149 mg, 0.549 mmole, 55%). ¹H NMR (400 MHz, CDCl₃): δ 6.34 (d, 1H *J* = 3.6 Hz), 6.32 (d, 1H, *J* = 4.0 Hz), 4.33–4.26 (m, 1H), 4.24 (dd, 1H, *J* = 11.6, 2.0 Hz), 4.05 (dd, 1H, *J* = 11.6, 7.6 Hz), 3.67 (dd, 1H, *J* = 10.4, 4.8 Hz), 3.59 (dd, 1H, *J* = 10.4, 6.0 Hz), 3.49 (t, 2 H, 6.4 Hz), 2.85 (t, 2H, *J* = 7.2 Hz), 1.67–1.50 (m, 4H), 1.49–1.30 (m, 4H). ¹³C NMR (100 MHz, CDCl₃): δ 141.7, 141.7, 99.9, 99.8, 77.4, 72.8, 72.1, 69.3, 66.4, 53.6, 41.8, 40.7, 32.5, 32.5, 30.3, 29.6, 26.8, 26.0, 25.9. HR-MS (FAB): calcd. for C₁₃H₂₁NO₃S+H⁺ 272.1320 [M+H⁺]; found 272.1321.

N,N'-bis[11-(2,3-dihydrothieno[3,4-b][1,4]dioxin-3-yl)methyl]-hexyl-1,4,5,8d-naphthalenetetracarboxydiimide. (Bis-EDOT-ND, 8b). Naphthalene dianhydride

(28.1 mg, 0.167 mmole), EDOT-NH₂ 7b (100.0 mg, 0.368 mmole), and zinc acetate (26.3 mg, 0.093 mmole) were mixed together in pyridine (10 mL). Following a similar purification procedure as that for 8a, 8b was obtained as an orange solid (52.0 mg, 80%) after flash chromatography (hexane:ethyl acetate = 3:1). ¹H NMR (400 MHz, CDCl₃): δ 8.75 (s, 4H), 6.33 (d, 2H, *J* = 3.6 Hz), 6.32 (d, 2H, *J* = 3.6 Hz), 4.32–4.26 (m, 2H), 4.23 (dd, 2H, *J* = 11.6, 2.4 Hz), 4.19 (t, 4H, *J* = 7.6 Hz), 4.05 (dd, 2H, *J* = 11.6, 7.6 Hz), 3.64 (dd, 2H, *J* = 10.4, 4.8 Hz), 6.59 (dd, 2H, *J* = 10.4, 6 Hz), 3.50 (t, 4H, *J* = 3.2 Hz), 1.77–1.64 (m, 4H), 1.61–1.50 (m, 8H), 1.50–1.38 (m, 4H). ¹³C NMR (100 MHz, CDCl₃): δ 163.0, 141.7, 141.7, 131.2, 126.9, 126.8, 99.9, 99.7, 72.8, 72.0, 69.3, 66.4, 41.0, 29.9, 29.5, 28.2, 27.0, 26.0. HR-MS (FAB): calcd. for C₄₀H₄₂N₂O₁₀S₂+H⁺ 775.2359 [M+H⁺]; found 775.2344.

11-(2,3-dihydrothieno[3,4-b][1,4]dioxin-3-yl)methyl-3,6,9-trioxaundecyl tosylate (EDOT-EG₄-OTs, 5c-OTs). A solution of EDOT-OH (1.72 g, 10.0 mmole) and 18-crown-6 (132 mg, 0.500 mmole) in anhydrous THF (10 mL) was added dropwise at 0°C into another air-free flask containing a suspension of sodium hydride (95%, 1.26 g, 50.0 mmole) in anhydrous THF (150 mL). The mixture was then added to tetraethylene glycol ditosylate (1.01 g, 20.0 mmole), and was refluxed overnight under N₂. After quenching the excess sodium hydride with water, THF was removed *in vacuo*. The reaction mixture was then washed with saturated NaCl_(aq) and extracted three times with CH₂Cl₂. The combined organic phase was dried with MgSO₄, and purified by flash chromatography (dichloromethane:ethyl acetate = 10:1). The product was dried under

vacuum to yield a light yellow liquid (1.00 g, 20%). ^1H NMR (400 MHz, CDCl_3): δ 7.80 (d, 1H, $J = 1.6$ Hz), 7.78 (d, 1H, $J = 1.2$ Hz), 6.32 (d, 1H, $J = 3.6$ Hz), 6.31 (d, 1H, $J = 3.6$ Hz), 4.35–4.29 (m, 1H), 4.24 (dd, 1H, $J = 11.6, 2.0$ Hz), 4.15 (t, 2H, $J = 4.8$ Hz), 4.11 (dd, 1H, $J = 14.4, 7.2$ Hz), 4.05 (dd, 1H, $J = 11.6, 7.6$ Hz), 3.76 (dd, 1H, $J = 10.4, 4.8$ Hz), 3.71–3.53 (m, 14H), 2.44 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) NMR (100 MHz, CDCl_3) 145.0, 145.0, 141.7, 141.6, 132.9, 130.0, 128.1, 99.8, 99.7, 72.7, 71.3, 70.8, 70.8, 70.7, 70.6, 70.7, 70.7, 70.6, 69.7, 69.4, 68.8, 66.2, 21.8. HR-MS (FAB): calcd. for $\text{C}_{22}\text{H}_{30}\text{O}_9\text{S}_2 + \text{H}^+$ $[\text{M} + \text{H}^+]$ 503.1404; found 503.1394.

2-((11-iodo-3,6,9-trioxaundecyloxy)methyl)-2,3-dihydrothieno[3,4-b][1,4]

dioxine (EDOT- EG₄-I, 5c-I). A solution of **5c-OTs** (1.00 g, 1.99 mmole) and sodium iodide (1.49 g, 10.95 mmole) was refluxed in acetone (20 mL) for 18 hours. The reaction mixture was then filtered, and acetone was removed *in vacuo*. It was then dissolved in CH_2Cl_2 , and washed with saturated $\text{Na}_2\text{S}_2\text{O}_5(\text{aq})$. After purification by flash chromatography (dichloromethane:ethyl acetate = 19:1), **5c-I** (400 mg, 43.6%) was obtained. ^1H NMR (400 MHz, CDCl_3) δ 6.33 (d, 1H, $J = 3.6$ Hz), 6.32 (d, 1H, $J = 3.6$ Hz), 4.36–4.29 (m, 1H), 4.25 (dd, 1H, $J = 11.6, 2.8$ Hz), 4.06 (dd, 1H, $J = 11.6, 7.2$ Hz), 3.77 (dd, 1H, $J = 10.6, 4.8$ Hz), 3.75 (t, 2H, $J = 5.2$ Hz), 3.71–3.63 (m, 13H), 3.26 (t, 2H, $J = 7.2$ Hz). ^{13}C NMR (100 MHz, CDCl_3) 141.7, 141.6, 99.9, 99.8, 72.8, 72.1, 71.3, 70.8, 70.8, 70.7, 70.7, 69.8, 66.3, 53.7. HR-MS (FAB): calcd. for $\text{C}_{15}\text{H}_{23}\text{IO}_6\text{S} + \text{H}^+$ $[\text{M} + \text{H}^+]$ 459.0333; found 459.0317.

2-((11-azido-3,6,9-trioxaundecyloxy)methyl)-2,3-dihydrothieno[3,4-b][1,4]

dioxine (EDOT-EG₄-N₃, 6c). A solution of **5c-I** (400 mg, 0.873 mmole) in DMF (5 mL) and an aqueous solution (5 mL) of sodium azide (227 mg 3.49 mmole) were mixed together and refluxed for 18 hours. DMF was removed by washing with saturated NH₄Cl_(aq). The reaction mixture was dissolved in CH₂Cl₂, washed with water, and dried with MgSO₄. The crude product was purified by flash chromatography (dichloromethane:ethyl acetate = 19:1) to yield **6c** as a viscous colorless liquid (212 mg, 0.568 mmole, 65%). ¹H NMR (400 MHz, CDCl₃): δ 6.33 (d, 1H, *J* = 4 Hz), 6.32 (d, 1H, *J* = 3.6 Hz), 4.35–4.29 (m, 1H), 4.25 (dd, 1H, *J* = 11.6, 2.4 Hz), 4.06 (dd, 1H, *J* = 11.6, 7.2 Hz), 3.76 (dd, 2H, *J* = 10.8, 5.2 Hz), 3.71–3.52 (m, 15H), 3.39 (t, 2H, *J* = 5.2 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 141.7, 141.6, 99.9, 99.8, 77.4, 72.8, 71.4, 70.9, 70.8, 70.8, 70.7, 70.2, 69.8, 66.3, 50.8. HR-MS (FAB): calcd. for C₁₅H₂₃N₃O₆S+H⁺ 374.1386 [M+H⁺]; found 374.1379.

2-((11-amino-3,6,9-trioxaundecyloxy)methyl)-2,3-dihydrothieno[3,4-b][1,4]

dioxine (EDOT-EG₄-NH₂, 7c). A solution of **6c** (100 mg, 0.268 mmole) in THF (3 mL) was mixed with triphenylphosphine (77.3 mg, 0.295 mmole), and was heated to 50° C for 1 hour. 3 mL of NaOH_(aq) (2 M) was subsequently added, and the reaction was stirred for another 2 hours. THF was removed by rotary evaporator, and the aqueous reaction

mixture was acidified to pH < 3. The aqueous phase was washed with CH₂Cl₂. NaOH was then added, and the resulting solution (pH > 10) was extracted with CH₂Cl₂. The organic layer was dried with Na₂SO₄, and the solvent was removed *in vacuo* to give a viscous **7c** as yellow liquid (80.0 mg, 86%). ¹H NMR (400 MHz, CDCl₃): δ 6.29 (d, 1H, *J* = 3.6 Hz), 6.28 (d, 1H, *J* = 3.2 Hz), 4.32–4.25 (m, 1H), 4.21 (dd, 1H, *J* = 11.6, 2 Hz), 4.02 (dd, 1H, *J* = 11.6, 7.6 Hz), 3.72 (dd, 2H, *J* = 10.4, 4.8 Hz), 3.67–3.57 (m, 15H), 3.47 (t, 2H, *J* = 5.2 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 141.6, 141.5, 99.7, 99.6, 73.0, 72.7, 71.2, 70.6, 70.6, 70.5, 70.2, 69.6, 66.1, 53.6, 41.6. HR-MS (FAB): calcd. for C₁₅H₂₅NO₆S+H⁺ 348.1481 [M+H⁺]; found 348.1478.

***N,N'*-bis[11-(2,3-dihydrothieno[3,4-*b*][1,4]dioxin-3-yl)methyl]-3,6,9-trioxaundecyl-1,4,5,8d-naphthalenetetracarboxydiimide. (Bis-EDOT-ND, **8c**).**

Naphthalene dianhydride (35.1 mg, 0.131 mmole), EDOT-NH₂ **7c** (100.0 mg, 0.288 mmole), and zinc acetate (20.2 mg, 0.092 mmole) were mixed together in pyridine (10 mL). Following a similar procedure as for the synthesis of **8a**, **8c** was obtained as an orange solid (11.0 mg, 30%) after flash chromatography (dichloromethane:ethyl acetate = 2:1). ¹H NMR (400 MHz, CDCl₃): δ 8.75 (s, 4H), 6.31 (d, 2H, *J* = 3.6 Hz), 6.29 (d, 2H, *J* = 3.6 Hz), 4.46 (t, 4H, *J* = 6 Hz), 4.34–4.27 (m, 2H), 4.23 (dd, 2H, *J* = 11.6, 2.4 Hz), 4.04 (dd, 2H, *J* = 11.6, 4.8 Hz), 3.84 (t, 4H, *J* = 5.6 Hz), 3.75 (dd, 2H, *J* = 10.4, 4.8 Hz), 3.72–3.64 (m, 10H), 3.64–3.56 (m, 16H). ¹³C NMR (100 MHz, CDCl₃): δ 163.1, 141.7, 141.6, 131.2, 126.9, 126.8, 100.2, 99.9, 99.8, 78.1, 72.8, 71.3, 70.8, 70.7, 70.7, 70.2, 69.8, 68.0,

66.3, 39.7. HR-MS (FAB): calcd. for $C_{44}H_{50}N_2O_{16}S_2+H^+$ 927.2680 $[M+H^+]$; found 927.2698.

8-azido-3,6-dioxaoctyl tosylate (TsO-EG3-N₃, 5d-N₃). To a solution of NaN₃ (3.25 g, 50 mmol) and NaI (0.30 g, 2 mmol), the chloride **5d-Cl** (1.46 mL, 1.69 g, 10 mmol) was added and the mixture heated at 80 °C over 18h. The solution was transferred into a separatory funnel and extracted with CH₂Cl₂ (5×). The combined organic layers were dried (MgSO₄) and the solution volume reduced to approx. 20-30 mL. Tosyl chloride (2.10 g, 11 mmol) and 4-dimethylaminopyridine (DMAP; 122 mg, 1 mmol) added, followed by dropwise addition of Et₃N (1.6 mL, 1.21 g, 12 mmol). After 18h, the solution was washed with 10% H₂SO_{4(aq)}, saturated NaHCO_{3(aq)}, dried (MgSO₄) and the volatiles removed in vacuum. The azide **5d-N₃** (3.10 g, 94%) was obtained as a colourless liquid after column chromatography (CombiFlash 40 g cartridge, 0 to 60% gradient of ethyl acetate in hexane over 20 minutes). The ¹H and ¹³C NMR data were in agreement with those previously reported.

2-((8-azido-3,6-trioxaoctyloxy)methyl)-2,3-dihydrothieno[3,4-b][1,4]dioxine (EDOT-EG3-N₃, 6d). To a solution of EDOT-OH (1.72 g, 10 mmol) and NaI (0.375 g, 2.5 mmol) and dry DMF (10 mL), NaH (60% in mineral oil; 600 mg, 15 mmol) were added against a weak back-flow of Ar and the mixture stirred over 20 minutes. A solution of **5d-N₃** (3.29 g) in DMF (10 mL) were added dropwise and the mixture stirred over 18h.

The mixture was partitioned between H₂O (300 mL) and diethyl ether (100 mL) and the organic layer was further washed with H₂O (5×). After drying (MgSO₄) and removal of the volatiles in vacuum, **6d** (2.94 g, 89%) were obtained after column chromatography (CombiFlash 40 g cartridge, 30 to 70% gradient of ethyl acetate in hexane over 20 minutes). ¹H NMR (400 MHz, CDCl₃): δ 6.35 (d, 1H, *J* = 3.6 Hz), 6.34 (d, 1H, *J* = 3.2 Hz), 4.36–4.31 (m, 1H), 4.26 (dd, 1H, *J* = 10.8, 1.6 Hz), 4.07 (dd, 1H, *J* = 10.8, 7.6 Hz), 3.77 (dd, 2H, *J* = 10.8, 5.2 Hz), 3.70–3.67 (m, 10H), 3.39 (t, 2H, *J* = 4.8 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 141.6, 141.5, 99.8, 99.7, 72.6, 71.2, 70.7, 70.6, 70.1, 69.6, 66.1, 60.4, 50.1. HR-MS (FAB): calcd. for C₁₃H₂₀N₃O₅S+H⁺ 330.1124 [M+H⁺]

2-((8-amino-3,6-dioxaoctyloxy)methyl)-2,3-dihydrothieno[3,4-b][1,4]dioxine

(EDOT-EG3-NH₂, 7d). A solution of **6d** (660 mg, 2.0 mmol) in THF (10 mL) and triphenylphosphine (577 mg, 2.2 mmol) was heated to 50 °C for 1h. NaOH_(aq) (2 M; 10 mL) was added, and the reaction mixture stirred for another 2h. THF was removed by rotary evaporator, and the aqueous reaction mixture was acidified to pH < 3. The aqueous phase was extracted with CH₂Cl₂ (3×) and the organic layers discarded. To the aqueous layer, NaOH was then added, and the solution (pH > 10) was extracted with CH₂Cl₂ (3×). The organic layer was dried with Na₂SO₄, and the solvent was removed *in vacuo* to give a viscous yellow liquid (388 mg, 86%). ¹H NMR (400 MHz, CDCl₃): δ 6.29 (d, 1H, *J* = 3.6 Hz), 6.28 (d, 1H, *J* = 3.2 Hz), 4.32–4.25 (m, 1H), 4.21 (dd, 1H, *J* = 11.6, 2 Hz), 4.02 (dd, 1H, *J* = 11.6, 7.6 Hz), 3.72 (dd, 2H, *J* = 10.4, 4.8 Hz), 3.67–3.57 (m, 15H), 3.47 (t, 2H, *J* = 5.2 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 141.5, 141.4, 99.7, 99.6, 73.4, 72.6, 71.2, 70.6,

70.5, 70.3, 69.6, 66.1, 41.7. HR-MS (FAB): calcd. for $C_{13}H_{21}NO_5S+H^+$ 304.1213 $[M+H^+]$; found 304.1210.

***N*-[3-(imidazol-1-yl)propyl]-*N'*-[8-((2,3-dihydrothieno[3,4-*b*][1,4]dioxin-3-yl)methoxy)-3,6-dioxaoctyl]-1,4,5,8-naphthalenetetracarboxydiimide (EDOT-ND-Im, **8d**).** Two alternative routes were attempted for the synthesis of **8d**. In a direct synthesis, a mixture of **7d** (50 mg, 0.17 mmol), naphthalene dianhydride (44.3 mg, 0.17 mmol), 1-(3-aminopropyl)imidazole (20.7 mg, 0.17 mmol), and zinc acetate (25.4 mg, 0.12 mmol) was refluxed in anhydrous pyridine (5 mL) for 15 hours. Upon removal of pyridine by vacuum distillation, the reaction mixture was partitioned between CH_2Cl_2 and an aqueous HCl solution (1 N), all the while maintaining a basic pH to ensure deprotonation of imidazole. The combined organic layer was dried with $MgSO_4$ and subjected to further purification by flash chromatography (dichloromethane: ethyl acetate = 1/10).

Alternatively, the two-step synthesis of **8d** began with conversion of naphthalene dianhydride to the monoimide monoanhydride. Naphthalene dianhydride (804.6 mg, 3 mmol, 200% excess) was slowly added to a solution of 1-(3-aminopropyl)imidazole (125.18 mg, 1 mmol) in dimethylacetate (30 mL), and the mixture was heated at 125 °C for 18 hours. Upon cooling to room temperature, 100 mL of chloroform was added to precipitate unreacted dianhydride. The precipitate was then removed by filtration, followed by removal of chloroform from the remaining filtrate by rotary evaporator. Water (150 mL) was then added, forming precipitate that was subsequently washed with

¹H NMR (400 MHz, CDCl₃): δ 8.74 (t, 4H, *J* = 8 Hz), 7.56 (m, 1H), 7.02 (m, 2H), 6.32 (d, 1H, *J* = 3.6 Hz), 6.31 (d, 1H, *J* = 3.6 Hz), 4.48 (t, 2H, *J* = 6 Hz), 4.32–4.25 (m, 1H), 4.23 (dd, 1H, *J* = 11.6, 2 Hz), 4.12 (t, 2H, *J* = 7.6 Hz), 4.05 (dd, 1H, *J* = 11.6, 7.2 Hz), 3.87 (dd, 1H, *J* = 10.4, 6 Hz), 3.71 (t, 2H, *J* = 5.6 Hz), 3.66–3.60 (m, 8H), 2.32 (q, 2H, *J* = 5.6 Hz), ¹³C NMR (400 MHz, CDCl₃): δ 163.10, 163.0, 141.7, 141.6, 137.4, 131.4, 131.2, 129.9, 126.8, 127.0, 126.9, 126.5, 118.8, 99.9, 99.8, 72.8, 71.4, 70.9, 70.7, 70.3, 69.8, 68.0, 66.3, 45.1, 39.8, 38.5, 29.5. HR-MS (FAB): calcd. for C₃₃H₃₂N₄O₉S+⁺ 661.1963 [M+H⁺]; found 661.1972.

***N*-[3-(imidazol-1-yl)propyl]-*N'*-[8-((2,3-dihydrothieno[3,4-*b*][1,4]dioxin-3-yl)methoxy)-3,6-dioxaoctyl]-1,4,5,8-naphthalenetetracarboxydiimide complex with Os(bpy)₂Cl₂ (EDOT-NTCDI-Os (9)).** **2a** (18.10 mg, 0.03 mmol) was added to a solution of **8d** (18.5 mg, 0.03 mmol) in ethylene glycol, and the mixture was heated to 180° C for 6 hours. The progress of the reaction was monitored by cyclic voltammetry. Upon completion, ethylene glycol was removed and the reaction mixture was extracted with chloroform and washed repeatedly with water. The product was obtained as dark purple paste (14.2 mg, 34% yield). HR-MS (FAB): calcd. for [C₅₃H₄₈ClN₈O₉OsS]⁺ 1199.2563 [M⁺]; found 1199.2571.

3.8.2 Electrochemical characterization

For characterization of compounds in series **3** and **4**, electrochemical tests were done on a CHI830 potentiostat from CH Instruments (Austin, TX). A Au disc working electrode, a Pt coil counter electrode (both from CH Instruments), and a nonleak Ag/AgCl (3.0 M KCl) reference electrode from Cypress Systems (Lawrence, KS) form the three-electrode system. A phosphate–buffer saline (PBS, pH 7.4) solution containing 0.15 M NaCl and 20 mM phosphate buffer was used as the supporting electrolyte.

For characterization of compounds **8a–c**, all electrochemical experiments were performed with an Autolab PGSTAT 32 potentiostat (Metrohm) in a glovebox from Innovative Technologies (Newburyport, MA). The one-chamber, three-electrode cell was made up of a quasi-internal Ag wire reference electrode (CH Instruments, Inc.) that is submerged in 0.01 M of AgNO₃/0.1 M of *n*Bu₄NPF₆ in anhydrous CH₃CN, a Pt button working electrodes, and a Pt coil counter electrode. Homo-polymerization was done on 10 mM of monomers **8a–c** in 0.1 M of *n*Bu₄NPF₆/ CH₂Cl₂ solution. For copolymerization,

two mixtures with different monomer ratios were prepared in 0.1 M of $n\text{Bu}_4\text{NPF}_6$ / CH_2Cl_2 . The first mixture contained 1 mM of **8a–c** and 10 mM of EDOT, and the other mixture contained 5 mM of **8a–c** and 5 mM of EDOT. Oxidative polymerization was carried out by repeated potentiodynamic scans between 0.3 and +1.3 V versus Ag/Ag^+ at a scan rate of 100 mV/s.

3.8.3 DNA binding study

UV-Vis spectrophotometry was done on an Agilent 8453 diode array spectrophotometer (Santa Clara, CA). Salmon sperm DNA was obtained from Q-bio Gene (Irvine, CA). Samples consist of mixtures of salmon sperm DNA and intercalators (mono- and bis-intercalators, **3**, **4**, **8**, **9**) at various ratios in 1x PBS buffer (pH 7.4) and were contained in a 1-cm path length quartz cuvette.

Viscometry study was done on an MFR 2100 Micro Fourier Rheometer from GBC Scientific Equipment (Dandenong, Australia). Salmon sperm DNA was sheared to ~500 kb by sonication.²⁵⁹ The dynamic viscosity was read for DNA solution in the presence of various intercalator molecules.

Circular dichroism spectroscopy was done on an Olis DSM 10 Spectrophotometer equipped with a Jobin Yvon SA DH-10 double grating monochromator (Photonitech Pte Ltd., Singapore). Salmon sperm DNA was sonicated before usage to break up the long DNA fragments. The CD of the DNA solution (1 mL, 200 μM base pairs) in buffer (1x PBS, pH 7.4) was recorded over the range 220–320 nm as small volumes of the reporter solution were titrated into the DNA. The resultant small increase in the final volume was less than or equal to 3%. Titrations were continued until no further change was observed in the CD spectrum, indicating that the saturation point of the DNA had been reached.

Fluorometry data were obtained on a Fluorolog®-3 spectrofluorometer from Jobin Yvon Inc. (Edison, NJ). Thiazole orange from Molecular Probes (Eugene, OR) and YOYO-3 from Sigma Aldrich (St. Louis, MO) were used as competitive binding agents for mono- and bis-intercalators, respectively. Excitation wavelength for YOYO-3 and TO are 620 nm and 509 nm, respectively. 1x TE buffer (pH 6.0) was used as buffer.

Both the gel electrophoresis cell Bio-Rad Mini Sub® GT Cell and ethidium bromide was obtained from Bio-Rad Laboratories (Hercules, CA). Hairpin oligonucleotides were custom-made by 1st Base Pte. Ltd. (Singapore) with the following sequence: 5'-AATTT-CCCC-AAATT, 5'-AATAT-CCCC-ATATT, and 5'-ATTTA-CCCC-TAAAT. Samples were loaded onto a 2.5% agarose gel (Seakem® LE) after addition of 10 vol% of glycerin. A potential of 90 V was applied for 15 minutes in TBE (49 mM tris-base, 49 mM boric acid, and 2 mM EDTA) running buffer. The gel was imaged using Chemi Genius gel documentation and analysis system (Syngene, Frederick, MD).

4 Transition-Metal Linked Intercalators for Nucleic Acid Detection

4.1 Introduction

The importance of nucleic acid detection for early diagnosis of diseases has been clearly established.¹⁶ As discussed in the literature review, several optical and electrochemical nucleic acid detection systems have been reported with exceptionally high sensitivity and selectivity.^{55, 108} However, the practical application of these assays is hampered by their complexity, mainly stemming from the need to pre-label the target nucleic acids. For this reason, the use of intercalators is beneficial for their ability to selectively recognize nucleic acid duplexes formed after hybridization of the label-free target DNA with the immobilized capture probes.

There have been numerous reports on the design and synthesis of electrochemical or luminescent intercalators as reporters in nucleic acid detection.^{116, 131, 134, 260, 261} These molecules are comprised of an intercalating unit and a small molecule or biomolecule capable of generating electrical or optical signals. Electrochemical intercalators are of particular interest because electrochemical detection is more cost-effective and capable of rapid, direct, and light-absorbing-tolerant detections. In addition, the detection devices are built with portable, robust, low-cost, and easy-to-handle electrical components, so electrochemical detection is suitable for field tests and point-of-care use.^{24, 60, 95, 117}

For this purpose, electroactive reporter molecules with novel structures and functionalities have been synthesized and characterized as described in Chapter 3. This chapter discusses the use of two transition-metal linked intercalators for direct detection

of DNA. These mono-intercalators—containing one intercalating unit per molecule—are functionalized with either Os (**3a**) or Ru (**3b**) complexes. Two detection schemes will be presented, utilizing the unique capacity of the reporters to intercalate specifically to double-stranded DNA (ds-DNA), i.e. recognizing DNA hybridization, and to generate catalytic electrochemical signal, i.e. providing signal amplification. Most of the data in this chapter has been reported in two journal publications.^{262, 263}

4.2 Detection scheme

Scheme 4-1: General detection scheme using transition-metal linked intercalators

Scheme 4-1 shows a generalized detection Scheme for **3a** and **3b**. The first four steps depict the construction of the biosensors: CP immobilization, electrode surface

passivation, target hybridization, and binding of reporter molecules through intercalation. These steps are common to all the assay strategies that will be discussed. On the other hand, the detection steps differ depending on the type of DNA label used. Although structurally very similar, the distinct redox potentials of these electroactive intercalators present different strategies for signal amplification, as will be discussed in the next sections. All potential values are referenced to Ag/AgCl.

4.3 Construction of nucleic acid biosensor

4.3.1 CP immobilization

Capture probes—made of 20-50 base-pair (bp) long thiol-functionalized oligonucleotides—were immobilized as a self assembled monolayer (SAM) on the electrode surface through thiol-Au chemistry.¹⁵⁰ The CP SAM was formed after immersion of clean Au electrode in a PBS solution of CP.

Surface density of the CP was assessed electrochemically using cationic redox molecules as indicators. In this method, proposed by Tarlov,¹²⁵ the CP-modified electrode was placed in an electrolyte solution containing multivalent cations rutheniumhexamine(III) $\text{Ru}(\text{NH}_3)_6^{3+}$. Due to electrostatic interactions, these cations bind to the anionic phosphate backbone of the oligonucleotides at a rate proportional to their charge (3+). The amount of bound $\text{Ru}(\text{NH}_3)_6^{3+}$ at the saturation point can be used to derive the amount of CP.

Figure 4-1: Chronocoulometric response of electrodes modified with mercaptododecane (—) and mixed CP/mercaptododecane monolayer (—) in the absence (dashed) and presence (solid) of $\text{Ru}(\text{NH}_3)_6^{3+}$.

Chronocoulometry was used to measure the amount of $\text{Ru}(\text{NH}_3)_6^{3+}$ under equilibrium conditions, with the representative experimental result presented in Figure 4-1. In a chronocoulometric experiment, the charge Q as a function of time is given by the integrated Cottrell expression:

$$Q = \frac{2nFAD_0^{1/2}C_0^*}{\pi^{1/2}}t^{1/2} + Q_{dl} + nFA\Gamma_0$$

where:

n = number of electrons per molecule for reduction,

F = Faraday constant (96,485 C/equiv),

A = electrode area (cm^2),

D_0 = diffusion coefficient (cm^2/s),

C_0^* = bulk concentration (mol/cm^3), and

Q_{dl} = capacitive charge due to double layer charging (C)

$nFA\Gamma_0$ = charge from the reduction of the redox marker per unit area (mol/cm^2)

The term $nF\Delta\Gamma_0$ represents the amount of redox marker confined near the electrode surface. It can be derived from the chronocoulometric experiment, where the intercept at $t = 0$ represents the sum of Q_{dl} and $nF\Delta\Gamma_0$. The surface excess term $nF\Delta\Gamma_0$ can thus be determined from the difference in charge at $t = 0$ for the identical potential step experiment in the presence and absence of redox marker, as depicted in Figure 4-1. Experimental data gives the value of $nF\Delta\Gamma_0$ at $(1.28-1.48)\times 10^{-6}$ C. Substituting $A = 7.07 \times 10^{-2} \text{ cm}^2$ and $n = 1$, the amount of adsorbed $\text{Ru}(\text{NH}_3)_6^{3+}$ was obtained: $\Gamma_0 = (1.88 - 2.17) \times 10^{-10} \text{ mol/cm}^2$.

Finally, the saturated surface excess of $\text{Ru}(\text{NH}_3)_6^{3+}$ is converted to CP surface density based on the relationship:

$$\Gamma_{DNA} = \Gamma_0 (z / m)$$

where Γ_{DNA} = probe surface density (mol/cm^2), m = number of bases in the probe DNA, and z = charge of the redox molecule (C). With $m = 50$ for the 50-bp long CP and $z = 3+$ for $\text{Ru}(\text{NH}_3)_6^{3+}$, CP density was obtained as $\Gamma_{DNA} = (1.13 - 1.30) \times 10^{-11} \text{ mol/cm}^2$ or $(6.80-7.82) \times 10^{12} \text{ molecules/cm}^2$.

4.3.2 Electrode surface passivation

The use of thiol monolayers¹⁵⁰ for surface passivation in electrochemical biosensors has been previously shown to be beneficial especially in reducing background signals.^{156, 157} Thiol molecules with long alkane chains form a compact monolayer that occupy the remaining electrode surface and prevent nonspecific adsorption of target oligonucleotides and reporter molecules.¹⁵⁵ Furthermore, capture probes in the mixed

CP/thiol monolayer are oriented at a better angle—as opposed to lying flat on the electrode surface—hence facilitating hybridization.

For the assays described in this chapter, a mixed CP/thiol monolayer was achieved by immersing the CP-coated electrode in a 10 mM ethanolic solution of mercaptododecane. Mercaptododecane was selected because it forms a compact monolayer with suitable thickness and hydrophilicity. Thiol concentration and duration of immersion is controlled to avoid replacement of bound CP by thiol molecules.

To ensure the effectiveness of the monolayer in minimizing background signal, cyclic voltammetry study was carried out to compare oxidation current of potassium ferricyanide in the presence and absence of thiol monolayer. The thiol monolayer formed is expected to be stable under the operational conditions of the electrochemical sensor.^{155, 158} as shown in Figure 4-2.

Figure 4-2: Cyclic voltammogram of potassium ferricyanide on electrodes with a monolayer of mercaptododecane (—), mixed monolayer of CP/mercaptododecane (—), and on bare Au electrode (—).

4.3.3 Target hybridization

Hybridization was carried out by depositing a droplet of solution containing target nucleic acid on the mixed monolayer-coated electrode. The hybridization temperature was set at $\sim 27^\circ\text{C}$ below the salt-adjusted melting temperature¹ of the perfectly matched CP-target duplex, i.e. a low stringency condition. It will later be shown that this condition provides acceptable specificity when combined with the multiple washing steps as described in the experimental section.

Hybridization efficiency of an oligonucleotide DNA target

Hybridization efficiency is defined as the fraction of CP that is actually hybridized upon the addition of a given concentration of matching target. For oligonucleotides samples (50-bp), the hybridization efficiency was evaluated at the high end of the dynamic range of target concentration (500 nM). The amount of hybridized DNA was calculated using Tarlov's method¹²⁵ as described in the previous section, and was found to be $(2.07\text{-}2.55)\times 10^{-12}$ mol/cm². On the other hand, surface density of CP was estimated to be 1.22×10^{-11} mol/cm², i.e. midrange of the values calculated from experimental data. Comparing these two quantities, the hybridization efficiency was thus found to be in the range of 17-21%.

For a 3 mm-diameter electrode, amount of hybridized DNA was worked out to be $(1.46\text{-}1.80)\times 10^{-13}$ mol. Considering that the sample droplet contain only one sequence, the

¹ $T_m = 81.5^\circ\text{C} + 16.6^\circ\text{C} \times (\log_{10}[Na^+] + [K^+]) + 0.41^\circ\text{C} \times (\%GC) - 675 / N$

Salt adjusted melting temperature, where N is the number of base in the oligonucleotide.

amount of target sequence in the 2.5 μL sample droplet was 1.25×10^{-12} mol. This means 12-14% of the target DNA was hybridized, comparable to the estimation found in the literature.^{152, 155}

Hybridization efficiency of a genomic mRNA target

Due to the significant difference in size, a separate study was done to study the hybridization efficiency of the mRNA target. Quartz crystal microbalance (QCM) was used to quantify the mass increase due to hybridization, as summarized in Table 4-1. The amount of hybridized mRNA was derived from the frequency change, given that the area of the quartz crystal resonator is 0.22 cm^2 and molecular weight of *TP53* is $3.9 \times 10^5 \text{ g/mol}$. Only $\sim 40 \text{ fmol}$ of *TP53*—or $\sim 1.6\%$ of the surface-bound CP—was hybridized, much less than the amount reported for short oligonucleotides (20-50-mers).^{154, 155} This lower hybridization efficiency is reasonable considering the much longer nucleic acid target.

Table 4-1: QCM data showing mass change upon hybridization of *TP53* mRNA target

	Δf (Hz)	Δm (ng)	<i>TP53</i> (fmol)
1	17	15	38
2	18	16	41
3	16	14	36
4	19	17	43

4.3.4 Binding of electroactive reporter

After the multiple washing steps to remove nonspecifically bound DNA, a droplet of intercalator solution was deposited on the electrode and left to bind to the hybridized

nucleic acid for 10 minutes. Another washing step was then carried out to remove nonspecifically bound reporter molecules. The final washing solution contains high salt concentration (NaCl-saturated) to remove most of reporter molecules that are electrostatically-bound to nucleic acid, while the 10% ethanol content served to minimize hydrophobic attraction of reporter molecules to the mercaptododecane-coated electrode surface. The amount of reporter molecules bound on DNA surface after washing should thus be proportional to that of hybridized DNA and represents the amount of complementary target sequence.

For subsequent discussions, the term ‘biosensor’ refers to a Au electrode with various elements immobilized on its surface according to the procedure described up to this point: immobilization of a mixed CP/thiol monolayer, hybridization of target DNA, and incubation with reporter molecules (**3a** or **3b**).

4.4 Application of Os-functionalized intercalators for nucleic acid detection

The mono-intercalator containing Os complex (**3a**) was used for detection of DNA oligonucleotides in two strategies: direct detection *via* the oxidation current of the reporters and amplified detection through catalytic oxidation of ascorbic acid by the reporters.

4.4.1 Loading of **3a** reporters

In order to correlate the signal from the reporters to the concentration of target nucleic acid, there should be excess amounts of reporter molecules for binding to nucleic acid duplexes throughout the dynamic range. It is therefore necessary to study the loading of these reporter molecules on the duplex-modified electrode surface.

The actual number of bound intercalator molecules can be estimated from the charge under oxidation current peak obtained from cyclic voltammetry. As shown in Figure 4-3 trace (—), a biosensor with 500 nM complementary target DNA and bound **3a** exhibited a current of 0.58 μA , corresponding to a charge of 5.01×10^{-7} C. Since two electrons are transferred per **3a** molecule, the amount of active and intercalated **3a** was calculated to be 2.6×10^{-12} mol. This amount represents <1% of molecule contained in the 2.5 μL assay droplet, ensuring the availability of excess **3a**. Considering that this analysis was based on a high target concentration (500 μM), the result confirmed that a maximum **3a** loading could be achieved throughout the dynamic range. Furthermore, since the amount of hybridized DNA on this electrode has been obtained with an average of 1.63×10^{-13} mol (8.1×10^{-12} mol basepairs), a ratio of one **3a** molecule per four DNA base pair was obtained. This value agrees well with that obtained from the solution-based UV-Vis binding study in section 3.6.

4.4.2 Direct detection by cyclic voltammetry

The first detection strategy was based on direct correlation of the reporter's redox signal to target DNA concentration; a strategy that has been previously utilized for other reporters. The target samples for this test were 50-bp complementary and noncomplementary oligonucleotides at various concentrations. The electrochemical

response of the biosensors with various target DNAs, observed through cyclic voltammetry, is shown in Figure 4-3.

Figure 4-3: (A) Cyclic voltammograms of biosensors with (—) 1.0 μM noncomplementary target DNA and (—) 10, (—) 200, and (—) 500 nM complementary target DNA. Voltammograms were recorded in PBS at a scan rate of 100 mV/s. (B) Calibration plot of voltammetric responses of biosensors with various concentrations of complementary target

Trace (—) in Figure 4-3 shows that for the noncomplementary DNA sample, one pair of minute voltammetric peaks was observed at the redox potential of **3a**. It was most probably generated by a small amount of **3a** bound through pure electrostatic interaction with CP. This indicates negligible nonspecific hybridization and effectiveness of the washing processes in removing background signal. Traces (—), (—), and (—) show the cyclic voltammograms of biosensors with complementary target DNA at increasing concentrations. A pair of redox peaks at $E_{1/2} = 0.16$ V, corresponding to the reversible redox reaction of $\text{Os}^{2+}/\text{Os}^{3+}$, was observed in each voltammogram. These peaks are significantly higher than that corresponding to a noncomplementary target and are

proportional to the concentration of complementary target. This is because the intercalating unit of the reporter molecules selectively interacts with the ds-DNA that has been formed upon hybridization of CP and matching targets, giving a greatly enhanced analytical signal compared to unhybridized capture probes.

The proportional increase of the redox signal to target concentration demonstrates that the intercalator selectively interacts with ds-DNA and forms very stable adducts of **3a**/ds-DNA. It also reveals the effectiveness of the proposed washing procedure in eliminating most of the non-DNA related uptake of reporter molecules. These results validate the direct detection strategy and the usage of **3a** as hybridization reporter in DNA biosensors. Subsequent cyclic voltammetry studies at even lower target concentrations revealed a detection limit of 1.5 nM (3.8 fmol) and a dynamic range up to 500 nM.

4.4.3 Mechanism of signal amplification

In the second assay, an amplification strategy was explored in which the Os complex of reporter molecules acts as a catalytic mediator for the oxidation of ascorbic acid (AA). The catalytic reaction, as illustrated in Scheme 4-2, produces higher currents that are still proportional to the concentration of complementary target DNA. This facilitates detection of target nucleic acid at lower concentrations and hence improves the detection limit.

Scheme 4-2: Catalytic oxidation of ascorbic acid by Os complex

Figure 4-4: Cyclic voltammograms of biosensors with 1.0 μM complementary target DNA: in PBS buffer containing 5.0 mM AA before (—) and after (—) **3a** incubation; and in blank PBS after **3a** incubation (—). Voltammograms were recorded at a scan rate of 100 mV/s.

To confirm this catalytic mechanism, the electrochemical signals of the biosensors under various conditions were studied through cyclic voltammetry as depicted in Figure 4-4. Trace (—) shows that in the absence of **3a** molecules, a large overpotential was needed for AA oxidation on the modified electrode surface. This is due to the reduced electron transfer efficiency through the layers of CP/thiol and target oligonucleotides assembled on the electrode surface. Upon addition of **3a**, as shown in trace (—), this overpotential was greatly reduced to result in the negative shift of E_p by as much as 690 mV to 0.16 V.

Comparing the two cyclic voltammograms, AA oxidation current rose more rapidly and the peak current was enhanced about 4-fold in the presence of AA. The increase of peak current and decrease of anodic overpotential demonstrate an efficient electrocatalysis of AA. The reduction of overpotential is a result of a kinetic effect that

can be attributed to the improvement in the reversibility of the electron transfer process.²⁶⁴ These significant improvements of voltammetric response after **3a** intercalation are thus proof of the genuine catalytic effect of the Os complex.^{80, 81}

Taking advantage of the successful amplification, the protocol was applied for detection of targets at lower concentrations. It was found that the catalytic current, observed through cyclic voltammetry, is proportional to the target DNA concentration in the range of 0.06-100 nM. A detection limit of 25 pM was found, 60-fold higher than that of the direct detection discussed in the previous section. Higher catalytic current was observable with higher AA concentration, but with very little improvement in detection limit due to a simultaneous increase in background signal.

4.4.4 Amplified detection by amperometry

On the basis of the above voltammetric studies, it was estimated that better analytical characteristics can be achieved in amperometry. Due to the catalytic effect, AA oxidation on the modified electrode takes place at a lower potential where possible interference is minimized and background signal is reduced. This leads to a potentially higher signal-to-noise ratio and lower detection limit.

In this amplified assay, the amperometric response was measured upon addition of AA to the electrolyte, with the potential set at the oxidation potential of **3a**. Typical amperometric responses of biosensors with various types of target DNA are presented in Figure 4-5 (A). As shown in trace (—), for a biosensor with 25 pM complementary target DNA, a current of 0.38 μ A was detected upon the addition of AA to the electrolyte solution. In contrast, a biosensor with 200 pM noncomplementary target DNA showed only a 0.012 μ A increment in AA oxidation current (—) under the same condition.

Detection of 25 pM target DNA is now possible, already surpassing the detection limit of the direct voltammetric method, with even lower target concentrations possible.

Figure 4-5: (A) Amperometric responses of biosensors with (—) 25 pM complementary target DNA, (—) 25 pM one-base mismatched DNA, (—) 25 pM two-base mismatched DNA, and (—) 200 pM noncomplementary DNA at a potential of 0.20 V upon addition of 5.0 mM AA into the electrolyte solution. (B) Amperometric response of (—) background current and (—) 600 fM complementary DNA at the detection limit.

Selectivity of the biosensor was further evaluated by comparing the results with one- and two-base mismatched target DNA. Current increments of 0.11 μA and 0.035 μA were detected for one- (—) and two-base mismatched (—) sequences, readily allowing discrimination between the perfectly matched and mismatched oligonucleotides. These results demonstrate the successful usage of **3a** as reporter in electrochemical nucleic acid biosensor with improved sensitivity and selectivity.

To capitalize on the amplification strategy, a much lower range of target concentrations were explored. A calibration plot was constructed based on the catalytic

oxidation current of 5.0 mM AA in PBS at potential of 0.20 V, as shown in Figure 4-6. A linear relationship between the amperometric oxidation current and the target DNA concentration was observed in the range of 1-300 pM with a correlation coefficient of 0.992. The detection limit—defined as a signal-to-noise ratio of 3—was found to be 600 fM, as shown in Figure 4-5 (B). This corresponds to 1.5 amol or 23 fg of oligonucleotide target in a 2.5 μ L sample droplet. Compared to the detection limit of 1.5 nM for direct voltammetry method, a 2500-fold improvement in sensitivity has been achieved. This result demonstrates the promising use of the novel electroactive intercalators in improving the sensitivity of the method while maintaining simplicity.

Figure 4-6: Calibration plot of the amperometric responses of biosensors with various concentrations of complementary target.

4.5 Application of Ru-functionalized intercalators for nucleic acid detection

A mono-intercalator containing Ru complex (**3b**) was used for direct detection of oligonucleotide (40 bp) and *TP53* gene (1182 bp). The biosensors in these assays were constructed according to the procedure described in section 4.3.

4.5.1 Direct detection of oligonucleotide

The calculation of hybridization efficiency and reporter loading for oligonucleotide sample gave similar values to those found for **3a**. In the first assay, the presence of the oligonucleotide sample was directly detected by measuring the oxidation current of bound electroactive reporters **3b**. Cyclic voltammograms of the biosensors after incubation and washing are shown in Figure 4-7.

Figure 4-7: Cyclic voltammograms of biosensors with (—) 200 nM of poly(T)₄₀ hybridized to a noncomplementary CP, and with 200 nM of (—) poly(AT)₂₀, (—) poly(AG)₂₀, and (—) poly(G)₄₀ hybridized to their respective complementary CPs. Voltammograms were recorded in PBS at a scan rate of 100 mV/s.

Each biosensor in Figure 4-7 was constructed with its own distinct CP sequence. For a biosensor with noncomplementary poly(T)₄₀ (—) target, a minute pair of voltammetric peaks were observed at the redox potential of **3b** ($E_{1/2} = 0.65$ V). This arises from **3b** molecules that are nonspecifically bound to the electrode surface through pure electrostatic interaction with the capture probes. For biosensors with matching CP/target pairs—poly(AT)₂₀, poly(AG)₂₀, and poly(G)₄₀—peak currents increased by as much as 100-fold to 0.4-0.9 μ A. Slight positive shifts of 8.0 ± 2.0 mV from 0.65 V were also observed due to the less efficient electron transfer to the modified electrode surface.

Interestingly, while the cathodic currents were fairly constant across the various complementary target sequences, a noticeable increment in anodic current was observed for complementary poly(AG)₂₀ (—) and poly(G)₄₀ (—) targets. This increment increased almost linearly with guanine content, indicating that guanine bases in the oligonucleotides are catalytically oxidized at 0.65 V by the intercalated **3b** molecules as illustrated in Scheme 4-3. This explanation is justifiable given the fact that guanine has the lowest oxidation potential out of the four DNA bases ($E^\circ \pm 1.3$ V vs. normal hydrogen electrode),²⁶⁵ making it susceptible to oxidation by numerous electrochemical systems including Ru complexes.

Scheme 4-3: Catalytic oxidation of guanine base by Ru complex

These results not only confirm the suitability of **3b** for DNA detection, but also present an opportunity for unique signal amplification method through catalytic oxidation of guanine base by the same reporter. This is especially useful for simplifying the assay since no other reagent was needed to generate the amplification.

4.5.2 Amplified detection of *TP53* gene

Genomic nucleic acids are longer and contain more guanine residue per molecule; they can thus generate higher catalytic currents through guanine oxidation. It is therefore logical to propose that a genomic nucleic acid sample can be detected more sensitively. The 1182-bp long *TP 53* gene was selected to demonstrate this hypothesis.

Table 4-2: QCM data showing mass change after the *TP53* mRNA hybridization and **3b** reporter binding.

	Δf (Hz)	Δm (ng)	3b (nmol/cm ²)
1	5.4	4.7	15
2	7.4	6.5	22
3	5.5	5.0	17
4	6.9	6.1	21

Unlike in previous assays, the sample solution in this assay contains a mixture of mRNA with the full length *TP53* mRNA being targeted for detection. First, reporter loading to the hybridized nucleic acid was studied using QCM measurements, with the results summarized in Table 4-2. The relatively high loading of **3b** reporter indicates that although hybridization only occurs at a short 20-bp segment of the target, the secondary structure of mRNA allows binding of more reporter molecules.

Figure 4-8: Cyclic voltammograms of DNA biosensors with (—) perfectly matched and (—) one-base mismatched capture probes for detection of *TP53* mRNA target in 1.0 μg of mRNA mixture. Voltammograms were recorded in PBS at a scan rate of 100 mV/s.

Figure 4-8 shows the representative cyclic voltammograms obtained from biosensors with mixed mRNA sample. Again, in this case the two biosensors contain different CP sequences. The considerably larger anodic peaks in both voltammograms indicate that more electrons are involved in the oxidation process. The contrast of anodic/cathodic peaks is also more pronounced than that observed for nucleotide samples (Figure 4-7), most probably due to the much longer *TP53* mRNA molecules bringing more guanine bases to the biosensor surface.

A biosensor with one-base mismatched capture probes was used as control to evaluate the selectivity of the assay. As shown in Figure 4-8 trace (—), the peak current for this biosensor was only ~40% of that for the biosensor with perfectly matched CP. Considering that the sample was a mixture of mRNA, this result shows excellent selectivity of the assay against one-base mutation and other mRNA sequences in biological samples.

Subsequent tests to quantify and establish the dynamic range of the assay called for samples with known concentration. Purified cDNA, obtained through reverse transcription of *TP53* mRNA, was therefore used for this purpose. A biosensor with noncomplementary capture probes was used as control in this experiment. It was found that the current increased linearly with cDNA concentration from 2.5 to 350 pM with a detection limit of 1.5 pM, corresponding to 0.60 ng/mL. Taking the 1.0 μ L sample volume into consideration, as little as 1.5 amol of *TP53* cDNA was successfully detected using the proposed method. Compared to the previous results of direct nucleic acid oxidation assays, the sensitivity of genomic nucleic acid assay was greatly improved by adopting the catalytic threading intercalator scheme.

4.6 Conclusions

Two electroactive intercalators (**3a** and **3b**) have been successfully used as reporters in various electrochemical detection schemes of nucleic acid sequences. In direct detection, the redox signal of these reporters could be directly correlated to target concentration. The detection limit for oligonucleotide targets was in the nM range.

Signal amplification, and thus improvement of assay sensitivity, was achieved by taking advantage of the metal complexes' catalytic nature. The Os complex in intercalator **3a** acted as catalytic mediator for oxidation of ascorbic acid. Measurement of amperometric oxidation current result in a detection limit of 600 fM for a 50-bp oligonucleotide target.²⁶³ The Os complex in this assay acts solely as a redox indicator reporting the presence of target nucleic acid.

In a different scheme, the Ru complex in intercalator **3b** catalytically oxidize the guanine bases of target DNA, enabling detection of 1182 bp-long *TP53* cDNA with a

detection limit of 1.5 pM.²⁶² In this case, the Ru complex plays a dual role as a reporter as well as a redox mediator for the intrinsic redox signal of guanine base. In both of the schemes, the detection limit was also improved because the current signals were observed at low potential regime (0.16 V for **3a** and 0.65 V for **3b**) where interference from other redox processes is minimized.

Aside from the improved sensitivity, these assays have also demonstrated sufficient selectivity against sequences with only one-base mismatch. This demonstrates the selectivity of intercalator binding as well as the effectiveness of the detection procedure in minimizing background signal. Furthermore, since these molecules recognize and bind to ds-DNA through intercalation, the need for pre-labeled target DNA or a second reporter probe is eliminated, hence reducing complexity and costs.

In conclusion, the findings in this chapter are of particular significance for the design of simple yet sensitive nucleic acid biosensors. The proposed schemes promise to greatly improve the sensitivity and practicality of electrochemical detection, which in turn enable the development of simple, low-cost, and portable systems for point-of-care molecular diagnosis.

4.7 Experimental

4.7.1 Materials

A phosphate buffered saline (PBS, pH 7.4), containing 0.15 M NaCl and 20 mM phosphate buffer, was used for CP immobilization and also as supporting electrolyte throughout the electrochemical analysis. TE buffer (pH 8.0) containing 10 mM Tris-HCl, 1.0 mM EDTA, and 0.10 M NaCl served as the hybridization buffer. And for final washing, a NaCl-saturated phosphate buffer (pH 7.4) containing 10% ethanol was used.

Hexaammineruthenium (III) chloride and ascorbic acid used in electrochemical analyses were purchased from Sigma Aldrich (St. Louis, MO).

Capture probes used in this work were custom-made by Alpha-DNA (Montreal, Canada). Oligonucleotide samples were also custom-made by 1st Base Pte Ltd (Singapore), with the sequences listed in Table 4-3. The mRNA sample containing the target *TP53* gene (1182 base pair) was extracted from human breast tissues using a Dynabeads® mRNA DIRECT™ Kit (DynaL Biotech ASA, Oslo, Norway) according to the manufacturer's protocol.

Table 4-3: Base sequences of CP and targets for detection using reporter **3a**

CP	5'-GCCAGCGTTCAATCTGAGCCATGATCAAACCTCTT CAAATGCCGATTAGGC-(A) ₆ -(CH ₂) ₆ -thiol
Complementary	5'-GCCTAATCGGCATTTGAAGAGTTTGATCATGGCT CAGAT TGAACGCTGGC
One-base mismatched	5'- GCCTAATCGGCATTTGAAGAGTTTGATCATGGC TCAGAATGAACGCTGGC
Two-base mismatched	5'-GCCTAATCGCATTTGAAGAGTTTGATCATGGCT CAGAATGAACGCTGGC
Non-complementary	5'-AGGCCTTAAGATACGCTATTAAGCTACTAGTTGG CCTAAAGCTGATTCCA

4.7.2 Electrochemical setup

Electrochemical experiments were carried out using a CH Instruments model 660C potentiostat coupled with a low current module (CH Instruments, Austin, TX). A conventional three-electrode system, consisting of a 3.0 mm-diameter Au working electrode, a nonleak-miniature Ag/AgCl reference electrode (Cypress Systems, Lawrence, KS), and a Pt wire counter electrode, was used in all electrochemical measurements. A

patterned hydrophobic film was used to contain the sample droplet on the Au electrode surface. All potentials reported in this work were referenced to the Ag/AgCl electrode.

Except for section 4.4.4, all analytical signals were observed through cyclic voltammetry with PBS buffer as supporting electrolyte and a scan rate of 100 mV/s. In section 4.4.4, the amperometric current of AA electrooxidation was measured in vigorously stirred PBS containing 5.0 mM AA. At low DNA concentrations, smoothing was applied after each amperometric measurement to remove random noise and electromagnetic interference

4.7.3 General protocol for biosensor construction

CP immobilization and surface passivation

The preparation and pretreatment of Au electrodes were as previously described.^{26,31} Gold working electrodes were first exposed to oxygen plasma for 20 minutes and then immediately immersed in absolute ethanol for 20 minutes to reduce the oxide layer, followed by rinsing in water and drying with a stream of nitrogen. A CP monolayer was adsorbed by immersing the Au electrode in a PBS solution of 100 µg/mL (5.4 µM) CP for 16–24 hours. After adsorption, the electrodes were copiously rinsed and soaked in PBS for 20 minutes, and rinsed again with water before being dried with a stream of nitrogen. To form the self-assembled thiol monolayer, the CP-coated electrodes were subsequently immersed in an ethanolic solution of 2.0 mg/mL 1-mercaptopdodecane (MD) for 4 hours. Excess MD molecules were then rinsed off and the electrodes were washed by immersion in a stirred ethanol for 10 minutes and followed by thorough rinsing with ethanol and water. The electrode was ready after blow drying with nitrogen.

Target hybridization and reporter binding

For hybridization, a CP coated electrode was placed in a moisture saturated environmental chamber with controlled temperature maintained at 60 °C. For oligonucleotide and cDNA samples, a 2.5 µL aliquot of hybridization solution containing various concentrations of the target DNA in TE buffer was uniformly spread onto the electrode and left to hybridize for 30 minutes. For mRNA mixture sample, 1.0 µg of mRNA mixture in TE was left to hybridize at 53 °C. The electrode was then rinsed thoroughly with a blank hybridization solution at the hybridization temperature. Next, the electrode was incubated at 25 °C for 10 minutes with a 5.0 µL aliquot of 100 µg/mL solution of intercalator **3a** or **3b** in hybridization buffer. This step allowed the intercalators to attach to the hybridized CP-target DNA. Upon air cooling, the electrode was held at room temperature for 10 minutes followed by a final washing step with NaCl-saturated phosphate buffer (pH 7.4) containing 10% ethanol.

5 Ethylenedioxythiophene Linked Intercalator for Nucleic Acid Detection

5.1 Introduction

Electroactive nucleic acid reporters that combine intercalative and electrocatalytic properties have been shown to enhance sensitivity while maintaining the simplicity of DNA detection assays.^{262, 263} Meanwhile, several reports have demonstrated substantial signal amplification through nanoparticle-mediated^{56, 107} or enzyme-catalyzed^{229, 230} material growth in conjunction with sandwich DNA assay. Combining this signal amplification strategy with the direct detection of DNA using electroactive intercalators, herein we propose a novel molecular approach of intercalator-mediated polymer growth to achieve sensitive DNA detection. The assay centers on the electroactive reporter **9** with a hybrid structure comprising a 3,4-ethylenedioxythiophene (EDOT) and an Os complex linked to a central unit of 1,4,5,8-naphthalenetetracarboxylic diimide (NTCDI) intercalator. Applying this detection strategy with an emphasis on minimizing background interferences, amperometric signal is expected to be in the μA to mA level. This is substantially higher than that obtained from direct voltammetric detection methods, potentially leading to lower detection limit and allowing ultrasensitive detection.

5.2 Detection scheme

Scheme 5-1: Detection scheme using EDOT-linked intercalators

As depicted in Scheme 5-1, the first four steps of the biosensor construction are identical to those described in section 4.3. Following appropriate washing steps, a proportionate amount of reporter molecule **9** is immobilized on the electrode surface through intercalation. Direct detection can be achieved by measuring the oxidation current of Os complex. The novel amplification strategy comes into play when the EDOT moieties in the reporter molecules act as ‘seeds’ for subsequent polymer growth. Upon addition of free monomers to the electrolyte solution and a carefully controlled electropolymerization process, the amount of polymer formed is positively related to that of reporter molecule **9**, and by extension, to the amount of nucleic acid target.

5.3 Construction of the nucleic acid biosensor

5.3.1 Electrode preparation

The Au electrodes used in this study were cleaned mechanically using alumina slurry and then electrochemically by applying cyclic potential. The detailed procedure is described in the experimental section. As shown in Figure 5-1, after several scans the electrode showed a characteristic anodic peak current near +1.3 V and a single cathodic peak near +0.8 V in dilute sulfuric acid solution.

Figure 5-1: Cyclic voltammogram of a Au electrode in 0.1 M H₂SO₄ at a scan rate of 100 mV/s.

5.3.2 Capture probe immobilization

Peptide nucleic acid (PNA)—DNA analogs with neutral peptide backbone—were used as capture probes in this assay. Unlike DNA, PNA capture probes do not interact electrostatically with the cationic reporters due to their neutrality.^{142, 144, 145} This leads to the reduction of background current from nonspecifically bound reporter molecules. PNAs also form more stable and specific hybrids with DNA,¹⁴³ allowing a better

selectivity against base mismatches. In addition, the enhanced duplex stability enables the biosensors to operate at lower salt concentrations where intercalator binding constant is enhanced.

The probe sequence was designed to target the N1 gene of avian flu virus. Cysteine was attached to the N-terminus of PNA capture probe to allow thiol-Au interaction with the electrode surface. A self-assembled capture probe monolayer was formed after overnight immersion of the electrode in PBS buffer solution containing 1 μ M PNA. Since the capture probes are not charged, their density cannot be determined using Tarlov's method. However, similar capture probe density is expected based on previously reported studies.

5.3.3 Electrode surface passivation

Passivation of the electrode surface by thiol monolayer has been shown to reduce background signal in electrochemical biosensors.^{156, 157} The performance of 1-mercaptododecane and 11-mercaptoundecanol—two candidate thiol molecules with similar lengths—was evaluated using cyclic voltammetry. A monolayer was first formed on separate electrodes, followed by the proposed steps for reporter binding and washing. The oxidation current in the absence of capture probe and target comes solely from nonspecifically-bound reporter molecules, and is thus indicative of the effectiveness of the monolayer in blocking the background signals.

A slightly higher background current was observed for the electrode passivated with 11-mercaptoundecanol, probably due to a less compact arrangement of alkyl chains. However, a pair of distinct redox peaks was detected at the oxidation potential of **9** for the electrode covered with a mercaptododecane monolayer, while virtually no distinguishable

peaks were found on the mercaptoundecanol-passivated electrode. This indicates that more reporter molecules are adsorbed on the mercaptododecane layer even after washing, probably due to the hydrophobic interaction between **9** and the long alkyl chain. Overall, the mercaptoundecanol layer is the better choice for electrode passivation. The polar hydroxyl end-group in mercaptoundecanol is also more resistant to oxidation, allowing a wider potential range for electroanalysis.²⁶⁶ Since both monolayers were of similar thickness, the kinetic of electron transfer across the monolayer is not significantly altered.¹⁵¹ The capture probe-immobilized electrodes for the assay were thus passivated by immersion in a 10 mM ethanolic solution of 11-mercaptoundecanol for 3 hours.

Figure 5-2: Cyclic voltammograms of **9** after incubation for 3 hours on electrodes passivated with 10 mM of (—) 1-mercaptododecane and (—) 11-mercaptoundecanol.

5.3.4 Target hybridization

Targets for this assay are DNA oligonucleotides (18 bp). Hybridization was carried out at 37 °C (~20 °C below the melting point), with Triton X-100 added to the hybridization buffer to further reduce nonspecific hybridization. Noncomplementary and two-base mismatched sequences were used as controls, while a nucleotide-free

hybridization buffer was used for ‘blank’ experiment. To remove the nonspecifically bound oligonucleotides and minimize background signals, multiple hybridization washes were carried out at various stringency levels as detailed in the experimental section.

5.3.5 Binding of electroactive reporters

Reporter binding was achieved by brief incubation of the electrodes with a droplet of hybridization buffer containing the reporter molecules. This was followed by a series of final washing steps with TE buffer containing 10% ethanol and saturated NaCl to eradicate nonspecific hydrophobic and electrostatic interactions, respectively. The term ‘biosensor’ in the subsequent discussions of this chapter refers to a Au electrode that has been modified according to the procedure described up to this point.

As shown in Figure 5-3, a biosensor with 1 μM complementary target DNA and bound reporter **9** exhibited a current of 0.12 μA , corresponding to a charge of 5.24×10^{-8} C. Since only one electron is transferred per reporter molecule, the amount of active and intercalated **9** was calculated as 5.43×10^{-13} mol. The slightly lower loading of reporter molecule **9**—compared to those for symmetrical transition-metal linked intercalators **3a** and **3b**—can be attributed to the lower positive charge and thus less stable binding of this reporter to DNA duplex as discussed in Chapter 3. There is also a possibility of lower density of immobilized PNA capture probe compared to DNA capture probe. This may lead to a lower amount of nucleic acid duplex and hence lower reporter loading. Nevertheless, this value ensures that excess reporter molecules were available to saturate DNA duplex with target concentrations up to 1 μM . It can then be deduced that the redox signal of the reporters is a direct indication of the amount of target DNA.

5.4 Application of EDOT-Os hybrid intercalator (9) for nucleic acid detection

Unlike the detection strategies described in Chapter 4, the hybrid structure of reporter molecule **9** used in this chapter enable signal generation from two different electroactive moieties. The $\text{Os}^{2+}/\text{Os}^{3+}$ redox process was used for direct detection; however more effort was focused on enhancing the assay sensitivity through the use of EDOT moiety in the subsequent amplification strategy. All the potential values are referenced to Ag/AgCl.

5.4.1 Direct detection by square wave voltammetry

Direct detection entails a direct correlation between electrochemical signal from reporter to the concentration of target oligonucleotide. Figure 5-3 presents a comparison of $\text{Os}^{2+}/\text{Os}^{3+}$ oxidation peaks observed in two different voltammetric methods. In both voltammograms, an oxidation peak was observed at the $\text{Os}^{2+}/\text{Os}^{3+}$ oxidation potential ($E_{1/2} = 0.14$ V). A significantly higher current was observed for the complementary target compared to the noncomplementary target at the same concentration, demonstrating selective binding of reporter molecules to capture probe/target duplexes. The detectable peak for the noncomplementary target might have been produced by reporter molecules **9** that were nonspecifically bound either through hydrophobic interaction with the passivation layer or electrostatic interaction with nonspecifically hybridized target DNA.

Cyclic voltammetric study, as shown in Figure 5-3 (A), revealed the behavior of the Os complex in **9** as an ideal, independent one-electron surface-confined redox system based on the reversibility of the pair of $\text{Os}^{2+}/\text{Os}^{3+}$ redox peaks. Square wave voltammetry (SWV) study, while unable to give such information, was able to enhance the voltammetric signals for the same set of samples. As shown in Figure 5-3 (B), more

defined peaks with ~ 3 -fold increase of current intensities were observed for both samples, and a higher contrast was achieved between biosensors with complementary and noncomplementary targets. These improvements are inherent advantages of SWV, in which the applied pulsed potential and sampling points are designed to negate nonfaradaic (charging) component of the current and remove background signal.

Figure 5-3: (A) Cyclic voltammograms of **9** on biosensors with 1 μM of (—) noncomplementary and (—) complementary oligonucleotide targets at a scan rate of 100 mV/s. (B) The corresponding signals observed in SWV. Voltammograms were recorded in aqueous 0.1 M LiClO_4 solution.

SWV is thus chosen for subsequent voltammetric measurements for the next set of samples with lower target concentrations of 20–100 pM, as shown in Figure 5-4 (A). A biosensor that was incubated in an oligonucleotide-free buffer at the hybridization step was used as the ‘blank’ experiment from which the background current was determined. Besides the noncomplementary target sequence, an oligonucleotide sample with two-base mismatch was used as an additional control to test the specificity of the assay.

At this lower concentration range, as shown in Figure 5-4 (A), the peak current obtained for complementary targets are still higher than that from noncomplementary target. However, the signal differences were very limited and the background current had become significant at this low current range. Even after subtraction of the background signal, taken as the current on the 'blank' sample, the peak current output of 100 pM complementary target was only ~3.5 fold of the signal from noncomplementary target of the same concentration (Figure 5-4 B).

Figure 5-4: (A) Square-wave voltammograms of **9** on biosensors with (—) no target, (—) 100 pM noncomplementary target, (—) 20 pM two-base mismatched target, (—) 20 pM complementary target, and (—) 100 pM complementary target. (B) Peak current from the corresponding biosensors after subtraction of signal from blank experiment.

5.4.2 Amplification strategy

The amplification strategy is based on a controlled polymer growth initiated by the reporter molecule **9**. In this regard, the reporter molecules behave as functionalized EDOT monomers. Hydroxymethyl-functionalized (EDOT-OH) was chosen as the

additional monomer primarily due to its solubility in aqueous solutions. The feasibility of co-polymerization of these two modified EDOTs was studied through repeated potential cycling as shown in Figure 5-5 (A). The cyclic voltammograms showed a negative shift of onset potential after the initial scan and an increase of charging current after each scan, indicating polymer growth on the electrode surface.

Figure 5-5: (A) Electropolymerization of 1.25 mM mixture of **9** (10%) and EDOT-OH (90%) by 8 cycles of potential scan. (B) Cyclic voltammogram of the resulting copolymer. Voltammograms were recorded on Au electrode in 0.1 M $n\text{Bu}_4\text{NPF}_6/\text{CH}_3\text{CN}$ at a scan rate of 100 mV/s.

Figure 5-5 (B) confirms the formation of the desired copolymer, with the cyclic voltammogram showing the redox activities of both components. A pair of redox peaks at $E_{1/2} = 0.22$ V represents the $\text{Os}^{2+}/\text{Os}^{3+}$ oxidation process in polymer matrix. This peak was superimposed on broad waves with no distinguishable peak, which is a typical characteristic of conducting polymers.²²⁷ A pair of redox peak was also observed at $E_{1/2} = -1.06$ V, signifying the reduction of NTCDI unit. At this negative potential range, the poly(EDOT) film is not electrochemically doped and thus has a lower conductivity and a

less efficient electron transfer kinetic. As a result, the NTCDI redox peak has a low intensity and we no longer observe the two-electron oxidation peaks.

Selective growth of polymer

The success of this amplification strategy hinges on the selective growth of polymer to represent the amount of target nucleic acid. It is therefore crucial to confirm that the electropolymerization is dependent on the electrode surface. In order to prove our hypothesis, the polymerization of EDOT-OH was studied on various electrodes representing the different stages of biosensor construction (Figure 5-6). The electropolymerization of poly(EDOT) and its derivatives, like other p-type conducting polymers, takes place by oxidation of the monomers. A lower concentration of 5 mM of EDOT-OH was chosen in order to achieve a better control of the electropolymerization.

Figure 5-6: Cyclic voltammograms of EDOT-OH oxidation on Au electrode with various modifications: SAM of mercaptoundecanol (—), mixed monolayer of capture probe/mercaptoundecanol and target DNA (—), and complete biosensor with mixed monolayer of capture probe/mercaptoundecanol, target DNA, and reporter molecule **9** (—).

Cyclic voltammograms in Figure 5-6 shows that the onset potential for monomer oxidation depends on the modification of electrode surface. In this case, a perfect thiol SAM forms a compact monolayer that limits electron transfer to and from the electrode surface such that monomer oxidation only occurs at potentials above 0.98 V (—). On the other hand, the less compact capture probe/thiol mixed monolayer allows oxidation to initiate at a lower potential (—).

On biosensors with existing EDOT moieties (in the form of bound reporter molecule **9**), monomer oxidation begins at a potential that is 40 mV lower (—) compared to an identical system without molecule **9** (—). This negative shift demonstrates the capacity of the reporter molecule to selectively enhance subsequent polymerization of EDOT, hence validating the proposed amplification strategy.

Beyond the onset potential, oxidation current increases drastically and it becomes extremely difficult to control the extent of electropolymerization. An optimum oxidation potential should thus be high enough to initiate polymerization but not too high to control the rate of polymerization. The key is to ensure that the amount of polymer formed can be positively related to the concentration of target nucleic acid.

5.4.3 Amplified detection through polymer growth

From this point onwards, the electroactivity of EDOT moiety in the reporter molecule—rather than that of Os complex—will be utilized. By attaching an EDOT monomer to a DNA intercalator in each reporter molecule, EDOT moieties were brought to the electrode surface at an amount proportional to the complementary target. Due to their immobilization onto the electrode surface, these EDOT molecules experience a more facile electron transfer and are therefore readily oxidized—presumably to form oligomers

or polymer of EDOT. This was achieved by applying cyclic potential between -0.2 and 1.0 V on the assembled biosensors in monomer-free electrolyte solution.

Figure 5-7: (A) Cyclic voltammogram of poly(EDOT) formed after repeated potential scans in blank electrolyte on biosensors with no target (—), 100 pM noncomplementary target (—), 20 pM two-base mismatched target (—), 20 pM complementary target (—), and 100 pM complementary target (—). (B) Amperometric response during electropolymerization of 5 mM EDOT-OH at 0.9 V on biosensors described in (A). An aqueous 0.1 M LiClO₄ solution was used as electrolyte.

The voltammetric response after this potential cycling was studied by cyclic voltammetry as shown in Figure 5-7 (A). Biosensors with complementary target show a greater increase of current upon the application of cyclic potential, indicating the formation of a greater amount of oligo/poly(EDOT) from the surface-bound EDOT monomers. To enhance the signal, 5 mM EDOT-OH was added to the electrolyte to grow the polymer film. After fine-tuning the conditions for electropolymerization, it was found that amperometric polymer growth at 0.90 V for 120 seconds served as the optimized condition as shown in Figure 5-7 (B). Applying higher potentials or prolonging the

polymerization time resulted in a significant increase of polymer growth on the control experiments, hence increasing the background signal.

Figure 5-8: (A) Cyclic voltammograms of poly(EDOT)s formed after seed-mediated electropolymerization of 5 mM of EDOT-OH on biosensors with no target (—), 100 pM noncomplementary target (—), 20 pM two-base mismatched target (—), 20 pM complementary target (—), and 100 pM complementary target (—). Voltammograms were recorded in an aqueous solution of 0.1 M LiClO₄ at a scan rate of 100 mV/s. (B) Current intensity of biosensors as described in (A) at 0.3 V after subtraction of signal from the blank experiment (no target).

Upon the amperometric polymerization, the resulting polymer was again studied by cyclic voltammetry as shown in Figure 5-8. The amount of polymer growth is positively related to the amount of pre-existing poly(EDOT) on the electrode surface, suggesting that these oligomers/ polymers indeed acted as ‘seeds’ for subsequent polymerization. The additional monomers tend to grow on these ‘seeds’ because the propagation step (addition of monomer to a growing polymer or oligomer) is generally less energetically demanding than the initiation step.²²⁷ This is due to the lower over-

potential requirement in the propagation step, given the improved electron transfer and availability of alternative stepwise polymer propagation steps besides the original radical-radical coupling.

The formation of poly(EDOT-OH) films resulted in larger peak current output (sub- μA level) in cyclic voltammograms, enabling satisfactory detection of 20 pM DNA target. After subtraction of the background signal, a greater signal contrast was observed between the biosensors with complementary targets and those with noncomplementary and two-base mismatched targets as illustrated in Figure 5-8 (B). This contrast is also much improved compared to the signal previously obtained from the $\text{Os}^{2+}/\text{Os}^{3+}$ redox couple. The contrast of peak current output between complementary noncomplementary DNA targets was >80 fold that obtained from SWV. Using the same method with various concentrations of complementary target, a calibration plot was obtained as presented in Figure 5-9.

Figure 5-9: Calibration plot of current response from biosensors with various concentrations of complementary target. Current intensity taken at 0.3 V after subtraction of the signal from blank biosensor (without target).

This amplification strategy benefits from the high conductivity of poly(EDOT) in the aqueous electrolytes, resulting in a high detection current. Background currents were reduced under the optimal condition due to the high energy barrier to polymerization in the absence of ‘seeds’. When combined, these two factors lead to a lower detection limit. In addition, due to the nature of the conducting polymer, the formation of poly(EDOT) can also be detected using other techniques such as quartz crystal microbalance, surface-plasmon resonance, fluorometry, and colorimetry.

5.5 Conclusions

An electroactive reporter with a hybrid structure linking an EDOT monomer and an Os complex to a central NTCDI intercalating unit has been utilized for direct and amplified detection of DNA. Direct detection was achieved by correlating target DNA concentration to the oxidation peak of Os complex observed in SWV. The amplified detection was based on the selective growth of conducting polymer on the biosensors, promoted by the presence of the reporter molecules that have been brought to the electrode surface through intercalation. An analytical procedure was designed, at which the amount of polymer formed was positively related to target DNA concentration. The experimental results demonstrate the validity of the proposed amplification scheme in amplifying the electrochemical signal and improving the selectivity against noncomplementary and mismatched targets, thus enabling target detection at lower concentrations. The combination of the novel reporter molecule and amplification strategy hence constitutes a potential approach for simple and sensitive detection of nucleic acids.

5.6 Experimental

5.6.1 Materials

PNA capture probes used in this work were custom-made by Applied Biosystems (Foster City, CA) while all other oligonucleotides were custom-made by 1st Base Pte Ltd (Singapore). The sequences are designed based on the N1 gene of avian flu virus.

Table 5-1: Base sequences of capture probe and targets for detection using reporter 9

Capture probe	N→ C: TTT GAG TCT GTT GCT TGG (EO) – Cys
Complementary	5'-CCA AGC AAC AGA CTC AAA
Two-base mismatch	5'-CCA AGG AAC AGA GTC AAA
Noncomplementary	5'-GGT TCG TTG TCT GAG TTT

PBS buffer (pH 7.4) was used for capture probe immobilization. Hybridization buffer was TE buffer (pH 8.0) containing 0.1 M NaCl and 0.01% Triton X-100. A NaCl-saturated TE buffer containing 10% ethanol was used for final washing of the electrodes before electrochemical analysis.

5.6.2 Methods

Electrochemical experiments were carried out using a CH Instruments model 660C potentiostat coupled with a low current module (CH Instruments, Austin, TX). A conventional three-electrode system, consisting of a 3.0-mm-diameter Au working electrode, a nonleak-miniature Ag/AgCl reference electrode with 3 M KCl (Cypress Systems, Lawrence, KS), and a Pt wire counter electrode, was used in all electrochemical measurements. An aqueous solution of 0.1 M LiClO₄ was used as the supporting

electrolyte in electrochemical analysis. All potentials reported in this work were referenced to the Ag/AgCl electrode.

5.6.3 Detection procedure

The Au electrode was first prepared by mechanical polishing with 0.5 μm alumina slurry, followed by ultrasonication. Electrochemical cleaning was subsequently done by repeated potential scanning from -0.3 to 1.5 V (vs. Ag/AgCl) in 0.1 M H_2SO_4 solution. Upon rinsing with water and drying with a stream of nitrogen, the electrode was ready to use.

Immobilization of PNA capture probe was achieved by immersing the Au electrode in a 1 μM solution of capture probe in PBS for 12 hours, leading to the formation of a self assembled monolayer of capture probe. After adsorption, the electrode was copiously rinsed and soaked in PBS and blown dry with a stream of nitrogen. To minimize nonspecific uptake of target DNA and intercalator, and improve the quality and stability of the capture probe monolayer, the capture probe-coated Au electrode was immersed in an ethanolic solution of 1 mM 11-mercaptoundecanol (MUD) for 3 hours. Unreacted MUD was rinsed with ethanol and then with water. Upon blow drying with nitrogen, the electrode was ready for the next step.

The hybridization of target DNA was done in a moisture-saturated chamber maintained at 37 $^{\circ}\text{C}$. A 2.5 μL aliquot of hybridization solution containing the target DNA was uniformly spread onto the capture probe-coated electrode and left to hybridize for 4 hours. To remove nonspecifically bound DNA from the electrode surface, a series of high- and low- stringency washes was carried out.

The electrode was first washed in a stirred hybridization buffer (blank, with 0.01% Triton X-100) at 37 °C for 5 minutes, followed by immersion in blank TE buffer at room temperature for 1 minute, and finally a brief wash in water. A 3.0 μ L aliquot of 100 μ M solution of **9** in TE buffer was then added to the electrode surface, allowing it to incubate at room temperature for 15 minutes. After being thoroughly rinsed with the final washing solution, the electrode was ready for the electrochemical analysis.

The electrochemical analysis was achieved in three steps. First, the redox reaction of $\text{Os}^{2+}/\text{Os}^{3+}$ was observed through SWV in 0.1 M solution of $\text{LiClO}_{4(\text{aq})}$. Next, the electrode was subjected to five cycles of potential scan from -0.2 to 1.0 V to form oligoEDOTs. These serve as ‘seeds’ for subsequent polymerization. In the final step, the electrode was immersed in a 0.1 M aqueous solution of LiClO_4 containing 5.0 mM of EDOT-OH. A constant potential was applied at 0.10 V for 120 seconds to allow polymerization of the EDOT-OH monomers. Lastly, after a brief washing with water, the electrode was analyzed in a blank electrolyte solution to quantify the copolymer film that has been formed.

6 Conclusions and Suggestions for Future Work

6.1 Conclusions

A series of electroactive molecules were designed to incorporate a DNA intercalating unit: 1,4,5,8-naphthalenetetracarboxylic diimide (NTCDI) and electroactive moieties: Os, Ru metal complexes, or EDOT. For reporter molecules containing Os or Ru complexes, a range of redox potential values were obtained by modifying the ligands. UV-Vis spectrophotometry, gel electrophoresis, viscometry, and circular dichroism spectroscopy were used to confirm the intercalative binding of these molecules to ds-DNA. Their binding constants were estimated through a fluorometric competitive binding assay, and were found to be comparable to or higher than that of the best commercial intercalators currently available. This binding is enhanced by positive charge that facilitates electrostatic attraction with the polyanionic DNA.

Three of these molecules were used in electrochemical detection schemes. The general scheme is based on the immobilization of capture probes on Au electrodes, followed by the hybridization of target nucleic acids and binding of reporter molecules. Direct detection was achieved by measuring the oxidation peak of Os or Ru complex in cyclic or square wave voltammetry. Enhanced sensitivity was achieved through various amplification strategies. The capacity of Os complex to catalytically oxidize ascorbic acid leads to sensitive amperometric detection of DNA oligonucleotides, while the compatibility of the electrocatalytic activity of Ru complex with guanine oxidation results in amplified detection of 1182 base-pair long *TP53* cDNA. In another detection scheme, the EDOT moieties on the reporter molecules act as ‘nuclei’ that selectively enhance

poly(EDOT) polymerization such that the amount of polymer formed could be positively related to target DNA concentration.

In conclusion, the unique combination of selective binding to hybridized nucleic acid and electrochemical activity for signal amplification allows the usage of the newly synthesized electroactive reporters toward simple and sensitive detection of nucleic acids.

6.2 Suggestions for future work

Electrochemical biosensors are at the forefront of research toward achieving extraordinarily sensitive detection of nucleic acid, as evident from the enormous research activity in this direction. The need for rapid, decentralized detection of pathogens presents a clear motivation for nucleic acid biosensors that are not only sensitive and accurate, but also simple, portable, and cheap. The main challenge in achieving point-of-care detection is to find an optimal balance between sensitivity and simplicity of the detection platform.

On the detection front, ongoing efforts are dedicated toward improving signal amplification, sensitivity, and detection limits should be accompanied by efforts in simplifying of the detection procedure. As with other bioaffinity sensors, special attention should be given to nonspecific binding or adsorption of mismatched sequences that ultimately determines the detection limit. Future endeavors should be aimed at improving the hybridization efficiency by compensating for steric constraints, and improve molecular and biological stability of probes. Almost all reports on the development of the nucleic sensors have dealt with relatively short, synthetic oligonucleotides target. Future studies may focus on the analysis of genomic DNAs directly isolated from organisms and organs, including human blood.

Aside from the detection platform, the realization of a functional detection system requires extensive developmental work, mainly involving the integration and automation

of the electronics and microfluidics components. An ideal device should combine the sample collection, DNA extraction, and amplification within the detection platform on a single microchip. Upon overcoming the challenges in detection technology and integration, it is reasonable to expect that electrochemical biosensors will become powerful tools for disease diagnostics in the near future.

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