New layered nanomaterials for sensing applications

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**NANYANG
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**NEW LAYERED NANOMATERIALS FOR
SENSING APPLICATIONS**

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SCHOOL OF PHYSICAL & MATHEMATICAL SCIENCES
NANYANG TECHNOLOGICAL UNIVERSITY

2017

NEW LAYERED NANOMATERIALS FOR SENSING APPLICATIONS

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Summary

This thesis explores various analytical detection techniques employing the use of novel nanomaterials. In particular, the design and application of electrochemical and fluorometric sensors based on graphene and black phosphorus are described. A total of **five (5) chapters** are presented in this thesis.

Chapter 1 is an introductory chapter and presents a brief overview of nanomaterials, with particular emphasis on two novel layered nanostructures: graphene and black phosphorus. It also discusses the common analytical transduction techniques applied in the development of chemical sensors. The significance of sensing techniques and the role of nanomaterials in sensing applications are also illustrated with two organic compounds: nitro-substituted aromatic explosives and deoxyribonucleic acid.

Chapter 2 discusses the application of electrochemically exfoliated graphene for trace electrochemical detection of nitroaromatic explosives.

Chapters 3 and 4 explore the application of nanomaterials in fluorescence-based DNA detection. The application of the graphene quantum dot as a nanoquencher for fluorescence resonance energy transfer-based DNA sensing is explored in **Chapter 3**, whereas **Chapter 4** highlights the application of the black phosphorus nanoparticle as a nanofluorophore in DNA detection.

Chapter 5 provides a brief outlook and concludes the thesis.

List of Publications

Ying Teng Yew, Adriano Ambrosi and Martin Pumera. Nitroaromatic explosives detection using electrochemically exfoliated graphene. *Sci. Rep.* **2016**, 6, 33276.

Ying Teng Yew, Adeline Huiling Loo, Zdeněk Sofer, Kateřina Klímová and Martin Pumera. Coke-derived Graphene Quantum Dots as Fluorescence Nanoquencher in DNA Detection. *Submitted*.

Ying Teng Yew, Zdeněk Sofer and Martin Pumera. Black Phosphorus Quantum Dots as a Novel Fluorescent Sensing Platform for Nucleic Acid Detection. *Submitted*.

List of Abbreviations

°C	Degree centigrade
AFM	Atomic force microscopy
BBS	Borate buffered saline
BPNP	Black phosphorus nanoparticle
cDNA	Complementary deoxyribonucleic acid
CV	Cyclic voltammetry
DLS	Dynamic light scattering
DMF	<i>N,N</i> -dimethylformamide
DNA	Deoxyribonucleic acid
DNT	2,4-Dinitrotoluene
DPV	Differential pulse voltammetry
<i>ds</i>	Double-stranded
EDA	Electron donor-acceptor
FAM	Carboxyfluorescein
FRET	Fluorescence resonance energy transfer
FT-IR	Fourier transform infrared spectroscopy
GC	Glassy carbon
GQD	Graphene quantum dot
hr	Hour (time)
LD ₅₀	Median lethal dose
LOD	Limit of detection
mDNA–3	Deoxyribonucleic acid with 3 mismatched bases
min	Minute (time)
mM	Millimolar
NAC	Nitroaromatic compound
ncDNA	Non-complementary deoxyribonucleic acid
nM	Nanomolar
nm	Nanometer
<i>p.a.</i>	Pro analysis

PL	Photoluminescence
ppm	Parts per million
R	Sample correlation coefficient (measure of linearity)
R^2	Coefficient of determination (measure of linearity)
rpm	Rotation per minute
SEM	Scanning electron microscopy
ss	Single-stranded
STEM	Scanning transmission electron microscopy
TEM	Transmission electron microscopy
TPa	Terapascals
TNT	2,4,6-Trinitrotoluene
Tris-HCl	Tris hydrochloride
UV–vis	Ultraviolet–visible
w/w	Mass fraction
XPS	X-ray photoelectron spectroscopy
XRD	X-ray powder diffraction
λ_{ex}	Excitation maxima

Chapter 1:

Introduction

1.1 Nanomaterials

1.1.1 Overview

1.1.2 Graphene: Discovery, Properties and Synthesis

1.1.3 Black Phosphorus: Discovery, Properties and Synthesis

1.2 Analytical Techniques in Chemistry

1.2.1 Overview

1.2.2 Electrochemical Detection

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1.1 Nanomaterials

1.1.1 Overview

Nanomaterials are a class of materials with a minimum of one critical dimension spanning less than 100 nm.¹ At the nanoscale, materials exhibit many new, unique and extraordinary properties and phenomena which promise a wide range of potential applications. The ability to manipulate materials at the atomic scale has attracted vast interest and unprecedented growth in the innovative research and commercial sectors, for the construction of novel nanostructures and nanodevices with enhanced features.

Nanomaterials are classified according to the number of possessed dimensions exceeding the nanoscale range of 100 nm.² Nanomaterials may be three-dimensional (e.g. bulk crystals), two-dimensional (e.g. thin films), one-dimensional (e.g. nanorods, nanotubes, and nanowires) or zero-dimensional (e.g. nanoclusters, nanoparticles and quantum dots). While a wide range of nanomaterials have been isolated thus far, two novel nanomaterials will be examined in detail in this thesis: graphene and black phosphorus.

1.1.2 Graphene: Discovery, Properties and Synthesis

Since its successful isolation by Novoselov and Geim in 2004,³ graphene has quickly incited immense research interest in the scientific community owing to its superior properties. Graphene is a one-atom-thick, two-dimensional sheet of carbon atoms aligned in a hexagonal crystal lattice structure. Each carbon atom in the cyclic, aromatic array is attached by sp^2 bonds. According to the International Union for Pure and Applied Chemistry (IUPAC), graphene is defined as *“a single carbon layer of graphite structure, describing its nature by analogy to a polycyclic aromatic hydrocarbon of quasi-infinite size”*.⁴

Figure 1. Structures of typical carbon allotropes. Reproduced from reference 5.

Graphene displays many fascinating and unique properties surpassing those of conventional materials. Possessing a specific surface area of $2630 \text{ m}^2 \text{ g}^{-1}$, the dimensional size of graphene exceeds graphite by over two orders of magnitude.⁶ Graphene also exhibits high thermal conductivity exceeding $5000 \text{ W m}^{-1} \text{ K}^{-1}$ at room ambient conditions,⁷ which is three orders of magnitude higher than that of amorphous carbon.⁸ With its high mechanical strength, graphene yields a Young's modulus of 1 TPa ,⁹ making it comparable to natural diamond in terms of hardness.¹⁰ The zero band gap behaviour characteristic of pristine graphene can also be readily fine tuned by surface modification to achieve different purposes.¹⁰⁻¹¹ Moreover, graphene displays extremely high electronic quality, achieving a theoretical electron mobility of up to $200000 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ under ambient conditions, for a carrier density of $1 \times 10^{12} \text{ cm}^{-2}$.¹² It also demonstrates high optical transmittance, absorbing only 2.3% of visible light.¹³

Below a lateral dimension of 100 nm , zero-dimensional graphene nanostructure exhibits strong quantum confinement effect and is termed as a graphene quantum dot (GQD).¹⁴ The introduction of a band gap into the emergent nanolight gives rise to its prominent fluorescence property, which is also influenced by the edge effect arising from the presence of functionalities onto the GQD.¹⁵ In addition to the intrinsic properties of graphene, GQD demonstrates many interesting and superior properties such as multicolour and tunable emission,¹⁶⁻¹⁸ broad excitation range,¹⁹ water solubility,²⁰⁻²¹ low cytotoxicity,²²⁻²³ excellent biocompatibility,²⁴ as well as photochemical²⁵⁻²⁶ and physicochemical stability.^{14,27}

The synthesis of graphene can be traced back to the use of a single pencil and adhesive tape.³ This mechanical exfoliation procedure has since been famously dubbed as the “scotch tape method”. In recent years, the scientific community has witnessed key advancements in the progress of graphene preparation, and they can be broadly classified into two categories: top-down and bottom-up.

The top-down synthesis approach typically involves the cutting, etching or exfoliation of bulk graphite and related carbon precursors into single graphene monolayers. These techniques include (i) mechanical, chemical or electrochemical exfoliation, (ii) post-reduction treatment by thermal, chemical or electrochemical means following a preliminary oxidation step, as well as (iii) the longitudinal unzipping of carbon nanotubes by mechanical, thermal or chemical means.²⁸⁻²⁹ Further cutting and exfoliation of bulk graphene derivatives then gives rise to the GQDs.³⁰⁻³¹ Fossil fuels such as coal³² and petroleum coke³³ have also emerged as abundant and affordable raw materials for the one-step top-down synthesis of GQDs in the recent years.

Conversely, the bottom-up synthesis approach involves the assembly of smaller carbon structures into graphene sheets. Fabrication methods include (i) epitaxial growth and (ii) chemical vapour deposition.³⁴ An additional method for GQD synthesis involves the cage-opening of fullerene molecules.³⁵⁻³⁶

1.1.3 Black Phosphorus: Discovery, Properties and Synthesis

The recent isolation of monolayer black phosphorus – phosphorene – in 2014 has brought prominence to this novel layered nanomaterial³⁷ and rekindled scientific interest in the bulk crystal. Similar to graphite, black phosphorus is a layered material stacked together by weak van der Waals forces and constituted of a single element. Within each layer, however, phosphorus atoms are covalently bonded by sp^3 bonds to construct a puckered hexagonal crystal lattice structure.

Figure 2. Structures of typical phosphorus allotropes. Reproduced from reference 38.

Black phosphorus displays many exceptional and remarkable characteristics that have fuelled vast research interest into the material, in particular its in-plane anisotropy. As a result of its atomic arrangement, black phosphorus crystal possesses a combination of zigzag and armchair directions within its lattice structure. This structural anisotropy in turn results in unusual in-plane anisotropic optical response, thermal transport and electrical conductivity properties.³⁹⁻⁴⁰ Unlike graphene, black phosphorus possesses an intrinsic layer-dependent band gap⁴¹ which can be further

fine tuned by surface modification,⁴² over a wide energy range (0.3 – 2 eV) exceeding that of other two-dimensional materials that have been isolated thus far.³⁹ With a high carrier mobility of $10000 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$,⁴³ the mobility of black phosphorus is two orders of magnitude higher than transition metal dichalcogenides such as GaSe, MoS₂, MoSe₂ and WSe₂,⁴⁴ although it has yet to surpass graphene. Black phosphorus is highly susceptible towards ambient degradation, owing to the presence of free lone pairs on the phosphorus atoms and bond angle of 102° which results in its chemical instability.⁴⁵ While it was previously established that black phosphorus exhibits inherently hydrophilic character which further exacerbates its degradation process,⁴⁶ a recent research paper published by Sutter *et al* in 2016 reported the hydrophobic nature of pristine black phosphorus and confirmed its chemical inertness in deaerated water.⁴⁷ Moreover, black phosphorus also demonstrates biocompatibility⁴⁸ and low cytotoxicity,⁴⁹ similar to that of graphene quantum dots.

While research interest in black phosphorus as a two-dimensional nanomaterial has surged over the past few years, the discovery and synthesis of bulk black phosphorus traces back to over a century ago. Black phosphorus was first synthesized by Bridgman in 1914 from the white allotrope at elevated pressure and temperature.⁵⁰ New methods of preparation have since surfaced, mostly involving the high temperature conversion of the red phosphorus allotrope into bulk black phosphorus with the use of a mineralization promoter,⁵¹ followed by subsequent chemical,⁵² mechanical⁵³ or electrochemical exfoliation⁵⁴ to obtain smaller nanostructures. Recent literature has also demonstrated the successful synthesis of air-stable black phosphorus using surface^{41,50} or chemical modification procedures.⁵⁵⁻⁵⁶

1.2 Analytical Techniques in Chemistry

1.2.1 Overview

Analytical techniques are methodologies utilized for the qualitative or quantitative determination of an analyte of interest.⁵⁷ They are often performed using a device known as a sensor, which can be broadly classified into three types: physical, chemical and biological.⁵⁸ Out of the three types of sensors, chemical and biological sensors are most commonly employed in chemical analysis.

Chemical sensors are self-contained devices that respond selectively to an analyte of interest through a chemical reaction,⁵⁹ whereas biosensors are a subset of chemical sensors that employ biological recognition elements.⁶⁰ Chemical sensors are made up of two components – a recognition element (the site where selective chemistry with the analyte occurs, resulting in signal response) and a transducer (the site where signal response is translated into a measurable physical quantity).⁶¹

Common transduction techniques utilized in chemical sensors include electrochemical, optical, thermometric, gravimetric and mechanical methods. Electrochemical sensing involves the occurrence of redox chemical reactions at the metal-aqueous interface with a solid electrode conductor.⁶¹ It is performed when the analyte of interest is an electroactive species. Optical transduction measures the interaction (e.g. absorption, luminescence, reflection, scattering or transmission) between photons of electromagnetic radiation with a sample containing the analyte of interest.⁶² It is a suitable transduction method when a light responsive species is present in a sample containing the analyte of interest. Gravimetric sensors measure changes in the mass of the sample deposited on a resonator surface, in the form of

frequency variations.⁶³ They are particularly useful as gas sensors due to their high frequency stability which enables the detection of mass changes down to a few nanograms.⁶⁴ Thermometric transduction is based on the measurement of temperature changes in a specific chemical reaction involving the analyte of interest, using a thermistor or a platinum resistance thermometer.⁶⁵ They are ideal for the detection of flammable gases.⁶⁶ In this thesis, the fabrication of electrochemical and fluorescence-based sensors based on new layered nanomaterials are described.

1.2.2 *Electrochemical Detection*

In electrochemical transduction, the analyte of interest is an electrochemically active species capable of undergoing reduction or oxidation with an electrode immobilized on the sensing device.⁶¹ An electrical signal correlated to the analyte concentration can then be obtained from the electrochemical reaction. Two types of transduction techniques are possible: potentiometry (measurement of potential difference) and amperometry (measurement of electric current).⁶⁷ Amperometry may be further subdivided into two classes: chronoamperometry (measurement of current as a function of time) and voltammetry (measurement of current as a function of applied potential).⁶⁸

In voltammetry, the applied potential of the working electrode is modulated in a stipulated manner with the simultaneous recording of the corresponding current response. The four main types of excitation signals include (i) linear scan (in linear sweep voltammetry), (ii) differential pulse, (iii) square wave and (iv) triangular (in cyclic voltammetry).⁶⁹ Linear scan waveform involves an increase in the applied voltage as a linear function of time. It is the simplest form of voltammetric technique and is advantageous for its highly rapid scan rates.⁷⁰ Differential pulse and square wave waveforms involve the measurement of current response at systematic time intervals. They eliminate background currents and provide scans with high sensitivities.⁷¹ Triangular waveform involves the repeated cycling of applied voltage over a specified potential window. It is typically performed when a complete analysis of the kinetics and thermodynamics of an electrochemical reaction is required.⁷²⁻⁷³

A basic electrochemical cell setup is constituted of three electrodes (reference, working and counter electrodes) immersed in a solution comprising the analyte of interest as well as an excess of supporting electrode made up of nonreactive chemical species. Voltage is applied to control the potential of the working electrode relative to the reference electrode, and the current signal passing between the working and counter electrodes is acquired.⁷⁴ Electrochemical redox reaction of the electroactive analyte species occurs on the working electrode surface.⁷⁵ Complete concentration polarization is assumed in voltammetry⁷⁶ – the limiting factor of redox reaction efficiency is the rate of analyte mass transport to the reactive sites on the electrode surface.

Electrochemical detection methods possess a multitude of advantages including high intrinsic sensitivity and selectivity,⁷⁷⁻⁷⁹ broad linear detection range,⁷⁷ rapid response,⁸⁰⁻⁸¹ low-cost instrumentation^{77,80} and portability.⁸¹ The fabrication of an electrochemical sensing platform based on voltammetric transduction will be explored in detail in **Chapter 2**.

1.2.3 Optical Detection

Optical transduction involves at least one species that is capable of emitting light upon excitation. Different excitation pathways give rise to different types of sensing techniques. Excited species may be produced from chemical reactions (in chemiluminescence), biochemical reactions (in bioluminescence) or electrochemical reactions (in electroluminescence). They can also be produced via light absorption (in photoluminescence).⁸²⁻⁸³ Photoluminescence (PL) may be further subdivided into two classes depending on their relaxation pathway: fluorescence (radiational transition between states of the same spin quantum numbers) and phosphorescence (radiational transition between states of different spin quantum numbers).⁸⁴ When direct sensing is not possible, the analyte of interest may be labelled with a fluorescent tag, also known as a fluorophore, for indirect detection. An optical signal correlated to the analyte concentration is then measured by the detector.

In fluorescence, the sample containing the analyte of interest is irradiated with a light source which promotes molecule transition from the ground electronic state S_0 to an excited electronic state S_1 or S_2 . At the electronic state S_2 , the molecule undergoes non-radiational vibrational relaxation to the S_1 state, which then returns to the S_0 state with simultaneous light emission. Fluorescence emission is then measured over a range of emission wavelengths to produce an emission spectrum.

Electronic excitation energy may also be transferred from an excited fluorescent species (donor) to a non-excited species (acceptor), via a process known as fluorescence resonance energy transfer (FRET).⁸⁵ Consequently, the acceptor molecule undergoes transition to an excited electronic state and can return to the

ground electronic state by radiational emission (fluorescence) or non-radiational transition in the form of heat (quenching)⁸⁶. FRET efficiency is highly dependent on three features: (i) degree of spectra overlap, (ii) distance (2 – 10 nm) and (iii) relative orientation between the donor and acceptor species.⁸⁷

Fluorescence detection possesses advantages including high sensitivity,⁸⁸⁻⁸⁹ high target recognition specificity,⁹⁰ favourable imaging properties⁹¹⁻⁹² and simplicity of operation.^{88,90} The fabrication of a fluorescence sensing platform based on FRET quenching will be examined in detail in **Chapters 3 and 4**.

1.2.4 Significance of Analytical Detection

In this thesis, the applications of nanostructures for sensing applications are illustrated using two analyte compounds: nitrogen-containing aromatic compounds (NACs) in **Chapter 2** and deoxyribonucleic acid (DNA) in **Chapters 3** and **4**.

NACs are energetic materials which possess the potential to undergo rapid self-propagating decomposition, resulting in a sudden release of heat and accumulated potential energy.⁹³⁻⁹⁴ Energetic materials are generally employed in the development of modern military explosives and munitions,⁹⁵⁻⁹⁷ with 2,4,6-trinitrotoluene (TNT) being one of the most representative explosive compounds. Another nitro-substituted aromatic explosive – 2,4-dinitrotoluene (DNT) – is frequently detected as an impurity of TNT in contamination sites as it is a highly volatile byproduct in commercial TNT production.⁹⁸⁻⁹⁹

Following the two World Wars, millions of tonnes of untreated chemical weapons were discarded into the oceans.¹⁰⁰ Over time, NACs leak from rusted ordnance and dissolve in seawater.¹⁰¹ These toxic contaminants eventually work their way up the marine food chain, manifesting as amplified adverse health effects in humans due to biomagnification. Nitroaromatic explosives poisoning may result in hair, skin and nails discolouration,¹⁰²⁻¹⁰³ cataract formation,¹⁰⁴ hepatic failure,¹⁰⁴ pancytopenia¹⁰⁵ and cancer development.¹⁰⁵ The median lethal dose (LD_{50}) values of TNT and DNT in male rats are 1320 mg kg^{-1} and 268 mg kg^{-1} , respectively.¹⁰⁶⁻¹⁰⁷ Therefore, the development of sensitive analytical platforms for the trace detection and quantification of nitroaromatic explosives, especially in seawater, is critical in assisting environmental remediation efforts and human health risk mitigation.

DNA is a biopolymer constituted of nucleotide monomers that are held in a chain by phosphodiester bonds. Each basic unit is made of three components – a pentose sugar known as deoxyribose which is attached to a nitrogenous base at the 1' position via a β -glycosidic linkage, and attached to a phosphate group at the 5' position via an ester bond.¹⁰⁸ The four naturally-occurring nitrogenous bases are adenine (A), thymine (T), cytosine (C) and guanine (G). Base pairing between two complementary single-stranded DNA oligonucleotides give rise to a double-stranded DNA helix. This involves the formation of hydrogen bonds between A–T, and between C–G.

DNA contains genetic information in the form of genes, each coding for a specific protein in a living organism. It is a hereditary material¹⁰⁹ that can be passed down onto successive generations. As such, DNA identification allows for paternity testing¹¹⁰ as well as ancestry determination.¹¹¹ DNA is also unique to an individual,¹¹⁰ hence DNA fingerprinting allows for suspect identification in forensic analysis¹¹² and victim identification in a mass fatalities incident.¹¹³

The genetic variation among individuals of the same species is partly due to the occurrence of occasional mutations in our genetic code.¹¹⁴ At times, DNA mutations have deleterious effects on an individual and manifest as genetic disorders, for example Alzheimer's disease. DNA detection can therefore allow for early medical diagnosis¹¹⁵⁻¹¹⁸ and assist in pharmaceutical drug development.¹¹⁹ Moreover, military applications,¹²⁰ food quality assurance¹²¹ and environmental monitoring¹²² also make up some of the myriad of applications available with the development of sensitive analytical platforms for selective DNA detection.

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Chapter 2:

Graphene – An Electrochemical Sensor for Trace Nitro-Aromatic Explosives Detection

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2.5 References

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Author Contributions:

Yew Y. T. performed the electrochemical experiments. A. Ambrosi synthesized and characterized the electrochemically exfoliated graphene materials. M. Pumera conceptualized and supervised the project. All authors contributed to discussion and manuscript preparation.

2.1 Introduction

This chapter illustrates the application of graphene nanostructures for the electrochemical detection of nitrogen-containing aromatic compounds (NACs) – 2,4-dinitrotoluene (DNT) and 2,4,6-trinitrotoluene (TNT).

Among the available analytical transduction methods for NAC detection outlined in *Sub-Section 2* of **Chapter 1**, electrochemical detection offers the additional possibility of in-field, real-time analysis which is crucial for long-term environmental monitoring. A recent research paper published by Dong *et al.* also revealed a wider linear detection range obtained with electroanalytical TNT detection in comparison to fluorometric TNT analysis, based on carbon quantum dots.¹

Electroanalytical detection of NACs is made possible by the electroactive nature of the nitro substituents ($-\text{NO}_2$), which can undergo stepwise reduction to a hydroxylamine group ($-\text{NHOH}$) via a 4-electron process, followed by successive reduction to an amine group ($-\text{NH}_2$) via a 2-electron process.²⁻⁴ Scheme 1 illustrates the reduction pathway demonstrating the full reduction of TNT. The electrochemical reduction process initiates in the *ortho* position with respect to the methyl substituent ($-\text{CH}_3$), continues with the second *ortho* position and finally proceeds onto the *para* position.³⁻⁴

Scheme 1. Electrochemical reduction pathway demonstrating the complete reduction of a nitroaromatic compound, exemplified here with TNT.

Graphene is a suitable candidate for the trace electroanalytical detection of NACs⁵⁻⁸ due to its electron-rich carbon sp^2 network and large surface area. The large sp^2 network of graphene facilitates the adsorption of electron-deficient nitroaromatic compounds onto the graphene surface, via π - π electron donor-acceptor (EDA) stacking interactions,⁹⁻¹⁰ where they can then undergo electrochemical reduction.

In this chapter, graphene probes were synthesized from the electrochemical exfoliation of graphite foil using two different electrolytes under anodic conditions – LiClO_4 and Na_2SO_4 . Their electroanalytical performance for the detection of DNT and TNT explosives were investigated and compared.

2.2 Results and Discussion

2.2.1 Structural Characterizations

The structural morphologies and chemical compositions of the two graphene materials were first analyzed and compared with the use of various characterization techniques. These techniques include scanning transmission electron microscopy (STEM), atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and Raman spectroscopy.

STEM was conducted to examine the structural morphology of the graphene materials (Figure 1).¹¹ Partially transparent graphene flakes with lateral dimensions of 3 – 10 μm are observed, reflecting a high degree of exfoliation resulting in few-layered graphene sheets. Both graphene materials exhibit high quality, as demonstrated by the smooth edges of the graphene flakes with few surface corrugations.

Figure 1. STEM images of (A) G-LiClO₄ and (B) G-Na₂SO₄ and. Scale bars: 1 μm .

XPS was performed to analyze the surface elemental composition and bonding arrangements within the graphene materials (Figure 2).¹²⁻¹³ C/O ratios of 4.0 and 8.8 were obtained for G-LiClO₄ and G-Na₂SO₄, respectively. High-resolution XPS (C1s) spectra revealed the presence of O–C=O (289.2 eV), C=O (287.9 eV) and C–O (286.7 eV). This indicates that both graphene materials possess oxygen functionalities in the form of carboxylic, carbonyl, epoxy and hydroxyl groups. Notably, a higher content of carbonyl and hydroxyl oxygen functionalities are present in G-LiClO₄ as compared to G-Na₂SO₄.

Figure 2. High-resolution C1s XPS spectra of (A) G-LiClO₄ and (B) G-Na₂SO₄.

Raman spectroscopy was conducted to examine the degree of disorder in the graphene materials (Figure 3).¹⁴ D/G ratios of 1.00 and 0.95 were obtained for G-LiClO₄ and G-Na₂SO₄, respectively. This reflects a similar degree of structural defects present in both graphene materials.

Figure 3. Raman spectra of (A) G-LiClO₄ and (B) G-Na₂SO₄.

2.2.2 *Electrochemical Characterizations*

Electrochemical analysis of the graphene materials were conducted following their morphological and chemical characterization studies. Specifically, cyclic voltammetry (CV) measurements were obtained in 20 mM borate buffer solution (BBS, pH 9.2) in order to ascertain the presence of intrinsic reduction peaks for the graphene materials (Figure 4A). The term “intrinsic” refers to the inherent electrochemistry of a material – whether it can be oxidized or reduced. Examining the intrinsic electrochemical properties of the graphene materials facilitates the isolation of reduction signals attributed to the NAC of interest, with no signal contribution from the graphene material itself. CVs were also recorded in seawater in order to evaluate the influence of background interferences as well as to assess the practical utility of the analytical sensor for environmental samples (Figure 4B). The seawater solution was prepared as a mixture of natural seawater and 200 mM BBS in the volume ratio of 9 : 1, in order to attain a resulting BBS concentration of 20 mM equivalent to that of the pure BBS system, while ensuring that seawater was only subjected to minimal dilution. All electrolyte solutions were maintained at a pH level within the range of 8 – 10 so as to achieve the most sensitive and stable NAC reduction signal response in subsequent experiments.⁷

A broad, intense reduction peak was observed for G-LiClO₄ at –819 mV in BBS and at –724 mV in seawater. Conversely, a low-intensity reduction peak was obtained for G-Na₂SO₄ at –655 mV in BBS which was not apparent in seawater. The matrix effect was more prominent in G-Na₂SO₄, evident from the sharp increase in current intensity beyond –1200 mV of the CV cathodic wave in the seawater system.

Nevertheless, the voltammograms recorded in both BBS and seawater demonstrated a high degree of similarity within the 0 mV to –1000 mV potential range, reflecting the absence of redox-active compounds that may interfere with the reduction of NACs in seawater. The larger intrinsic reduction signal of G-LiClO₄ measured in both BBS and seawater corresponds to the presence of a larger concentration of oxygen functionalities on G-LiClO₄ that may undergo irreversible electrochemical reduction, as compared to G-Na₂SO₄. The inherent reduction peak intensities of the graphene materials G-LiClO₄ and G-Na₂SO₄ concur well with their respective C/O ratios of 4.0 and 8.8.

Figure 4. Cyclic voltammograms of (A) blank buffer and (B) seawater for bare GC, G-LiClO₄ and G-Na₂SO₄. Conditions: (A) BBS (20 mM, pH 9.2) and (B) seawater solution (pH 8.8) constituted of natural seawater (pH 8.0) and BBS (200 mM, pH 9.2) prepared in a volume ratio of 9 : 1. Scan rate: 100 mV s⁻¹.

2.2.3 Graphene in the Electrochemical Detection of 2,4-Dinitrotoluene (DNT)

CV measurements were first performed in BBS to determine the two reduction peak potentials of DNT on the electrochemically exfoliated graphene materials (Figure 5A). CV scans were then performed in seawater to simulate real-time environmental assessment of DNT (Figure 5B). Two intense reduction peaks were obtained for DNT reduction in both BBS and seawater systems – denoted as *a* and *b*, in increasing (more negative) reduction potential values. Peak *b* occurs at the same cathodic peak potential as the reduction of surface oxygen functionalities intrinsic to the exfoliated graphene materials. Hence, peak *a* is selected for the subsequent elucidation of DNT sensing capabilities of the graphene materials. The voltammograms revealed relatively similar cathodic current intensities corresponding to peak *a* in both BBS and seawater systems, with a shift in the cathodic peak potential to lower values in the seawater system as compared to the BBS system, for all three electrode surfaces (Table 1).

Figure 5. Cyclic voltammograms of DNT (20 ppm) in (A) BBS and (B) seawater for bare GC, G-LiClO₄ and G-Na₂SO₄. Conditions: (A) BBS (20 mM, pH 9.2) and (B)

seawater solution (pH 8.8) constituted of natural seawater (pH 8.0) and BBS (200 mM, pH 9.2) prepared in a volume ratio of 9 : 1. Scan rate: 100 mV s⁻¹.

Table 1. Cathodic peak potentials (mV) of DNT (20 ppm) in (A) BBS and (B) seawater for G-LiClO₄ and G-Na₂SO₄.

Material	Cathodic peak potential (mV)	
	Peak <i>a</i>	Peak <i>b</i>
(A) BBS		
Bare GC	-492	-645
G-LiClO ₄	-528	-694
G-Na ₂ SO ₄	-501	-660
(B) Seawater		
Bare GC	-462	-606
G-LiClO ₄	-416	-580
G-Na ₂ SO ₄	-435	-594

Subsequent trace analysis of DNT was conducted using a more sensitive electrochemical technique – differential pulse voltammetry (DPV) – in order to obtain voltammograms of higher resolution. The mass of graphene electrocatalyst was first optimized by drop-casting different masses of the graphene material onto the electrode surface, and recording the DPVs of the electrode system in varying concentrations of DNT (4, 8 and 12 ppm). As presented in Figure 6, both graphene materials demonstrated the largest cathodic current intensity corresponding to peak *a* for a coating mass of 1 µg. Consequently, an optimum electrocatalyst mass of 1 µg was applied for subsequent voltammetric experiments.

Figure 6. Cathodic current intensities (μA) based on differential pulse voltammetry measurements of DNT (4, 8 and 12 ppm) for (A) G-LiClO₄ and (B) G-Na₂SO₄. Conditions: BBS (20 mM, pH 9.2). Data is based on the first DNT reduction signal (peak *a*).

DPVs were then recorded in BBS and seawater for varying concentrations of DNT (0, 4, 8, 12, 16 and 20 ppm) (Figure 7). Analysis of the DPVs revealed that both graphene materials exhibited larger reduction peak heights in the seawater system, as compared to the BBS system. Notably, peak current was doubled at all concentrations for G-LiClO₄ upon replacing the BBS system with seawater. Among all three electrode surfaces, G-LiClO₄ also consistently displayed the largest cathodic current intensities in both BBS and seawater for all DNT concentrations.

Figure 7. Differential pulse voltammograms of varying DNT concentrations (0, 4, 8, 12, 16 and 20 ppm) in (left panel) BBS and (right panel) seawater for (A, D) bare GC electrode, (B, E) G-LiClO₄ and (C, F) G-Na₂SO₄. Conditions: (A – C) BBS (20 mM, pH 9.2)

and (D – F) seawater solution (pH 8.8) constituted of natural seawater (pH 8.0) and BBS (200 mM, pH 9.2) prepared in a volume ratio of 9 : 1. Scan rate: 100 mV s⁻¹.

Corresponding calibration curves are produced based on the signal response of peak *a* from Figure 7, and presented in Figure 8 as a linear function of DNT concentration. The sensitivity of a sensing platform corresponds to the slope of the plotted calibration curve. Therefore, G-LiClO₄ presented the highest electrode system sensitivity in both BBS and seawater, which is followed by G-Na₂SO₄, and lowest for bare GC (Table 2). It is noteworthy that G-LiClO₄ also exhibited the largest reduction current intensities across all DNT concentrations. Overlapping of the calibration curves of G-Na₂SO₄ with bare GC is indicative of the former electrode system only having a higher signal response than bare GC beyond 16 ppm of DNT in BBS, and beyond 10 ppm of DNT in seawater.

In the BBS system, the limit of detection (LOD) was lowest for G-LiClO₄, followed by G-Na₂SO₄, then bare GC. The *R*² value was highest for the G-Na₂SO₄, followed by G-LiClO₄ which gave a comparable *R*² value, and lowest for bare GC. However, in the seawater system, bare GC gave rise to the lowest LOD and highest *R*² value, followed by G-LiClO₄, then G-Na₂SO₄ which displayed the highest LOD and lowest *R*² value.

Comparing the DNT sensing performance of the graphene electrochemical sensors in both BBS and seawater systems, one can observe significant improvements in the electrode system sensitivities and *R*² values for both graphene materials in the latter system. Notably, system sensitivity was doubled for G-LiClO₄ when switched from BBS to the seawater system.

Figure 8. Calibration curve of DNT (4, 8, 12, 16 and 20 ppm) in (A) BBS and (B) seawater for bare GC, G-LiClO₄ and G-Na₂SO₄. Conditions: (A) BBS (20 mM, pH 9.2) and (B) seawater solution (pH 8.8) constituted of natural seawater (pH 8.0) and BBS (200 mM, pH 9.2) prepared in a volume ratio of 9 : 1. Data is based on the first DNT reduction signal (peak *a*).

Table 2. Sensitivities (nA ppm⁻¹), R^2 and LOD (ppm) of bare GC, G-LiClO₄ and G-Na₂SO₄ towards DNT detection in (A) BBS and (B) seawater. Data is based on the first DNT reduction signal (peak α). Standard deviations are stated in parenthesis.

Material	Sensitivity (nA ppm ⁻¹)	R^2	LOD (ppm)
A) BBS			
Bare GC	-165 (±50)	0.729	11.26
G-LiClO ₄	-289 (±43)	0.917	2.73
G-Na ₂ SO ₄	-197 (±30)	0.918	5.43
B) Seawater			
Bare GC	-133 (±10)	0.983	3.17
G-LiClO ₄	-552 (±58)	0.968	4.35
G-Na ₂ SO ₄	-284 (±41)	0.942	5.97

2.2.4 Graphene in the Electrochemical Detection of 2,4,6-Trinitrotoluene (TNT)

Similar to the electrochemical sensing of DNT, cyclic voltammetry scans were performed in BBS and seawater separately for the analysis of TNT (Figure 9). Three intense reduction peaks were uncovered in both BBS and seawater systems – denoted as *c*, *d* and *e*, in increasing reduction potential values. Further analysis of the CV scans revealed coinciding cathodic peak potentials for peak *e* with the reduction of surface oxygen functionalities present on the graphene materials. It should also be noted that peaks *d* and *e* were not apparent for bare GC and G-LiClO₄ in BBS, as well as for bare GC and G-Na₂SO₄ in seawater. Consequently, peak *c* is selected for the analytical comparison of subsequent experimental results for TNT sensing. Similar to DNT detection, a shift in the reduction peak potential of peak *c* to lower values was observed for all three electrode surfaces when the BBS system was replaced with the seawater system (Table 3). Comparable cathodic current intensities corresponding to peak *c* was also observed in both BBS and seawater systems, with the exception of G-LiClO₄ exhibiting a marked increase in current intensity in the seawater system.

Figure 9. Cyclic voltammograms of TNT (20 ppm) in (A) BBS and (B) seawater for bare GC, G-LiClO₄ and G-Na₂SO₄. Conditions: (A) BBS (20 mM, pH 9.2) and (B) seawater solution (pH 8.8) constituted of natural seawater (pH 8.0) and BBS (200 mM, pH 9.2) prepared in a volume ratio of 9 : 1. Scan rate: 100 mV s⁻¹.

Table 3. Cathodic peak potentials (mV) of TNT (20 ppm) in (A) BBS and (B) seawater for G-LiClO₄ and G-Na₂SO₄.

Material	Cathodic peak potential (mV)		
	Peak <i>c</i>	Peak <i>d</i>	Peak <i>e</i>
(A) BBS			
Bare GC	-389	-555	-702
LiClO ₄	-394	-567	-714
Na ₂ SO ₄	-392	-543	-670
(B) Seawater			
Bare GC	-343	-499	Undetectable
LiClO ₄	-279	-440	-565
Na ₂ SO ₄	-311	-465	-587

Due to poor peak separation using cyclic voltammetry, differential pulse voltammetry was performed in BBS and seawater for a range of TNT concentrations (0, 4, 8, 12, 16 and 20 ppm) (Figure 10). Both graphene materials presented enhancements in reduction peak heights in the seawater system as compared to the BBS system, with G-LiClO₄ displaying an increase in current intensities by more than three times in seawater for all concentrations. G-LiClO₄ also demonstrated the largest reduction peak current in both BBS and seawater systems for all concentrations.

Figure 10. Differential pulse voltammograms of varying TNT concentrations (0, 4, 8, 12, 16 and 20 ppm) in (left panel) BBS and (right panel) seawater for (A, D) bare GC electrode, (B, E) G-LiClO₄ and (C, F) G-Na₂SO₄. Conditions: (A – C) BBS (20 mM, pH 9.2)

and (D – F) seawater solution (pH 8.8) constituted of natural seawater (pH 8.0) and BBS (200 mM, pH 9.2) prepared in a volume ratio of 9 : 1. Scan rate: 100 mV s⁻¹.

Corresponding calibration curves are produced based on the signal response of peak *c* from Figure 10, and presented in Figure 11 as a linear function of TNT concentration. In the BBS system, bare GC demonstrated the highest sensitivity, followed by G-LiClO₄ with comparable electrode system sensitivity, then G-Na₂SO₄ (Table 4). In the seawater system, G-LiClO₄ displayed the highest system sensitivity, which is followed by bare GC, and lowest for G-Na₂SO₄. Interestingly, G-LiClO₄ also exhibited the largest reduction current intensities across all TNT concentrations, similar to its electrochemical performance in DNT sensing. Overlapping of the calibration curves of G-Na₂SO₄ with bare GC is indicative of the former electrode system only having a higher signal response than bare GC at TNT concentrations less than 2 ppm in BBS, and less than 16 ppm in seawater.

In the BBS system, the limit of detection (LOD) was lowest for bare GC, followed by G-Na₂SO₄, then G-LiClO₄. The *R*² value was highest for bare GC, followed by G-LiClO₄, and lowest for G-Na₂SO₄. However, in the seawater system, G-LiClO₄ gave rise to the lowest LOD and highest *R*² value, followed by bare GC, then G-Na₂SO₄ with the highest LOD and lowest *R*² value.

Significant improvements in the system sensitivities and *R*² values were apparent for both electrode systems in seawater, in comparison to the TNT sensing performance of the graphene materials in their corresponding BBS systems. Notably, G-LiClO₄ exhibited a two-fold increase in system sensitivity following a switch from the BBS system to the seawater system.

Similar to DNT sensing, G-LiClO₄ demonstrated superior electrochemical performance than G-Na₂SO₄ for the electrochemical detection of TNT. The improved electroanalytical signal observed for G-LiClO₄ is contrary to expected results: a lower C/O content, corresponding to higher concentration of surface oxygen functionalities, theoretically results in (i) increased water competition for analyte on the graphene surface which impedes π - π stacking interactions¹⁵ as well as (ii) reduced conductivity¹⁶, thereby lowering the current response of the electrode system. The contradictory experimental results obtained therefore signals that EDA stacking interactions cannot be considered solely; a competition of different factors must be at play influencing the overall electrochemical performance of the graphene materials. Other works have also demonstrated this phenomenon: the determining factor in a competition of concomitant factors, each capable of influencing the electrochemical detection of an electroactive molecule, is specific for the molecule of interest.¹⁷⁻¹⁸

The enhanced electrochemical performance demonstrated by G-LiClO₄ may be attributed to the electrostatic interactions between oxygen functionalities on the graphene surface and the electron-withdrawing -NO₂ groups of NAC,¹⁹ dominating over EDA stacking interactions. G-LiClO₄ possesses a larger amount of surface oxygen functionalities than G-Na₂SO₄ which promotes the efficient adsorption of NACs onto the graphene material, thereby enhancing its electrochemical signal response.

Figure 11. Calibration curve of TNT (4, 8, 12, 16 and 20 ppm) in (A) BBS and (B) seawater for bare GC, G-LiClO₄ and G-Na₂SO₄. Conditions: (A) BBS (20 mM, pH 9.2) and (B) seawater solution (pH 8.8) constituted of natural seawater (pH 8.0) and BBS (200 mM, pH 9.2) prepared in a volume ratio of 9 : 1. Data is based on the first TNT reduction signal (peak c).

Table 4. Sensitivities (nA ppm⁻¹), R^2 and LOD (ppm) of bare GC, G-LiClO₄ and G-Na₂SO₄ towards TNT detection in (A) BBS and (B) seawater. Data is based on the first TNT reduction signal (peak c). Standard deviations are stated in parenthesis.

Material	Sensitivity (nA ppm ⁻¹)	R^2	LOD (ppm)
A) BBS			
Bare GC	-212 (±24)	0.950	4.08
G-LiClO ₄	-205 (±35)	0.893	6.74
G-Na ₂ SO ₄	-155 (±029)	0.879	6.54
B) Seawater			
Bare GC	-246 (±22)	0.976	3.76
G-LiClO ₄	-617 (±30)	0.993	2.03
G-Na ₂ SO ₄	-167 (±15)	0.975	3.85

2.2.5 Selectivity Performance of Graphene in the Electrochemical Detection of Nitroaromatic Compounds

The selectivity performance of the proposed electrochemical sensing platform was assessed by monitoring changes in the voltammetric signal with the addition of increasing DNT concentrations in seawater containing 8 ppm of TNT. Three intense reduction peaks were uncovered – denoted as *f*, *g* and *h*, in increasing reduction potential values (Figure 12). Analysis of the DPV scans revealed that the reduction current intensity of peak *f* demonstrated little variation following an increase in DNT concentration. This indicates that peak *f* is independent of DNT, thus it can be solely assigned to TNT. The selective electrochemical detection of TNT in the presence of DNT is therefore possible in our proposed sensing platform. Interestingly, we can also observe that the cathodic peak potential and intensity of peak *f* follow closely to that of peak *c* for an equivalent TNT concentration, in all electrode systems.

Further analysis revealed that peaks *g* and *h* demonstrated high resemblance to peaks *a* and *b* for the corresponding DNT concentration, with negligible influence from peaks *d* and *e* produced by TNT, respectively. The observed phenomenon is even more prominent for G-LiClO₄ owing to its enhanced electrochemical performance. The electrochemical sensing platform proposed in this work demonstrates the ability to selectively detect and quantify DNT and TNT in a NAC mixture.

Figure 12. (Left panel) Differential pulse voltammograms of varying DNT concentrations (0, 4, 8, 12, 16 and 20 ppm) in seawater containing TNT (8 ppm) for (A) bare GC, (B) G-LiClO₄ and (C) G-Na₂SO₄. Differential pulse voltammograms of (middle panel) varying DNT concentrations (0, 4, 8, 12, 16 and 20 ppm) and (right panel) TNT (8 ppm) in seawater for (D, G) bare GC, (E, H) G-LiClO₄ and (F, I) G-Na₂SO₄ are shown for comparison. Conditions: seawater solution (pH 8.8) constituted of natural seawater (pH 8.0) and BBS (200 mM, pH 9.2) prepared in a volume ratio of 9 : 1. Scan rate: 100 mV s⁻¹.

2.3 Conclusions

This chapter elucidated the application of graphene synthesized from the electrochemical anodic exfoliation of graphite foil in different electrolytes (LiClO_4 and Na_2SO_4) as an electrochemical sensing platform for the trace detection of DNT and TNT in borate buffer. The practicality, reliability and feasibility of the proposed electrode systems in real-time sample analysis were also examined with seawater.

Remarkable electrochemical sensing abilities including high sensitivity, high linearity and low limit of detection was attained by G- LiClO_4 for the detection of both types of nitro-substituted aromatic explosives investigated in this study. G- LiClO_4 consistently exhibited superior electrochemical performance in comparison to G- Na_2SO_4 , which only displayed comparable signal responses to that of the unmodified GC electrode. Charge electrostatic interactions between surface oxygen functionalities on the graphene material and the $-\text{NO}_2$ groups of the NAC analyte was determined to be the most significant factor influencing the electron transfer behaviour of the graphene probe towards NACs reduction, dominating over the opposing effects of π - π stacking interactions with the aromatic moiety.

Enhanced sensitivity and linearity were also exhibited by the G- LiClO_4 electrochemical sensor in seawater, which also demonstrated the ability to discriminate and quantify the two NAC analytes in a sample mixture. The electrochemical sensing platform investigated in this study displays promising potential for the application of graphene-based nanomaterials with high oxygen contents for highly sensitive and selective real-time, in-situ electroanalytical detection of nitroaromatic compounds.

2.4 Experimental

2.4.1 Materials

N,N-dimethyl formamide (DMF) and acetonitrile were obtained from Merck, Singapore. 2,4-dinitrotoluene (DNT) and sodium tetraborate decahydrate were procured from Sigma-Aldrich, Singapore. DNT was prepared as a stock solution in acetonitrile (1000 ppm) for experiment use. 2,4,6-trinitrotoluene (TNT) was procured from AccuStandard, Connecticut, USA as an analytical standard solution prepared in acetonitrile (1000 ppm), and used as received. Borate buffer solution (BBS, pH 9.2, 20 mM) was prepared by dissolving sodium tetraborate decahydrate in milli-Q water. Filtered seawater (pH 8.0) was obtained from a local aquarium shop, and mixed with BBS (200 mM, pH 9.2) in a ratio of 9 : 1 to obtain an electrolyte mixture of pH 8.8. Milli-Q water with a resistivity of 18.2 MΩ cm was used for all experiments.

2.4.2 Instrumentation

Voltammetry measurements were recorded using a μ Autolab Type III electrochemical analyzer (Electrochemie, The Netherlands) under ambient conditions. All experiments were performed in a 1 mL and 5 mL electrochemical cell for TNT and DNT with a three-electrode configuration, respectively. A glassy carbon (GC, diameter of 3 mm) served as the working electrode and a platinum electrode served as the auxiliary electrode. All electrochemical potential measurements were reported with respect to the Ag/AgCl reference electrode. The working, auxiliary and reference electrodes were purchased from CH Instruments, Texas, USA.

Scanning transmission electron microscopy (STEM) was performed with a Jeol JSM 7600F instrument (Jeol, Japan) at an accelerating voltage of 30 kV. X-ray

photoelectron spectroscopy (XPS) was conducted using a Phoibos 100 spectrometer (SPECS, Germany) using a monochromatic magnesium X-ray radiation source. Raman spectroscopy spectra were recorded by a CCD detector using a confocal micro-Raman LabRam HR instrument (Horiba Scientific, Japan), under backscattering geometry mode.

2.4.3 Synthesis of Electrochemically Exfoliated Graphene Materials

Graphite foil of size 3×1 cm was immersed into the corresponding electrolyte solution (0.5 M), at an immersion depth of 1 cm. The graphite working electrode was connected to the anode with copper tape, which was assembled at a distance of 2 cm away from a platinum foil cathode. Direct current voltage of +2 V was applied to the anode for 2 min, which was then increased to +10 V until complete exfoliation was achieved. The exfoliated graphene material was washed with milli-Q water several times, then collected using suction filtration and dried at 40 °C in a vacuum oven for 48 hrs.

2.4.4 Voltammetry Analysis Procedure

Exfoliated graphene materials were suspended in DMF (1 mg mL^{-1}), followed by sonication for 2.5 hrs. The suspension ($1 \text{ }\mu\text{L}$) was deposited onto the GC electrode surface via drop-casting and the graphene material was immobilized upon solvent evaporation under a heat lamp. Material immobilization was repeated to obtain one to three layers of randomly distributed graphene material on the GC electrode. The electrode surface is renewed by polishing with alumina polishing powder ($0.05 \text{ }\mu\text{m}$) on a polishing pad following each measurement.

All cyclic voltammetry (CV) experiments were carried out at a scan rate of 100 mV s^{-1} . All differential pulse voltammetry (DPV) experiments were conducted at a step potential of 5 mV and modulation amplitude of 25 mV , and the raw data was subjected to baseline correction at a peak width of 0.01 , using the moving average algorithm. Measurement reproducibility was ensured with triplicate measurements throughout the experiments. Buffer and seawater solutions were not degassed prior to electrochemical measurements to simulate real-time environmental pollution assessment. All electrochemical potentials in this chapter are reported vs. the Ag/AgCl reference electrode.

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Chapter 3:

Graphene Quantum Dots – A Broad-Spectrum Nanoquencher for Fluorometric DNA Detection

3.1 Introduction

3.2 Results and Discussion

3.2.1 *Structural, Chemical and Photophysical Characterizations*

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3.5 References

The results in this chapter were submitted for publication.

Author Contributions:

Yew Y. T. and A. Loo H. performed the experiments. Z. Sofer and K. Klímová synthesized and characterized the graphene quantum dots. M. Pumera conceptualized and supervised the project. All authors contributed to discussion and manuscript preparation.

3.1 Introduction

This chapter illustrates the application of graphene nanostructures for the fluorescence spectroscopic detection of deoxyribonucleic acid (DNA).

Among the available analytical transduction techniques for DNA detection highlighted previously in *Sub-Section 2* of **Chapter 1**, fluorometric detection allows for extremely low detection limits necessary for the highly sensitive ultratrace detection of DNA oligonucleotides.¹

Fluorescence-based DNA detection is realized with the spontaneous and specific self-assembly of complementary DNA sequences. Such homogeneous fluorescence-based sensors typically exist as either fluorescence resonance energy transfer (FRET) assays or quenching (static quenching or FRET quenching) assays.² A DNA capture probe serves as the recognition component, with one end attached to a fluorescent dye and another end modified with a quencher molecule. In the absence of a target molecule (i.e. complementary DNA sequence), the donor and acceptor molecules are in close proximity, resulting in photoluminescence (PL) quenching. Upon the introduction of a target molecule, spontaneous self-assembly of corresponding base pairs with the DNA probe forces the quencher molecule to separate from the fluorophore molecule, resulting in fluorescence recovery.

The analytical performance of FRET sensors is highly dependent on the choice of donor-acceptor pair in the detection assay. In order to circumvent this limitation, nanomaterials can serve as the quencher molecule, since they possess the capacity to quench over a range of emission wavelengths.³ Additionally, nanoquenchers are capable of enhancing the signal-to-noise ratio of FRET genosensors.⁴

Graphene quantum dots are apposite candidates for fluorometric DNA detection due to their versatility and large sp^2 network. The intrinsic advantages of nanomaterials support the use of GQDs as FRET acceptors. Additionally, the broad excitation range coupled with the tunable emission of GQDs allows the simple selection of an excitation wavelength that corresponds well with the absorption spectrum of an acceptor molecule while avoiding its direct excitation. This thereby limits the background interference of fluorescence sensing assays and supports the use of GQDs as FRET donors as well. Therefore, GQDs can function as either FRET acceptors or donors depending on the sensing platform desired. In addition, electron delocalization within the large sp^2 network inherent to graphene-based nanostructures facilitates the adsorption of single-stranded DNA onto the nanomaterial surface, via non-covalent π - π stacking interactions and hydrophobic interactions with DNA nucleobases.⁵⁻⁷ The application of graphene quantum dots as FRET donors in DNA sensing has been well-explored in other works.⁸⁻⁹ However, exploration of GQDs as potential FRET acceptors in DNA detection still remains at a preliminary stage.

In this chapter, the application of graphene quantum dots as nanoquenchers in the development of a fluorescence-based nucleic acid detection platform was explored and presented as a proof of concept. Graphene quantum dots were synthesized from the facile chemical oxidation of a cost-effective fossil fuel derivative – calcined petroleum coke.

3.2 Results and Discussion

3.2.1 Structural, Chemical and Photophysical Characterizations

Comprehensive characterizations were first performed to analyze the structural, chemical and photophysical properties of GQDs. GQD characterizations were performed using transmission electron microscopy (TEM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FT-IR), Raman spectroscopy, UV–vis absorption spectroscopy and fluorescence emission spectroscopy.

TEM was performed to probe the structural morphology of the GQD particles (Figure 1).¹⁰ Moderate to severe aggregation of GQDs is observed following sample drying for TEM imaging. Spherical dots are evident from the TEM images.

Figure 1. TEM image of GQDs. Scale bar: 50 nm.

DLS was performed to obtain the size distribution profile of the GQDs in water (Figure 2).¹¹⁻¹² The hydrodynamic diameters of the GQDs demonstrate broad size distributions, with sizes mainly concentrating at 55 nm. The generally large lateral dimensions of the as-derived GQD particles may be attributed to the layered morphology of GQDs.

Figure 2. DLS particle size distribution of GQDs.

Wide-scan and high-resolution XPS of C1s were conducted to analyze the surface chemical composition and bonding configurations within the GQD materials (Figure 3).¹³⁻¹⁴ Wide-scan XPS results showed that the GQDs contained mainly C and O elements, with a C/O ratio of 1.63 (Figure 3A). The extensive oxygen functionalities present on GQDs highlights the formation of additional hydrogen-bonding interactions with the DNA nucleobases during the adsorption of single-stranded DNA onto the GQDs.¹⁵ High-resolution XPS of C1s uncovered the presence of C=C, C-C, C-O, C=O, and O-C=O peaks, suggesting the presence of hydrophobic regions as well as hydrophilic hydroxyl, carbonyl and carboxylic acid functionalities in the GQDs (Figure 3B). The large abundance of hydrophilic moieties in the GQDs greatly

enhances the solubility of GQDs in aqueous solutions and supports their applications in aqueous systems.

Figure 3. (A) Wide-scan and (B) high-resolution C1s XPS spectra of GQDs.

FT-IR was performed to ascertain the functional groups present in the GQDs (Figure 4).¹⁶ The FT-IR spectrum displayed peaks at approximately 3432 cm^{-1} , 1724 cm^{-1} , 1621 cm^{-1} and 1068 cm^{-1} , corresponding to the stretching vibrations of O–H, C=O, C=C and C–O, respectively. The peak at 1437 cm^{-1} may be attributed to the C–OH or C–H bending modes. The FT-IR data further confirms the presence of hydrophilic carboxyl and hydroxyl functional groups in the GQDs.

Figure 4. FT-IR spectrum of GQDs.

Raman spectroscopy was performed to evaluate the density of defects in GQDs (Figure 5).¹⁷ A D/G ratio of 0.44 was obtained, suggesting a possibly low degree of

structural defects present in the as-synthesized GQDs. However, the enhanced intensity of the G-band is also partly attributed to the instrumentation (He–Cd UV laser, 325 nm) operated for Raman spectroscopy. High cross-section of G-band resulted in signal intensification in comparison to the D-band.¹⁸

Figure 5. Raman spectrum of GQDs.

Fluorescence emission and ultraviolet–visible (UV–vis) absorption spectroscopy were performed to evaluate the optical properties of the GQDs (Figure 6).¹⁹ Negligible PL emission was observed over the entire UV–vis region for a range of applied excitation wavelengths (λ_{ex}) spanning from 230 – 600 nm, while broad absorption bands spanning UV and near-visible light regions were observed. This verifies the non-fluorescent nature of the GQDs and supports their potential application as nanoquenchers in fluorescence-based biosensing platforms. The spikes in fluorescence emission which shift with a change in λ_{ex} may be attributed to Rayleigh–Tyndall scattering.²⁰

Figure 6. Fluorescence emission spectra (solid lines) at 230 – 600 nm excitation wavelengths, λ_{ex} , and UV–vis absorption (dashed line) spectrum of GQDs. Conditions: λ_{ex} = 230, 350, 450 and 490 nm are presented.

3.2.2 Graphene as a Nanoquencher for Fluorometric DNA detection

Scheme 1 illustrates the schematic representation of DNA biosensor system based on GQD as a nanoquencher. In (A), FAM-Lprobe adsorbs onto the GQD surface following incubation, via three types of bonding interactions: (i) π - π stacking interactions, (ii) hydrophobic interactions and (iii) hydrogen-bonding interactions. The favoured adsorption process of single-stranded DNA onto GQD results in FRET quenching of FAM fluorophore by the GQD quencher. In (B), the initial addition of the cDNA target to FAM-Lprobe promotes the hybridization of both single-stranded oligonucleotide sequences, resulting in the formation of a double-stranded DNA complex (*ds*-cDNA-FAM-Lprobe) which is then incubated with the GQD nanoquencher. Since the DNA nucleobases are shielded within the phosphate backbone, the *ds*-cDNA-FAM-Lprobe assembly has reduced affinity for GQD and does not adsorb onto the quencher surface, consequently retaining the PL signal of the FAM dye as opposed to (A).

Scheme 1. Schematic representation (not to scale) of the proposed DNA detection strategy based on GQD as a nanoquencher.

GQDs were then incubated with FAM-Lprobe to confirm the successful adsorption of the fluorophore molecules onto the quencher GQD surface. Quencher volume was optimized for a utilized concentration of 40 nM FAM-Lprobe, by recording changes in the fluorescence signal of the FAM fluorophore upon exposure to increasing volumes of GQDs for 20 min (Figure 7). A gradual increase in the quenching efficiency of GQDs on FAM was observed with the incubation of higher concentrations of GQDs, indicating successful fluorescence quenching of FAM, which tapers off at around 100 μ M. Consequently, an optimum volume of 100 μ M was applied for subsequent fluorometric assays.

Figure 7. Fluorescence quenching of FAM-Lprobe (40 nM) with increasing volumes of GQDs (0, 20, 40, 60, 80, 100 and 120 μ L). Conditions: Tris-HCl buffer (20 mM, pH 7.4), $\lambda_{\text{ex}} = 490$ nm.

Prior to investigating the sensing performance of GQDs for cDNA target detection, a control study was first conducted in order to investigate the effects of interference by the cDNA target on the detected fluorescence signal (Figure 8A). No apparent influence on the fluorescence emission was observed by cDNA following hybrid formation with FAM-Lprobe (comparing brown line with blue line). Conversely, fluorescence quenching was evident following the adsorption of FAM-Lprobe onto GQDs (comparing brown line with purple line). This is therefore confirmation that the PL emission intensity is solely influenced by FAM-Lprobe, and that cDNA has no direct effect on the detected PL intensity.

The sensing performance of the proposed DNA detection strategy was then evaluated over a range of cDNA target concentrations spanning across 4 pM to 400000 pM. The corresponding calibration graph based on the fluorescence intensity of FAM-Lprobe was plotted as a linear-logarithmic function of cDNA concentration (Figure 8B). Higher fluorescence intensity of FAM-Lprobe was achieved with higher concentrations of cDNA, tapering off at 4000 pM. This corresponds to the formation of larger amounts of the *ds*-cDNA-FAM-Lprobe hybrid complex which exhibit poor physical adsorption onto GQDs, thereby resulting in a lower extent of fluorescence quenching and giving rise to a greater extent of fluorescence intensity retention. The linear dynamic range of detection ($R = 0.89$) extends from 4 pM to 4000 pM. Low limits of detection (LOD) and quantification (LOQ) of 9.2 pM and 30.5 pM are attained, respectively.

Figure 8. (A) Fluorescence emission spectra of FAM-Lprobe (40 nM) following cDNA target (4000 pM) hybridization, in the presence and absence of GQDs. (B) Calibration curve of FAM-Lprobe (40 nM) with increasing concentrations of cDNA target (4, 40, 400 and 4000 pM), in the presence of GQDs. Conditions: Tris-HCl buffer (20 mM, pH 7.4), $\lambda_{\text{ex}} = 490 \text{ nm}$.

In order to evaluate the selectivity of the proposed DNA sensing platform, the fluorescence responses of FAM-Lprobe towards complementary (cDNA), three-bases mismatched (mDNA-3) and non-complementary (ncDNA) nucleic acid sequences were examined and compared, following incubation with GQDs for 20 min. Fluorescence recovery of up to 63% was achieved with the introduction of cDNA, as compared to only 56% and 51% in the presence of mDNA-3 and ncDNA, respectively (Figure 9). The results obtained illustrate the ability of the detection assay to distinguish complementary DNA from a mismatched or non-complementary sequence, as well as the ability to discriminate multiple nucleotide length polymorphisms from the cDNA of interest.

Figure 9. Fluorescence emission spectra of FAM-Lprobe upon incubation with cDNA (4000 pM), mDNA-3 (4000 pM) and ncDNA (4000 pM), in the presence of GQDs. Conditions: Tris-HCl buffer (20 mM, pH 7.4), $\lambda_{\text{ex}} = 490$ nm.

3.3 Conclusions

This chapter elucidated the application of graphene quantum dots synthesized from calcined petroleum coke as a FRET acceptor in fluorescence-based nucleic acid detection. GQDs derived from a facile, one-step synthesis method were successfully employed as nanoquenchers in the development of a fluorescent sensing platform for DNA detection, for the first time.

This detection strategy exploits the differences in affinities of labelled single-stranded and double-stranded DNA oligonucleotides towards spontaneous self-assembly with GQD particles. The proposed detection assay was able to detect DNA concentrations with LOD and LOQ values as low as 9.2 pM and 30.6 pM respectively, and demonstrated a large linear dynamic detection range over three orders of magnitude from 4 pM to 4000 pM with good linearity ($R = 0.89$). The DNA probe was also capable of realizing triple nucleotide polymorphism determination; nevertheless further experimentation using different quencher systems may be beneficial towards improving the target specificity performance of the detection assay.

The cost-effectiveness and large abundance of fossil fuel precursors makes these GQDs a promising nanomaterial for the development of fluorescence-based detection platform for genosensing applications. The reported findings would pave the way towards the identification of alternative nanomaterials for the development of inexpensive fluorescence-based nanobiosensors with high selectivity and sensitivity for DNA sensing.

3.4 Experimental

3.4.1 Materials

Calcinated petroleum coke (1 – 2 cm, source: Slovakia), was purchased from Resorbent S.R.O (Ostrava, Czech Republic). Sulfuric acid (96 %, w/w), nitric acid (68, w/w) and sodium hydroxide (>99%, *p.a.*) were obtained from Penta (Prague, Czech Republic). Dialysis membranes (Visking, MWCO 14 kDa) were purchased from P-LAB (Prague, Czech Republic). Tris(hydroxymethyl)aminomethane, sodium chloride, potassium chloride and hydrochloric acid (37%, w/w) were obtained from Sigma-Aldrich (Singapore). Magnesium chloride hexahydrate was obtained from Quality Reagent Chemical (Auckland, New Zealand). Deionized water (16.8 MΩ cm) was used for GQD synthesis and dialysis. Tris-HCl buffer (20 mM, pH 7.4) was prepared by dissolving NaCl (100 mM), KCl (5 mM) and MgCl₂ (5 mM) in milli-Q water (18.2 MΩ cm), and was used for all spectroscopic detection experiments. DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore) and purified using high-performance liquid chromatography. The DNA oligonucleotide sequences are listed as follows:

FAM-Lprobe:

5' [6FAM] ACC AGG CGG CCG CAC ACG TCC TCC AT 3 ';

Complementary DNA target, cDNA:

5' ATG GAG GAC GTG TGC GGC CGC CTG GT 3 ';

Three-base mismatched DNA, mDNA–3:

5' ATG GAG GAC GTG ATA GGC CGC CTG GT 3 ';

Non-complementary DNA, ncDNA:

5' AAA AAA AAA AAA AAA AAA AAA AA 3'.

3.4.2 Instrumentation

Transmission electron microscopy (TEM) was performed using a JEOL JEM-1010 instrument at an accelerating voltage of 80 kV. Images were captured using SIS MegaView III digital camera (Soft Imaging Systems) and analyzed with AnalySIS v2.0 software. Samples were dried prior to measurements.

Dynamic light scattering (DLS) measurement was conducted at 20 °C with a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, England).

Wide-scan and high-resolution C1s X-ray photoelectron spectroscopy (XPS) were performed using an ESCAProbeP spectrometer (Omicron Nanotechnology Ltd, Taunusstein, Germany) using a monochromatic aluminium X-ray radiation source (1486.7 eV). Samples were prepared for XPS measurements by drop-casting the GQD solution on gold coated silicon wafers and dried in a vacuum oven (50 °C).

Fourier transform infrared spectroscopy (FT-IR) spectrum was measured by a MCD D* detector using a NICOLET iS50R spectrometer (Thermo Scientific, Waltham, MA, USA) affixed with a Smart SAGA Specular Reflectance accessory. Samples were prepared for XPS measurements by drop-casting the GQD solution on gold coated silicon wafers and dried in a vacuum oven (50 °C).

Raman spectroscopy was recorded by a CCD detector using an inVia Raman microscope (Renishaw, England), under backscattering geometry mode.

GQD concentration was calculated using a LiquiTOC II analyzer (Elementar Analysensysteme, Hanau, Germany).

UV–Vis absorbance spectroscopy measurements were performed at room temperature using a Cary 100 UV–Vis spectrophotometer. All measurements were performed using a quartz glass cuvette.

Photoluminescence spectroscopy scans were recorded at room temperature using a Varian Cary Eclipse fluorescence spectrophotometer. λ_{ex} of the nucleic acid detection experiments involving GQDs was fixed at 490 nm, and the fluorescence emission spectra were measured across the range of 200–900 nm. All measurements were conducted with a quartz glass cuvette.

3.4.3 Synthesis of Graphene Quantum Dots

Concentrated sulfuric acid (60.0 mL), nitric acid (22.0 mL) and the calcined carbon precursor (300 mg) were added into a reaction flask, then sonicated for 2 hrs. The obtained reaction mixture was then heated at 100 °C for 24 hrs under stirring, then allowed to cool undisturbed to room temperature. The mixture was then poured into a beaker over ice (100 g), and the pH was adjusted to 6.0 using sodium hydroxide solution (3 M). The solution was then filtered with a nylon filter (0.45 μm) and maintained at 0 °C for 24 hrs. Crystallized sodium sulfate was then removed using suction filtration, and the filtrate was dialysed for 5 days in distilled water until the conductivity dropped below 20 μS . The dialysate was concentrated using a vacuum evaporator, affording the GQDs solution (142 mg L^{-1} , 100 mL).

3.4.4 Preparation of DNA Sensing System Based on Graphene

For assay development, cDNA prepared in varying concentrations was added to 40 nM of FAM-Lprobe in 1 mL of Tris-HCl buffer. The mixture was heated at 50 °C for 30 min with continuous agitation at 400 rpm, then cooled to room temperature over 30 min. The solution mixture was then incubated with 100 µL of GQDs for 20 min at room temperature. Spectroscopic analysis was performed using 1 mL of the prepared sample.

For selectivity evaluation, 100 µL of GQDs was incubated with 40 nM of FAM-Lprobe in 1 mL of Tris-HCl buffer for 20 min. 4000 pM of the DNA of interest (cDNA, mRNA or ncDNA) was added into the mixture, which was then heated at 50 °C for 30 min with continuous agitation at 250 rpm. Spectroscopic analysis was performed using 1 mL of the prepared sample upon cooling the sample mixture to room temperature over 30 min.

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Chapter 4:

Black Phosphorus Quantum Dots – A Novel Nanosensor for Fluorescence-Based DNA Detection

4.1 Introduction

4.2 Results and Discussion

4.2.1 Structural, Chemical and Photophysical Characterizations

4.2.2 Black Phosphorus as a Nanofluorophore for Fluorometric DNA Detection

4.3 Conclusions

4.4 Experimental

4.4.1 Materials

4.4.2 Instrumentation

4.4.3 Synthesis of Black Phosphorus Nanoparticles

4.4.4 Preparation of DNA Sensing System Based on Black Phosphorus

4.5 References

The results in this chapter were submitted for publication.

Author Contributions:

Yew Y. T. conceptualized the project and performed the experiments. Z. Sofer synthesized and characterized the black phosphorus nanoparticles. M. Pumera directed and supervised the project. All authors contributed to discussion and manuscript preparation.

4.1 Introduction

In **Chapter 3**, the application of graphene quantum dot as a nanoquencher for the development of a fluorescence-based DNA detection assay was examined in detail. The exploration of suitable nanomaterials as potential candidates for fluorometric DNA sensing applications is continued in **Chapter 4**.

This chapter illustrates the application of black phosphorus nanostructures for the fluorescence spectroscopic detection of deoxyribonucleic acid (DNA).

Despite the promising properties exhibited by black phosphorus as discussed in *Sub-Section 1.3* of **Chapter 1**, exploration of the novel nanomaterial for potential biomedical applications remains limited at this stage.¹⁻² This lack of interest predominantly stems from the susceptibility of black phosphorus towards photobleaching under ambient conditions,³ limiting its practical use. However, the successful synthesis of air-stable black phosphorus nanostructures in recent studies can overcome this major limitation allowing for the development of fluorescence-based biosensing devices employed by black phosphorus.

In this chapter, the application of pristine black phosphorus nanoparticle (BPNP) as a fluorophore in the development of a novel fluorescence-based nucleic acid detection platform was investigated and presented as a proof of concept. BPNPs were synthesized from the red allotrope using a high pressure phase transformation, which is followed by shear force milling in *N,N*-dimethylformamide (DMF).

4.2 Results and Discussion

4.2.1 Structural, Chemical and Photophysical Characterizations

Comprehensive characterizations were first performed to analyze the structural, chemical and photophysical properties of BPNPs. BPNP characterizations were performed using transmission electron microscopy (TEM), X-ray powder diffraction (XRD), X-ray photoelectron spectroscopy (XPS), Raman spectroscopy and fluorescence emission spectroscopy.

TEM was performed to study the structural morphology⁴ and crystallinity⁵ of the black phosphorus material. Few layered nanoparticles with lateral dimensions of approximately 100 nm are observed (Figure 1A). The orthorhombic crystalline nature of the as-synthesized black phosphorus can be observed using high-resolution TEM (HR-TEM) in Figure 1B and is further confirmed by the selected area electron diffraction (SAED) pattern shown in the inset of Figure 1A.

Figure 1. (A) TEM and (B) HR-TEM images of BPNPs. Inset: SAED pattern of BPNP.

Scale bar: (A) 100 nm and (B) 10 nm.

XRD was performed to study the crystal structure of the BPNPs.⁶ The obtained diffractogram further affirms the orthorhombic crystal structure of the BPNPs. The weak reflections present in the diffractogram are attributed to the (0k0) reflections (Figure 2).⁷

Figure 2. XRD pattern of BPNPs.

High-resolution XPS of P2p was conducted to determine the types of chemical bonding present in BPNPs (Figure 3).⁸ Two peaks at 129.7 eV and 134.6 eV were observed, and they are assigned to elemental P⁰ and oxidized P, respectively. The peak at 134.6 eV may be attributed to the partial surface oxidation of BPNPs.⁹ Moreover, predominance of the peak at 129.7 eV corresponding to 2p energy binding was also observed. The obtained high-resolution XPS spectrum is in good agreement with the literature.¹⁰

Figure 3. High-resolution P2p XPS spectrum of BPNPs.

Further structural characterization of BPNPs was conducted with the use of Raman spectroscopy (Figure 4). The A_g^1 , B_{2g} and A_g^2 optical photons characteristic of black phosphorus materials were observed at 360.7 cm^{-1} , 438.6 cm^{-1} and 465.7 cm^{-1} . The locations of the three Raman active modes correspond well with literature values.¹¹

Figure 4. Raman spectrum of BPNPs.

Fluorescence emission spectroscopy was performed to evaluate the optical properties of the BPNPs (Figure 5).¹² Maximum PL emission was observed at 527 nm, corresponding to an excitation wavelength (λ_{ex}) of 200 nm. This verifies the

fluorescent nature of the BPNPs and supports their potential application as nanofluorophores in fluorescence-based biosensing platforms. BPNPs were excited at 200 nm for subsequent experiments in this study.

Figure 5. Fluorescence emission spectra of BPNPs at 200 – 230 nm excitation wavelengths, λ_{ex} . Conditions: $\lambda_{\text{ex}} = 200, 210, 220$ and 230 nm.

4.2.2 Black Phosphorus as a Nanofluorophore for Fluorometric DNA Detection

Scheme 1 illustrates the schematic representation of DNA biosensor system based on BPNP as a nanofluorophore. In (A), Dabcyl-Lprobe adsorbs onto the BPNP surface following incubation, likely via dispersive forces and hydrophobic interactions.¹³ The favoured adsorption process of single-stranded DNA onto BPNP results in PL quenching of BPNP by the dark quencher label, dabcyl. In (B), the initial addition of the cDNA target to dabcyl-Lprobe promotes the hybridization of both single-stranded oligonucleotide sequences, resulting in the formation of a double-stranded DNA complex (*ds*-cDNA-dabcyl-Lprobe) which is then incubated with the BPNP fluorophore. The *ds*-cDNA-dabcyl-Lprobe assembly has reduced affinity for BPNP and does not adsorb onto the fluorophore surface, consequently retaining the PL signal of BPNP as opposed to (A).

Scheme 1. Schematic representation (not to scale) of the proposed DNA detection strategy based on BPNP as a nanofluorophore.

BNPs were then incubated with dabcyl-Lprobe to confirm the successful adsorption of the quencher molecules onto the fluorescent nanoparticle surface. Quencher concentration was optimized for a utilized volume of 75 μL BPNP, by recording

changes in the fluorescence signal of the nanofluorophore upon exposure to increasing concentrations of dabcyl-Lprobe for 20 min (Figure 6). A gradual increase in the quenching efficiency of dabcyl on BPNPs was observed with the incubation of higher concentrations of dabcyl-Lprobe, indicating successful fluorescence quenching of BPNPs, which tapers off at around 160 nM. Consequently, an optimum concentration of 160 nM was applied for subsequent fluorometric assays.

Figure 6. Fluorescence quenching of BPNP with increasing concentrations of dabcyl-Lprobe (0, 40, 100, 160 and 200 nM). Conditions: Tris-HCl buffer (10 mM, pH 7.2), λ_{ex} = 200 nm.

Prior to investigating the sensing performance of BPNPs for cDNA target detection, a control study was first conducted in order to investigate the effects of interference by the cDNA target on the detected fluorescence signal (Figure 7A). No apparent fluorescence emission was observed by cDNA following hybrid formation with dabcyl-Lprobe (comparing blue line with green line). Conversely, fluorescence quenching was evident following the adsorption of dabcyl-Lprobe onto BPNPs (comparing brown line with purple line). This is therefore confirmation that the PL

emission intensity is solely influenced by the BPNPs, and that cDNA has no direct effect on the detected PL intensity.

The sensing performance of the proposed DNA detection strategy was then evaluated over a range of cDNA target concentrations spanning across 4 pM to 400000 pM. The corresponding calibration graph based on the fluorescence intensity of BPNPs was plotted as a linear-logarithmic function of cDNA concentration (Figure 7B). Higher fluorescence intensity of BPNPs was achieved with higher concentrations of cDNA, tapering off at 4000 pM. This corresponds to the formation of larger amounts of the *ds*-cDNA-dabcyl-Lprobe hybrid complex which exhibit poor physical adsorption onto BPNPs, thereby resulting in a lower extent of fluorescence quenching and giving rise to a greater extent of fluorescence intensity retention. The linear dynamic range of detection ($R = 0.91$) extends from less than 4 pM to 4000 pM. Low limits of detection (LOD) and quantification (LOQ) of 5.9 pM and 19.7 pM are attained, respectively.

Figure 7. (A) Fluorescence emission spectra of *ds*-cDNA-dabcyl-Lprobe hybrid (4000 pM) and BPNP, in the presence and absence of dabcyl-Lprobe (160 nM). (B) Calibration curve of BPNP with increasing concentrations of cDNA target (4, 40, 400

and 4000 pM), in the presence of dabcyl-Lprobe (160 nM). Conditions: Tris-HCl buffer (10 mM, pH 7.2), $\lambda_{\text{ex}} = 200$ nm.

In order to evaluate the selectivity of the proposed DNA sensing platform, the fluorescence responses of BPNPs towards complementary (cDNA), three-bases mismatched (mDNA-3) and non-complementary (ncDNA) nucleic acid sequences were examined and compared, following incubation with dabcyl-Lprobe for 20 min. Fluorescence recovery of up to 45% was achieved with the introduction of cDNA, as compared to only 37% and 27% in the presence of mDNA-3 and ncDNA, respectively (Figure 8). The results obtained illustrate the ability of the detection assay to distinguish complementary DNA from a mismatched or non-complementary sequence, as well as the ability to discriminate multiple nucleotide length polymorphisms from the cDNA of interest.

Figure 8. Fluorescence emission spectra of BPNP upon incubation with cDNA (400 pM), mDNA-3 (400 pM) and ncDNA (400 pM), in the presence of dabcyl-Lprobe. Conditions: Tris-HCl buffer (10 mM, pH 7.2), $\lambda_{\text{ex}} = 200$ nm.

4.3 Conclusions

This chapter elucidated the application of black phosphorus nanoparticle as a novel nanomaterial in fluorescence-based nucleic acid detection. BPNPs derived from a facile, one-step synthesis method were successfully employed as nanofluorophores in the development of a fluorescent sensing platform for DNA detection, for the first time.

This detection strategy exploits the differences in affinities of labelled single-stranded and double-stranded DNA oligonucleotides towards spontaneous self-assembly with BPNPs. The proposed detection assay was able to detect DNA concentrations with LOD and LOQ values as low as 5.9 pM and 19.7 pM respectively, and demonstrated a large linear dynamic detection range from less than 4 pM to 4000 pM with good linearity ($R = 0.91$). The DNA probe was also capable of realizing triple nucleotide polymorphism determination.

This chapter demonstrates a proof of concept that black phosphorus possesses promising potential for biosensing applications, in particular as DNA nanobiosensors. The results presented in this chapter may promote the investigation of BPNP as a fluorescence-based detection platform for other target molecules including proteins, enzymes and inorganic ions. The reported findings would be significant in encouraging further research work into the development of biomedical devices and technologies based on black phosphorus.

4.4 Experimental

4.4.1 Materials

Red phosphorus (99.999%) was obtained from Sigma-Aldrich (Prague, Czech Republic). *N,N*-dimethylformamide (DMF) was purchased from PENTA (Prague, Czech Republic). Tris(hydroxymethyl)aminomethane, sodium chloride and hydrochloric acid (37%, w/w) were purchased from Sigma-Aldrich (Singapore). Magnesium chloride hexahydrate was purchased from Quality Reagent Chemical (Auckland, New Zealand). Tris-HCl buffer was prepared by dissolving NaCl (150 mM) and MgCl₂ (5 mM) in milli-Q water (18.2 MΩ cm), and was used for all spectroscopic detection experiments. DNA oligonucleotides were procured from Sigma-Aldrich (Singapore). The oligonucleotide sequences are listed as follows:

Dabcyl-Lprobe:

5' [Dabcyl] ACC AGG CGG CCG CAC ACG TCC TCC AT 3' ;

Complementary DNA, cDNA:

5' ATG GAG GAC GTG TGC GGC CGC CTG GT 3' ;

Three-base mismatched DNA, mDNA-3:

5' ATG GAG GAC GTG ATA GGC CGC CTG GT 3' ;

Non-complementary DNA, ncDNA:

5' AAA AAA AAA AAA AAA AAA AAA AAA AA 3' .

4.4.2 Instrumentation

High-resolution transmission electron microscopy (HR-TEM) was performed with an EFTEM Jeol 2200 FS microscope (Jeol, Japan). An acceleration voltage of 200 keV was used for measurement. The sample was prepared by drop casting the suspension (1 mg mL⁻¹ in water) on a TEM grid (Cu; 200 mesh; Formvar/carbon) and drying at 60 °C for 12 h.

X-ray powder diffraction (XRD) data were collected at room temperature on a Bruker D8 Discoverer powder diffractometer with parafocusing Bragg–Brentano geometry using Cu K α radiation (λ = 0.15418 nm, U = 40 kV, I = 40 mA). The data was scanned over the angular range of 10–80° (2 θ) with a step size of 0.02° (2 θ). Data evaluation was performed in the software package EVA.

X-ray photoelectron spectroscopy (XPS) was performed with an ESCAProbeP spectrometer (Omicron Nanotechnology Ltd, Taunusstein, Germany) using a monochromatic aluminium X-ray radiation source (1486.7 eV). Samples were prepared for XPS measurements by drop-casting the BPNP solution on gold coated silicon wafers and dried in a vacuum oven (50 °C).

Raman spectrum was recorded using a CCDdetector using an inVia Raman microscope (Renishaw, England), under backscattering geometry mode using DPSS laser (532 nm, 50 mW), through a 50 $\times$ objective and using 2.5 mW laser power.

Photoluminescence spectroscopy scans were recorded at room temperature using a Varian Cary Eclipse fluorescence spectrophotometer. λ_{ex} of the nucleic acid detection experiments involving BPNPs was fixed at 200 nm, and the fluorescence emission

spectra were measured across the range of 200–900 nm. All measurements were conducted with a quartz glass cuvette.

4.4.3 Synthesis of Black Phosphorus Nanoparticles

Red phosphorus (10 g) was wrapped in graphite foil and placed in high pressure/high temperature uniaxial pressing apparatus of 1" size. The sample was compressed at 6 GPa and heated at 600 °C for 30 min, at a rate of 100 °C min⁻¹, then allowed to cool to room temperature at a rate of 100 °C min⁻¹. The graphite foil was removed by polishing. Pulverization of the obtained black phosphorus in agate mortar followed by sieving was carried out to separate particles with sizes below 0.5 mm. Black phosphorus powder was then dispersed in DMF by ultrasonication (9 mg mL⁻¹; 400 W; 15 min). Milling by share force milling apparatus at 17000 rpm was then performed in a glass jacketed vessel at 15 °C for 1 hr, under argon atmosphere.

4.4.4 Preparation of DNA Sensing System Based on Black Phosphorus

For assay development, cDNA prepared in varying concentrations was added to 160 nM of dabcyL-Lprobe in 1 mL of Tris-HCl buffer. The mixture was heated at 50 °C for 30 min with continuous agitation at 400 rpm, then cooled to room temperature over 30 min. The solution mixture was then incubated with 75 µL of BPNPs for 20 min at room temperature. Spectroscopic analysis was performed using 1 mL of the prepared sample.

For selectivity evaluation, 75 µL of BPNPs was incubated with 160 nM of dabcyL-Lprobe in 1 mL of Tris-HCl buffer for 20 min. 400 pM of the DNA of interest (cDNA, mDNA–3 or ncDNA) was added into the mixture, which was then heated at 50 °C for 30 min with continuous agitation at 250 rpm. Spectroscopic analysis was performed

using 1 mL of the prepared sample upon cooling the sample mixture to room temperature over 30 min.

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Chapter 5:

Outlook and Conclusion

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The emergence of novel nanomaterials such as graphene and black phosphorus in the recent years has generated much impetus for the fundamental understanding of these layered nanostructures for their application in nanodevices and nanosystems. Chemical sensors utilizing nanotechnology are at the forefront of environmental health and biomedical research applications. The design and fabrication of nanosensors based on electrochemical and fluorometric transduction techniques for the detection of NAC explosives and DNA were introduced in this thesis.

Investigation of graphene materials obtained from electrochemical exfoliation for the electrochemical sensing of NAC explosives was conducted in **Chapter 2**. The presence of additional oxygen functionalities onto G-LiClO₄ proved to be critical in promoting electrostatic interactions with the –NO₂ groups of the NAC analyte, thereby assisting in the electrochemical reduction and detection of NACs on electrochemically exfoliated graphene materials. The examination of nanostructures for the fabrication of fluorescence sensing platforms for DNA detection was subsequently explored in **Chapters 3** and **4**. Possessing the ability to function as a broad-spectrum nanoquencher, graphene quantum dots obtained from calcined petroleum coke precursors were successfully employed in the development of cost-effective DNA sensing assays in **Chapter 3**. The application of black phosphorus nanoparticles in DNA detection was also presented as a proof of concept in **Chapter 4**, offering infinite possibilities for the utilization of the novel layered nanostructure in biomedical applications.

In this thesis, different transduction techniques were adopted for the examination of novel layered nanomaterials in sensing applications. The reported findings challenge

conventional understanding of the processes involved in analyte detection by graphene nanostructures, as well as expand the spectrum of applications offered by black phosphorus nanostructures. Optimization with a range of donor fluorophores and acceptor quenchers for graphene quantum dots and black phosphorus nanoparticles, respectively, would be beneficial in the identification of the most suitable donor-acceptor pairs in the fabrication of target recognition specific FRET-based sensors. Further studies into the application of graphene and black phosphorus obtained from different synthesis routes would also be vital to achieve nanosensors with high sensitivity for ultra-trace detection applications.

Nonetheless, exploration of graphene and black phosphorus in sensing applications remain primitive as novel detection strategies continue to emerge. With continued research, these layered nanomaterials may in time redefine the limitations of current nanotechnology-enabled sensors.