Nanomaterials for electrochemical biosensing applications and fundamental studies

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NANOMATERIALS FOR ELECTROCHEMICAL
BIOSENSING APPLICATIONS AND FUNDAMENTAL
STUDIES

ADELINE LOO HUI LING

2016

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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BIOSENSING APPLICATIONS AND FUNDAMENTAL
STUDIES**

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SCHOOL OF PHYSICAL & MATHEMATICAL SCIENCES
NANYANG TECHNOLOGICAL UNIVERSITY

2016

Nanomaterials for Electrochemical Biosensing Applications and Fundamental Studies

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As what Friedrich Nietzsche, a German philosopher, says, “That which does not kill us, makes us stronger”. This journey has not been smooth-sailing for sure, but I am thankful for the numerous obstacles and hurdles that were presented to me. For it is these challenges which gave me the chance to learn important lessons, be it in the context of scientific research or in life. Nevertheless, these obstacles would not have been overcome without the help of the following people.

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Finally, I would like to urge candidates who are currently in the midst of their journey, be diligent and courageous in the pursuit of science, even if you are the only one exploring a particular direction. Follow your passion and fulfil your chase for knowledge, never do it for the glory and allow yourself to take the easy way out, or be constrained by your peers and the environment.

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Summary

Nanomaterials represent the key building blocks of nanotechnology. Due to the extensive research performed on them, a wide array of nanomaterials in different sizes, shapes and compositions are now available. In this thesis, we focus on three classes of nanomaterials, specifically graphene and its analogues, transition metal dichalcogenides, and carbon quantum dots. Attributable to their attractive physical and chemical properties, these nanomaterials are ideal candidates for signal generation and transduction in biosensing applications. Nonetheless, despite of the numerous studies conducted in an attempt to employ these nanomaterials for biosensors development, some areas remain unexplored. In particular, there is still inadequate understanding of the fundamental working principles of biosensors, and potential novel biosensing systems which are yet to be designed. In view of these, the three classes of nanomaterials in study were exploited to gain deeper insights on the fundamental principles and to explore novel biosensing systems.

Specifically, graphene and its analogues were utilized to attain better understandings on the effects of DNA immobilization methods on its subsequent hybridization efficiency, and to validate the mechanism behind physical adsorption of DNA. After which, a total of four novel biosensing applications were demonstrated with graphene oxide and chemical vapour deposition fabricated graphene.

With transition metal dichalcogenides, the inherent charge transfer resistance of these nanomaterials was first characterized before using them to investigate their interactions with DNA. Thereafter, the influence of different transition metals, in these nanomaterials, on their performance as a fluorescence quencher for DNA detection was

studied. Finally, a new approach of using molybdenum disulfide for DNA detection was established.

Last of all, for the case of carbon quantum dots, the pioneering work of adopting them as the fluorescence quencher for DNA detection was illustrated.

List of Publications

[1-3] Review articles

1. Bonanni, A.; **Loo, A. H.**; Pumera, M., Graphene for Impedimetric Biosensing. *Trac-Trend. Anal. Chem.* 2012, *37*, 12-21.
2. Pumera, M.; **Loo, A. H.**, Layered Transition Metal Dichalcogenides (MoS₂ and WS₂) for Sensing and Biosensing. *Trac-Trend. Anal. Chem.* 2014, *61*, 49-53.
3. Ambrosi, A.; Chua, C. K.; Latiff, N. M.; **Loo, A. H.**; Wong, C. H. A.; Eng, A. Y. S.; Bonanni, A.; Pumera, M., Graphene and its Electrochemistry - An update. *Chem. Soc. Rev.* 2016, *45*, 2458-2493.

[4-9] Research articles: Graphene and its analogues

4. **Loo, A. H.**; Bonanni, A.; Pumera, M., Soldering DNA to Graphene via 0, 1 and 2-point Contacts: Electrochemical Impedance Spectroscopic Investigation. *Electrochem. Commun.* 2013, *28*, 83-86.
5. **Loo, A. H.**; Bonanni, A.; Pumera, M., An Insight into the Hybridization Mechanism of Hairpin DNA Physically Immobilized on Chemically Modified Graphenes. *Analyst* 2013, *138*, 467-471.
6. **Loo, A. H.**; Bonanni, A.; Pumera, M., Thrombin Aptasensing with Inherently Electroactive Graphene Oxide Nanoplatelets as Labels. *Nanoscale* 2013, *5*, 4758-4762.
7. **Loo, A. H.**; Bonanni, A.; Pumera, M., Inherently Electroactive Graphene Oxide Nanoplatelets as Labels for Specific Protein-Target Recognition. *Nanoscale* 2013, *5*, 7844-7848.

8. **Loo, A. H.**; Bonanni, A.; Pumera, M., Mycotoxin Aptasensing Amplification by using Inherently Electroactive Graphene Oxide Nanoplatelet Labels. *ChemElectroChem* 2015, 2, 743-747.
9. **Loo, A. H.**; Ambrosi, A.; Bonanni, A.; Pumera, M., CVD Graphene based Immunosensor. *RSC Adv.* 2014, 4, 23952-23956.

[10-13] Research articles: Transition metal dichalcogenides

10. **Loo, A. H.**; Bonanni, A.; Sofer, Z.; Pumera, M., Exfoliated Transition Metal Dichalcogenides (MoS₂, MoSe₂, WS₂, WSe₂): An Electrochemical Impedance Spectroscopic Investigation. *Electrochem. Commun.* 2015, 50, 39-42.
11. **Loo, A. H.**; Bonanni, A.; Sofer, Z.; Pumera, M., Transitional Metal/Chalcogen Dependant Interactions of Hairpin DNA with Transition Metal Dichalcogenides, MX₂. *ChemPhysChem* 2015, 16, 2304-2306.
12. **Loo, A. H.**; Bonanni, A.; Ambrosi, A.; Pumera, M., Molybdenum Disulfide (MoS₂) Nanoflakes as Inherently Electroactive Labels for DNA Hybridization Detection. *Nanoscale* 2014, 6, 11971-11975.
13. **Loo, A. H.**; Bonanni, A.; Pumera, M., Strong Dependence of Fluorescence Quenching on the Transition Metal in Layered Transition Metal Dichalcogenide Nanoflakes for Nucleic Acid Detection. *Analyst* 2016, 141, 4654-4658.

[14] Research article: Carbon quantum dots

14. **Loo, A. H.**; Sofer, Z.; Bousa, D.; Ulbrich, P.; Bonanni, A.; Pumera, M., Carboxylic Carbon Quantum Dots as a Fluorescence Sensing Platform for DNA Detection. *ACS Appl. Mater. Interfaces* 2016, 8, 1951-1957.

[15-16] Research articles: Other contributions

15. Urbanova, V.; Hola, K.; Bourlinos, A. B.; Cepe, K.; Ambrosi, A.; **Loo, A. H.**; Pumera, M.; Karlicky, F.; Otyepka, M.; Zboril, R., Thiofluorographene - Hydrophilic Graphene Derivative with Semiconducting and Genosensing Properties. *Adv. Mater.* 2015, 27, 2305-2310.
16. Chua, C. K.; **Loo, A. H.**; Pumera, M., Nanostructured MoS₂ Nanorose/Graphene Nanoplatelet Hybrids for Electrocatalysis. *Chem. Eur. J.* 2016, 22, 5969-5975.

Scientific conferences:

1. 7th Singapore International Chemistry Conference, Singapore, 2012.
2. NANO KOREA 2014 Symposium, Seoul, South Korea, 2014.
3. Advanced Materials & Nanotechnology 7, Nelson, New Zealand, 2015.
4. 66th Annual Meeting of the International Society of Electrochemistry, Taipei, Taiwan, 2015.

Thesis Organization

This thesis is organized into **fourteen chapters**. At the beginning, a literature review discussing the general features of biosensing is presented in **Chapter 1**. Thereafter, the thesis is divided into **three sections**, based on the class of nanomaterials.

The first section, **Section A**, covers graphene and its analogues, with **Chapter 2** outlining the characteristics of these nanomaterials. Subsequently, **Chapters 3 and 4** examine fundamental concepts of biosensing based on graphene and its associated nanomaterials. With a holistic knowledge of the scientific background, the utilization of graphene and its analogues for various biosensing applications will then be demonstrated in **Chapters 5 and 6**.

Similarly for **Section B** which encompasses transition metal dichalcogenides, a description of the properties of these nanomaterials is first provided in **Chapter 7**. This is then followed by fundamental studies which are evaluated in **Chapters 8 and 9**, before directing the focus to the biosensing applications of transition metal dichalcogenides in **Chapters 10 and 11**.

For **Section C** which reports on carbon quantum dots, a broad overview of this class of nanomaterials is presented in **Chapter 12**. **Chapter 13** then illustrates the application of carbon quantum dots for biosensing purpose.

A summary of the findings in this thesis and the future outlook of this research field are collated in **Chapter 14**.

In the **Appendices**, the published version of the results depicted in **Chapters 3 - 6, 8 - 11, and 13** can be referred.

Index of Abbreviations

CMG	Chemically modified graphene
GPO	Graphite oxide
GO	Graphene oxide
CRGO	Chemically reduced graphene oxide
TRGO	Thermally reduced graphene oxide
ERGO	Electrochemically reduced graphene oxide
GONP	Graphene oxide nanoplatelet
CQD	Carbon quantum dot
cCQD	Carboxylic CQD
TMD	Transition metal dichalcogenide
CVD	Chemical vapour deposition
PVT	Physical vapour transport
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
XPS	X-ray photoelectron spectroscopy
FT-IR	Fourier transform infrared spectroscopy
DLS	Dynamic light scattering
AFM	Atomic force microscopy
EIS	Electrochemical impedance spectroscopy
CV	Cyclic voltammetry
DPV	Differential pulse voltammetry
HER	Hydrogen evolution reaction
ORR	Oxygen reduction reaction
HET	Heterogeneous electron transfer
R_{ct}	Charge transfer resistance
mM	Millimolar (10^{-3} mol/L)
μM	Micromolar (10^{-6} mol/L)
nM	Nanomolar (10^{-9} mol/L)
pM	Picomolar (10^{-12} mol/L)
fM	Femtomolar (10^{-15} mol/L)
PBS	Phosphate buffered saline
SSC	Saline sodium citrate
DNA	Deoxyribonucleic acid
ssDNA	Single-stranded DNA
dsDNA	Double-stranded DNA
HpDNA	Hairpin DNA
INO-Hprobe	Inosine-substituted hairpin DNA probe
Biotin-probe	Biotinylated DNA probe
Apt	Aptamer
IgG	Immunoglobulin G

Anti-IgG	Antibody for IgG
THR	Thrombin
BSA	Bovine serum albumin
Av	Avidin
OTA	Ochratoxin A
DEP	Disposable electrical printed
RPM	Revolution per minute
°C	Degree centigrade
hr	Hour
min	Minute

Chapter 1

Literature Review on Biosensing

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1. Biosensors

Figure 1. Schematic illustration of a biosensor.

A biosensor is defined as a device which is capable of detecting biological interactions with high sensitivity and selectivity. As illustrated in Figure 1, a biosensor is comprised of two vital components, a biological recognition layer and a transducer layer. The biological recognition layer contains receptors which have unique selectivity towards their corresponding target analytes. These target analytes are often of biological origin, such as DNA, proteins, or cells. In addition to that, target analytes can also be simple molecules, provided that a receptor with the particular selectivity is available. Based on the type of target analytes, receptors can be nucleic acids, antibodies or enzymes. On the other hand, the transducer layer plays the role of translating the biological interactions at the biological recognition layer into a signal which can be processed

subsequently. In some cases, labels are introduced into the biosensing system after the biological recognition process. This additional procedure is usually performed with three aims. The first rationale behind the additional step is to enhance the signal intensity and improve the sensitivity. Similarly, labelling can also aid in increasing the overall selectivity of the biosensor. Last of all, labels may be added for the purpose of generating a signal in order for the biological recognition process to be monitored. Interesting to note, for the past decade, nanomaterials have been widely used as the transducer layer in the development of biosensors. In these nanomaterials-based biosensors, two types of signals are generally employed, and they are electrochemical and fluorescence signals.

1.1. Signal

1.1.1. Fluorescence

For the case of fluorescence signal, it is measured by fluorescence spectroscopy. For biosensors belonging to this category, the principle of detection is often based on a simple quenching mechanism.¹⁻³ Traditionally, such biosensing systems usually comprise of a fluorophore and a quencher molecule attached to the opposite ends of a DNA probe molecule. In the absence of the target molecule, the fluorophore and quencher molecules are in close proximity and fluorescence is suppressed. Upon the occurrence of hybridization with the target molecule, the quencher molecule becomes physically separated from the fluorophore molecule and fluorescence is released. However, biosensors of such design suffer from one major shortcoming, which is they require careful selection of fluorophore-quencher pair in order to ensure optimum efficiency.²

To circumvent such drawback, nanomaterials have been employed as the quenchers in recent years as they are capable of quenching various fluorophores of different emission wavelengths and improving the signal-to-noise ratio.^{4, 5} An example of such approach is demonstrated by Zhang and co-workers.⁶ In their study, they exploited the weaker interactions which double-stranded DNA (dsDNA) has with molybdenum disulfide (MoS₂) nanosheets as compared to single-stranded DNA (ssDNA), for the detection of DNA. The employed protocol first involves the introduction of a fluorophore-labelled ssDNA probe into a solution containing MoS₂ nanosheets, with the fluorescence rapidly quenched through the interactions between MoS₂ nanosheets and fluorophore label. Subsequently, upon the addition of a complementary ssDNA target, hybridization was resulted and a dsDNA duplex was formed. Due to the weaker interactions which dsDNA has with MoS₂ nanosheets, the dsDNA duplex desorbed itself from the nanosheets and the fluorescence signal was recovered.

In a similar report, using the same approach, the detection of thrombin protein is illustrated.⁷ For this instance, a fluorophore-labelled aptamer was introduced to the MoS₂ nanosheets solution before adding in the thrombin. Upon specific binding to thrombin, the aptamer altered its configuration and the resulting aptamer-thrombin complex desorbed itself from the MoS₂ nanosheets owing to weaker interactions, and fluorescence recovery ensued.⁸

In addition to MoS₂, other nanomaterials such as graphene oxide (GO)^{9, 10} have also been utilized. Fan and co-workers¹¹ demonstrated that due to the high quenching efficiency and large planar surface of GO, multiple DNA probes labelled with different dyes could be simultaneously quenched, leading to a multi-colour biosensor for the detection of multiple DNA targets in a homogeneous solution. In another study by Seo and co-

workers,¹² an immuno-biosensor based on GO for pathogen detection was reported. In their study, rotavirus-specific antibody was first immobilized on GO. Thereafter, the target cell was captured by the immobilized antibody before being sandwiched by a detection antibody which was conjugated with a gold nanoparticle (AuNP). The binding event was monitored by observing the fluorescence quenching which resulted from energy transfer between GO and AuNP.

Nevertheless, despite the wide applicability of fluorescence spectroscopy, it does not display huge potential for miniaturization and rapid response.

1.1.2. Electrochemical

To that aim, electrochemical techniques which detect for electrochemical signals are promising candidates. Electrochemical techniques encompass chronoamperometry, differential pulse voltammetry (DPV), square wave voltammetry (SWV), stripping voltammetry and electrochemical impedance spectroscopy (EIS). Among these, voltammetry and amperometry belong to the class of potentiostatic techniques whereby the electrode potential is regulated and applied to drive an electron transfer reaction, thus producing a measurable current. Specifically to amperometry, the produced current is monitored over time after a fixed potential is applied. On the other hand, for voltammetry, the produced current is monitored against varying potential. For the series of voltammetric techniques, the difference in them lies in their respective excitation waveform.^{13, 14} For the case of EIS, it is the study of an electrochemical system in response to an applied oscillating potential over a wide range of frequencies.^{15, 16} In recent years, EIS is reported to be highly suitable for monitoring changes occurring at the interface of electrode and electrolyte.¹⁷⁻²¹ Therefore, EIS can be advantageously utilized for the monitoring of biological recognition events.²²⁻²⁴ In addition, EIS does not

require any additional signal-generating labels to detect the changes at the interface, thus allowing label-free detection of target analytes. Furthermore, due to the small voltage perturbation employed during EIS measurement, EIS is not destructive and does not lead to any alterations to the target analytes.¹³ Published examples of such approach will be discussed in depth in the later parts of this chapter.

1.2. Transducer

Moving on, the next component of a biosensor to be discussed is the transducer layer. For the past decade, there has been an emerging trend of using nanomaterials as the transducer layer. In particular, there are three classes of nanomaterials which are of enormous interest. These nanomaterials are graphene and its analogues, transition metal dichalcogenides (TMDs), and carbon quantum dots (CQDs). Such a trend can be credited to the extraordinary properties which they exhibit. For example, the large specific surface area presented by them allows the anchoring of more biological receptors during the fabrication of biological recognition layer. Furthermore, there is also a greater available surface area for heterogeneous electron transfer (HET). This in turn brings about the potential to enhance the sensitivity and selectivity of the biosensor. Apart from the large specific surface area, these nanomaterials often display superior electron conductivity which can aid in faster HET and lead to enhanced sensitivity. The three different classes of nanomaterials will be addressed in detail in the subsequent chapters, namely Chapter 2, 7, and 12.

1.3. Biological recognition layer

In order for a biosensor to perform its basic function of detecting a specific target analyte, the biological recognition layer plays a determining role. The biological recognition layer contains receptors which have unique selectivity towards a particular target analyte. These receptors may be nucleic acids, antibodies, and enzymes, as depicted in Figure 2.

Figure 2. The different types of biological recognition elements which can be employed in the development of biosensors.

To fabricate the biological recognition layer on nanomaterials which serve as the transducer, various methods are available, depending on the type of receptors and nanomaterials.

1.3.1. Nucleic acids as biological receptors

With nucleic acids employed as the biological recognition element, different immobilization techniques have been formulated to construct the biological recognition layer on the surface of nanomaterials. As nanomaterials often have different properties

and functional moieties present on their surfaces, it is crucial to choose the most appropriate immobilization technique in order to ensure the stability and the desired orientation of the immobilized nucleic acid probes.²⁵ Immobilization methods which are widely adopted are:

Physical adsorption - With physical adsorption, the immobilization of nucleic acid probes onto nanomaterials exploits the hydrophobic and π - π interactions between nucleic acid probes and the nanomaterials. It is important to highlight that the resulting orientation of the immobilized nucleic acid probes is random.

Covalent attachment - With covalent attachment, the immobilization process involves the formation of a covalent bond between the nanomaterials and the nucleic acid probes. To achieve a covalent bond, the inherent functional groups on the surface of the nanomaterials are often exploited. On the other hand, the nucleic acid probes are modified with a functional group on one terminal, with NH_2 being the most commonly used modification.

Cross-linking - For cross-linking method, it often requires an organic linker molecule which facilitates the covalent immobilization of nucleic acid probes onto the surface of nanomaterials. A linker molecule is usually employed in cases whereby the nanomaterials do not have sufficient inherent functional groups on their surface for direct covalent attachment of the nucleic acid probes. As such, linker molecules have dual properties in general, consisting of hydrophobic regions for conjugation to the basal plane of nanomaterials by van der Waals forces and functional groups for the formation of covalent linkages with nucleic acid probes containing a modified terminal.

Biotin-avidin interaction - Biotin-avidin immobilization technique itself denotes a form of biological recognition process. Avidin is a protein which has the ability to bind up to four biotin molecules with a high degree of affinity and specificity. Interesting to note, the biotin-avidin complex is the strongest known non-covalent interaction between a protein and a ligand. Using this technique, prior modification of the nanomaterials' surface with a layer of avidin molecules is necessary before the addition of biotinylated nucleic acid probes. Upon the introduction, biotinylated nucleic acid probes will bind to the avidin molecules by affinity, resulting in the immobilization of nucleic acid probes.

Electrostatic attraction - For electrostatic attraction, immobilization of nucleic acid probes is achieved through the negatively charged phosphate backbone of nucleic acids, thus rendering the nucleobases exposed for effective hybridization. On this point, this signifies that the surface of nanomaterials has to be positively charged, or modified in such a way to confer a certain degree of positive charge to it. Therefore, parallel to the cross-linking approach, an organic molecule with dual properties has to be adopted.

With nucleic acids functioning as the biological receptors, nanomaterials-based biosensors for the detection of various target analytes have been proposed, with their working concepts explained in the following subsections, corresponding to the nature of the target analytes.

1.3.1.1. Nucleic acids analysis

For the purpose of detecting nucleic acids with nucleic acids also serving as the biological recognition element, Mukherjee *et al.*²⁶ designed a biosensor for the detection of mycobacterium tuberculosis gene, with chemically reduced graphene oxide (CRGO) functioning as the sensing platform. A ssDNA probe was first covalently immobilized onto CRGO surface, with methylene blue (MB) serving as an electroactive hybridization

indicator. Due to the greater affinity which MB has for ssDNA as compared to dsDNA, a smaller DPV peak current was derived after hybridization with the tuberculosis gene. A detection range of 2.872×10^{-3} - 2.872×10^5 ng μ L⁻¹ was obtained for the fabricated biosensor, with good selectivity against single-base mismatch and non-complementary nucleic acids.

In another study, Benvidi *et al.*²⁷ demonstrated an impedimetric biosensor for the sensing of amelogenin gene, also using CRGO as the transducing platform, and covalent DNA probe immobilization. The proposed biosensor presented a broad detection range of 1×10^{-20} - 1×10^{-14} M, with a limit of detection (LOD) of 3.2×10^{-21} M. Furthermore, the biosensor also displayed good specificity against non-complementary nucleic acid. Thereafter, the same group attempted to improve the analytical performance of their biosensor in another study.²⁸

Using CRGO as the transducer, Zhang *et al.*²⁹ presented an impedimetric biosensor for the detection of hepatitis B virus. In this study, the nucleic acid probe was covalently immobilized onto CRGO by cross-linking. To facilitate the cross-linking process, CRGO was first modified with tryptamine and then glutaraldehyde. The sensing platform demonstrated a detection range of 1×10^{-12} - 1×10^{-7} M, with a LOD of 5.2×10^{-13} M. In addition, the biosensor also showed superior selectivity against single-base mismatch and non-complementary nucleic acids.

Apart from CRGO, thermally reduced graphene oxide (TRGO) can be employed as the transducer as well. In 2014, Rasheed and Sandhyarani³⁰ published a work on employing TRGO as the sensing platform for the detection of breast cancer 1 (BRCA1) gene. The proposed sensor uses a sandwich detection strategy. The capture DNA probe first underwent physical adsorption onto TRGO while AuNPs were covalently conjugated to a

reporter DNA probe. Upon exposure to the BRCA1 gene, the capture DNA probe hybridized with half of the gene while the AuNPs-conjugated reporter DNA probe hybridized with the remaining half. As a result, a sandwich complex was produced and the oxidation signal of AuNPs was utilized for BRCA1 gene detection with chronoamperometry. A detection range of 1 fM - 1 nM was attained and selectivity was exhibited with three-base mismatch and non-complementary nucleic acids.

Following CRGO and TRGO, electrochemically reduced graphene oxide (ERGO) was also utilized for the aim of nucleic acids detection. Li *et al.*³¹ presented a human immunodeficiency virus 1 (HIV1) gene biosensor based on an ERGO-graphene double-layer surface. The ssDNA probe was physically immobilized onto the sensing platform, before undergoing hybridization with HIV1 gene. Herein, the analytical signal was derived from the DPV peak current of ferricyanide. It was observed that the peak current decreased upon the exposure to HIV1 gene, owing to the formation of dsDNA which contains more negative charges, and resulted in greater electrostatic repulsion with ferricyanide. The sensing interface achieved a detection range of 10^{-12} - 10^{-7} M, with a LOD of 1.58×10^{-13} M and selectivity against non-complementary nucleic acid.

Moving on, other than the reduced analogues of GO; other forms of graphene materials have also been applied to nucleic acids biosensing. These materials include thiofluorographene³², GO itself³³ and GO-graphite hybrid.^{34, 35} Urbanova *et al.*³² reported on the proof-of-concept of using thiofluorographene as a platform for impedimetric nucleic acid sensing. Thiofluorographene was first obtained by substituting fluorine atoms in fluorographene with nucleophilic sulfhydryl groups. Subsequently, physical adsorption was adopted to immobilize the ssDNA probes before hybridization reaction. A lower impedimetric signal was attained after the hybridization process due to the

partial removal of immobilized ssDNA probes upon the formation of dsDNA which has weaker interactions with thiofluorographene. It was shown that thiofluorographene could distinguish between complementary, single-base mismatch and non-complementary nucleic acids, while fluorographene did not have such capability.

On another note, Sun *et al.*³³ developed a biosensor for the sensing of miRNA-21, which represents a diagnostic tool for lung cancer. In their report, GO was utilized as the transducing platform, with silver nanoparticles (AgNPs)-labelled nucleic acid probes physically adsorbed on the surface. In the presence of miRNA-21, hybridization took place, forming a duplex structure which was then released from GO. Hence, the stripping current of AgNPs was exploited as the analytical signal and a detection range of 100 fM - 1 nM was acquired, with a LOD of 60 fM. Selectivity of the biosensor was also observed for up to three mismatch sites.

For the application of GO-graphite hybrid, two separate studies have been published. Firstly, Congur *et al.*³⁴ fabricated an impedimetric biosensor for detecting miRNA-34a, which is related to Alzheimer disease and cancers. In this study, pencil graphite electrode modified with GO was adopted as the sensing surface. Interesting to note, their sensing approach is to hybridize the nucleic acid probes with the miRNA-34a targets before exposing the sensing surface to the formed duplexes. Due to the NH₂ terminal of the nucleic acid probe, the formed duplex underwent covalent bonding with the GO-graphite hybrid surface. The biosensor achieved a detection range of 0 – 10 µg mL⁻¹, with a LOD of 1.9 µg mL⁻¹, and selectivity against other miRNAs.

In the second study conducted by Zhang *et al.*³⁵, scaly GO-graphite fibre hybrid electrode was utilized as the biosensing platform for the impedimetric detection of nucleic acid. GO sheets were synthesized in situ at the surface of graphite fibres by acid oxidation to

yield scaly GO-graphite fibre hybrid electrode. The partially peeled GO sheets provide numerous binding sites for the immobilization of DNA probes, and a greater surface area for enhanced electron transfer. Herein, DNA probes underwent physical adsorption on the GO-graphite fibre hybrid prior to hybridizing with its complementary DNA target. Due to the lower affinity towards dsDNA as compared to ssDNA, detection of DNA target was achieved. The range of detection was determined to be 0.01 - 1 nM, with a LOD of 5.6 pM and selectivity demonstrated against single-base mismatch and non-complementary nucleic acids.

1.3.1.2. Other biological molecules analysis

In addition to nucleic acids analysis, detection of smaller biological molecules is also attainable with nucleic acids functioning as the biological recognition element. Wu *et al.*³⁶ reported an aptasensor for the detection of adenosine triphosphate (ATP) using ATP aptamer as the recognition element. Aptamers are synthetic single-stranded nucleic acids which are in vitro selected through the systematic evolution of ligands by exponential enrichment (SELEX) process to bind to specific biological target analytes. They exhibit several advantages, including high specificity, easy synthesis, simple structure and long-term stability. Herein, CRGO was utilized as a label and its electrocatalytic property towards ascorbic acid (AA) oxidation, derived from its defective sites, was exploited. For the fabrication of the aptasensor, ATP aptamer with a SH terminal was first covalently linked to gold electrode. In the absence of ATP, CRGO could bind to ATP aptamer through π - π stacking interactions, leading to a greater DPV oxidation current and a lower oxidation potential of AA. On the other hand, in the presence of ATP, the immobilized aptamer formed a complex with ATP and CRGO was released from the biosensing interface, resulting in a sharp decrease in oxidation current

and an increase in oxidation potential of AA. The changes in oxidation current and oxidation potential of AA were both employed as the analytical signals for the detection of ATP. With oxidation current as the analytical signal, a low LOD of 11.7 pM and a wide detection range of 0.05 nM - 1.0 μ M were attained. On the other hand, using oxidation potential as the analytical signal, a LOD of 25.0 pM with detection range of 0.05 - 10.0 nM was acquired. In addition, the aptasensor also displayed excellent selectivity for ATP over other similar molecules.

1.3.1.3. Heavy metals analysis

Nucleic acids playing the role of a biological recognition element can contribute to the monitoring of environmental safety by detecting for pollution by heavy metals. Zhang *et al.*³⁷ developed a biosensor for mercury (Hg^{2+}) detection based on thymine-mercury-thymine (T- Hg^{2+} -T) interactions and CRGO as the transducing platform. A single-stranded, ferrocene-tagged T-rich DNA probe was first physically adsorbed onto the surface of CRGO, with the redox peaks of ferrocene utilized as the analytical signal with DPV. In the presence of Hg^{2+} , the ferrocene-labelled T-rich DNA probe could hybridize with another ssDNA, forming a dsDNA complex through Hg^{2+} -mediated coordination of T- Hg^{2+} -T base pairs. Owing to the weaker affinity between CRGO and dsDNA complexes, the ferrocene labels drew away from the surface of CRGO and the resulting redox peaks were substantially diminished. The proposed biosensor displayed a LOD of 5 pM, with a detection range of 25 pM - 10 μ M. Furthermore, high selectivity of the biosensor was also validated.

In another study, Tang *et al.*³⁸ also reported a biosensor for Hg^{2+} sensing, exploiting T- Hg^{2+} -T interactions, but ERGO as the biosensing platform. A ssDNA was first covalently immobilized onto the sensing platform by cross-linking. Upon the exposure to Hg^{2+} ,

stable duplex complexes were formed through strong T-Hg²⁺-T bonding with two immobilized DNA strands in close proximity. The captured Hg²⁺ subsequently catalyzed the formation of AuNPs. Since the extent of AuNPs formation is proportional to the concentration of Hg²⁺, Hg²⁺ concentration was directly quantified by stripping voltammetry of the formed AuNPs. The proposed biosensor demonstrated a range of detection of 0.5 - 120 nM, with a LOD of 0.06 nM. Similar to the previous work, excellent selectivity against other metal ions was also exhibited.

1.3.2. Antibodies as biological receptors

For the construction of biological recognition layer with antibodies, additional considerations have to be exercised as antibodies are a type of protein. As compared to nucleic acids, proteins are much more sensitive to their physiological environments and can be easily denatured or degraded by physical and chemical effects. Most importantly, it is crucial to maintain the inherent conformation of an antibody during the immobilization process and ensure its correct orientation. The common immobilization strategies are:

Physical adsorption - With physical adsorption, antibodies are conjugated onto the surface of nanomaterials through van der Waals' forces. Physical adsorption represents the simplest and most direct form of immobilization method as compared to the other strategies. However, such method often results in random orientation of the immobilized antibodies.

Covalent attachment - Based on the covalent attachment method, antibodies are linked to the surface of nanomaterials through the formation of covalent bonds, usually an amide bond. To form an amide linkage, an antibody with a NH₂ terminal and the inherent oxygen-containing functional groups, such as carboxylic acid or aldehyde, on

the surface of the nanomaterials are often exploited. Upon activation by carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) chemistry, amide bonds are generated and antibodies are covalently attached to the surface of nanomaterials. Apart from amide bond, other types of covalent bonds are also plausible, subjected to the type of functional moieties which are available on the antibodies and nanomaterials. Weighing against physical adsorption, covalent attachment allows better control over the orientation of the immobilized antibodies.

Cross-linking - Cross-linking immobilization approach makes use of linker molecules to facilitate the covalent attachment of antibodies to the surface of nanomaterials. This method is commonly utilized in cases whereby there are no or inadequate quantities of the desired functional groups present on the surface of the nanomaterials. In addition, cross-linking can also be exploited to introduce spacing, thereby improving the accessibility of the antibodies to the target analytes.

Biotin-avidin interaction - The principle behind this immobilization technique lies in the exceptional affinity between biotin and avidin molecules. Each avidin molecule is capable of binding with up to four molecules of biotin. To employ this immobilization strategy, prior modifications of the nanomaterials and antibodies are imperative. Specifically, the nanomaterials have to be modified with avidin molecules while the antibodies have to be conjugated to a biotin molecule each. Upon exposure to each other, the biotinylated antibodies will bind to the avidin molecules by affinity, thereby immobilizing the antibodies onto the nanomaterials successfully.

The subsequent subsections will elucidate how biosensors adopting antibodies as the biological recognition element can be applied for the detection of various target analytes.

1.3.2.1. Biomarkers analysis

The detection of biomarkers with antibodies serving as the biological recognition element signifies a popular and effective clinical diagnostic tool.³⁹⁻⁴³ Among the various diagnostic methods currently available, immunoassays remain the most ideal owing to their advantages of affordability, selectivity, and sensitivity. In recent times, nanomaterials have also been widely involved in the fabrication of immunosensors for the detection of vital biomarkers.

In a study adopting chemical vapour deposition (CVD) grown graphene as the transducing platform, Jin *et al.*⁴⁴ designed a voltammetric immunosensor for carcinoembryonic antigen (CEA) detection. CVD graphene is an example of graphene materials which has been attracting research interest in the recent years due to the demand for graphene materials of high quality. For this report, it is interesting to note that the capture antibodies were first covalently immobilized onto magnetic beads before attracting them onto the CVD graphene platform using an external magnetic field. After the incubation with CEA, a sandwich strategy was employed, using AuNPs conjugated with detection antibodies and horseradish peroxidase (HRP) enzymes. The HRP/H₂O₂ catalytic system then provided the analytical signal which was monitored with cyclic voltammetry. Based on the biosensing approach, the detection range was concluded to be 5 - 60 ngmL⁻¹, with LOD of 5 ngmL⁻¹ and superior specificity.

Other than CVD graphene, reduced analogues of GO, such as CRGO, have also been extensively used for immunosensing. Wang *et al.*⁴⁵ fabricated an impedimetric immunosensor for the detection of prostate specific antigen (PSA). In the work, CRGO was employed as the transducing material and it was functionalized with silk peptide and glutaraldehyde to facilitate the covalent immobilization of anti-PSA via the cross-

linking procedure. Based on the fabricated sensor, $0.1 - 5.0 \text{ ngmL}^{-1}$ and $5.0 - 80.0 \text{ ngmL}^{-1}$ were determined as the linear detection ranges, with LOD at 0.053 ngmL^{-1} . Finally, selectivity against other proteins was also observed.

In two other studies, CRGO functionalized with tetraethylene pentamine (CRGO-TEPA) was utilized. In the first study, Yuan *et al.*⁴⁶ demonstrated a voltammetric immunosensor for the simultaneous detection of C-reactive protein (CRP) and CD40 ligand (CD40L), based on a sandwich assay. Herein, CRGO-TEPA was employed as a label, with metal ions conjugated to it and detection antibodies covalently immobilized on it through cross-linking with glutaraldehyde. On the other hand, the respective recognition antibodies were covalently attached onto the transducer layer. Detection of CRP and CD40L were achieved by measuring the signal derived from their respective metal ions on the CRGO-TEPA labels using DPV. With the proposed sensing interface, detection range was assessed to be $0.05 - 100 \text{ ngmL}^{-1}$, with a LOD of 16.7 pgmL^{-1} and 13.1 pgmL^{-1} for CRP and CD40L, accordingly. Furthermore, specificity was also exhibited by the immunosensor.

In the second study, Wu *et al.*⁴⁷ utilized CRGO-TEPA as the transducing platform to design a voltammetric immunosensor for the simultaneous detection of two cervical cancer biomarkers, CEA and squamous cell carcinoma antigen (SCCA). The respective capture antibodies were covalently immobilized on CRGO-TEPA and a sandwich assay was employed. Two different redox mediators, neutral red and thionine, were used as the labels to produce the analytical signals which were then monitored with DPV. Based on the designed immunosensing approach, CEA and SCCA could be detected in the range of $0.05 - 20 \text{ ngmL}^{-1}$ and $0.03 - 20 \text{ ngmL}^{-1}$ respectively. In addition, LOD of CEA and SCCA was concluded to be 0.013 ngmL^{-1} and 0.010 ngmL^{-1} , correspondingly. Acceptable selectivity parameters were also observed in the immunosensor.

As an extension from CRGO, CRGO nanoribbons were demonstrated by Shi *et al.*⁴⁸ to be an useful platform for the concurrent detection of interleukin-6 (IL-6) and matrix metalloproteinase-9 (MMP-9). In their report, IL-6 and MMP-9 capture antibodies were immobilized onto CRGO nanoribbons by physical adsorption to fabricate the biological recognition layer. Upon incubation with the target biomarkers, a sandwich assay was employed, with two different metals acting as labels for the individual biomarker. Quantification of the target biomarkers was then attained with stripping voltammetry. The developed immunosensor exhibited a detection range of 10 fg mL^{-1} - $1 \text{ } \mu\text{g mL}^{-1}$, with a LOD of 5 fg mL^{-1} for MMP-9. For IL-6, the detection range was determined to be 1 pg mL^{-1} - $1 \text{ } \mu\text{g mL}^{-1}$, with the LOD at 0.1 pg mL^{-1} . In addition, good specificity was also displayed by the immunosensor.

Moving on from CRGO, other types of graphene materials have also been used in recent years. For example, Kailashiya *et al.*⁴⁹ presented a GO-based impedimetric immunosensor for platelet-derived microparticles (PMPs). In their study, the antibodies for PMPs were covalently immobilized onto GO surface and the biosensing platform acquired a detection range of $100 - 7000 \text{ counts } \mu\text{L}^{-1}$ with specificity.

In another work, Lim *et al.*⁵⁰ developed a graphene-based immunosensor for human chorionic gonadotropin (hCG), whereby AuNPs were utilized as electroactive labels. The capture antibodies were first physically adsorbed onto graphene, before exposing to hCG and sandwiched with detection antibodies which were conjugated with AuNPs. DPV was subsequently employed to measure the analytical signal arising from AuNPs, and the detection range was concluded to be $0 - 500 \text{ pg mL}^{-1}$, with a LOD of 5 pg mL^{-1} .

1.3.2.2. Nucleic acids analysis

Apart from biomarkers, the application of antibodies as a biological recognition element can be extended to nucleic acids analysis. Tran *et al.*⁵¹ exhibited the use of antibodies in the fabrication of a voltammetric immunosensor for the detection of miRNA-141. Herein, a composite constituting of CRGO and carbon nanotubes was employed as the transducing platform, and a ssDNA probe was first covalently attached onto the surface. Upon exposure to the target miRNA, hybridization was resulted and a DNA/RNA hybrid was formed. Subsequently, an antibody bound selectively to the DNA/RNA hybrid before a secondary antibody, which was conjugated to HRP, bound to the first antibody. The HRP/H₂O₂ catalytic system then led hydroquinone to be oxidized to benzoquinone, and the consequent reduction of benzoquinone back to hydroquinone at the modified electrode surface was exploited as the analytical signal using SWV. With the above immunosensing approach, the LOD was calculated to be 30 fM, with selectivity shown against non-complementary miRNA.

1.3.2.3. Other biological molecules analysis

With the aim of monitoring food safety, Narayanan *et al.*⁵² designed a voltammetric immunosensor for the detection of botulinum neurotoxin (BoNT). The proposed immunosensor utilized CRGO, functionalized with carboxylic acid groups, as the biosensing interface, with BoNT antibodies covalently immobilized on it. Upon the exposure to BoNT, a series of reactions followed, ending with the deposition of AgNPs. Hence, the stripping current of AgNPs was used for the quantification of BoNT present. The designed immunosensor was able to selectively detect BoNT in the range of 10 pgmL⁻¹ - 10 ngmL⁻¹, with the LOD at 5 pgmL⁻¹.

Besides food toxins, antibodies can also be employed in the biosensing of essential biological molecules such as cholesterol. Ali *et al.*⁵³ revealed an impedimetric lipid cholesterol immunosensor, with the capture antibodies covalently immobilized onto the CRGO transducing platform. To facilitate the covalent immobilization, CRGO was functionalized with amine groups beforehand. Based on the biosensing interface, a detection range of 5 - 120 mgdL⁻¹ and a LOD of 5 mgdL⁻¹ were attained.

Eissa *et al.*⁵⁴ reported on the utilization of CVD graphene for the development of an impedimetric immunosensor for ovalbumin. To fabricate the immunosensor, ovalbumin antibody was first covalently immobilized onto CVD graphene by the cross-linking approach, whereby CVD graphene underwent prior functionalization with carboxylic acid groups. The proposed immunosensor displayed a detection range of 1 pgmL⁻¹ - 100 ngmL⁻¹, with a LOD of 0.9 pgmL⁻¹, and selectivity against non-specific proteins.

1.3.3. Enzymes as biological receptors

Analogous to antibodies, special attention has to be dedicated to the fabrication of an enzymatic biological recognition layer since enzymes are also a kind of protein. The immobilized enzymes provide the selectivity for the biosensor, and catalyze the production of the electroactive species for detection. For the construction of an enzymatic biological recognition layer, the most important enzyme immobilization protocols⁵⁵ are:

Physical adsorption - Physical adsorption represents a fast and straightforward method, utilizing van der Waals' forces to immobilize enzymes onto the surface of nanomaterials. By taking this approach, there is almost no control over the orientation of the resulting immobilized enzymes.

Covalent attachment - Based on covalent attachment, inherent functional groups present on the surface of nanomaterials are exploited to form covalent linkages with the exposed side chains of amino acids utilized to build the enzymes. Depending on the type of covalent bonding formed, it may be possible to manipulate the final orientation of the immobilized enzymes. Most importantly, this method enables direct anchoring of the enzymes onto the nanomaterials platform. Therefore, direct transfer of electrons from the transducer layer to the enzyme is allowed.

Cross-linking - The cross-linking technique involves the usage of linker molecules to aid in the immobilization of enzymes onto nanomaterials through the formation of covalent bonds. Such approach is commonly employed in cases whereby the surface of nanomaterials does not contain, or contain insufficient amount of the preferred functional groups.

Electropolymerization - The employment of this method involves the mixing of the enzyme with a monomer molecule which can undergo polymerization. Thereafter, an electrochemical procedure is applied to initiate electropolymerization. The incorporation of the enzymes into the polymer matrix is mostly achieved through electrostatic interactions. One major advantage of this technique is that thin layers can be produced with superior control.

Layer by layer - The working principle behind the layer by layer immobilization technique is based on the attraction between oppositely charged ionic layers on the surface of the electrode. By combining layer by layer immobilization technique and nanomaterials, improvements in the response of enzymatic biosensors have been demonstrated.

Through the usage of an enzymatic biological recognition layer, the detection of small molecular targets has been investigated.

1.3.3.1. Small molecules analysis

In a study conducted by Seo's group,⁵⁶ graphite nanoparticles (NPs), consisting of several stacked graphene sheets, were employed as the transducer for the construction of an enzymatic biosensor for glucose detection. Graphite NPs were chosen due to their high conductivity, homogeneous spherical shape, and large surface area available for biomolecular conjugation. To attach glucose oxidase onto the surface of graphite NPs, graphite NPs were first modified with 1-pyrenebutyric acid NHS ester through π - π stacking interactions between the pyrene moieties and the graphite NPs. Subsequently, glucose oxidase was linked through the formation of an amide bond. Using amperometry to measure the produced H_2O_2 , the LOD of glucose was determined as 50 μM , with sensitivity of $72.9 \text{ pAmM}^{-1}\text{cm}^{-2}$.

Baptista-Pires *et al.*⁵⁷ reported an enzymatic biosensor for catechol using tyrosinase enzyme as a proof-of-concept receptor, and graphene materials as the transducing platforms. The biological receptor, tyrosinase enzyme, was immobilized onto GO and CRGO by physical adsorption and the biosensing performance of these two materials was evaluated. The principle of detection lies in tyrosinase enzyme catalyzing the oxidation of catechol to o-quinone. Consequently, the electrochemical reduction of o-quinone to catechol was exploited as the analytical signal using chronoamperometry. Crucial to note, the coupling between catalytic oxidation (catechol to o-quinone) and electrochemical reduction (o-quinone to catechol) shuttled catechol into a repetitive cycle, resulting in significant amplification of signal. From this study, it was deduced that CRGO had better analytical performance than GO. CRGO exhibited a LOD of 0.0103 nM,

and a sensitivity of $0.0898 \mu\text{A}\mu\text{M}^{-1}$, while GO displayed a LOD of 0.0711 nM , and a sensitivity of $0.0679 \mu\text{A}\mu\text{M}^{-1}$. The enhanced analytical performance by CRGO was attributed to its higher conductivity and its mode of interactions with the tyrosinase enzyme. Tyrosinase enzyme was physically immobilized onto CRGO by hydrophobic interactions while GO interacted with tyrosinase via electrostatic attractions. Hence, it was inferred that hydrophobic interactions for tyrosinase enzyme immobilization is more effective than with electrostatic attractions, as the latter adversely affects the activity of the immobilized enzyme.⁵⁸ Nonetheless, both forms of graphene materials gave rise to better analytical response for the detection of catechol, as compared to unmodified bare electrode.

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Section A

Graphene and its Analogues

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Chapter 2

Introduction to Graphene and its Analogues

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Since its successful isolation in 2004, graphene, which comprises of a single layer of carbon atoms densely arranged in a two-dimensional honeycomb matrix, has been the main research interest of several scientists.¹ The immense attention which graphene is garnering can be explained by the superior properties it exhibits. Some examples of these properties include high surface area, robust mechanical strength,² ease of functionalization and exceptional thermal³ and electron conductivity.⁴⁻⁸ Due to the above mentioned extraordinary characteristics, graphene has been dubbed as a “miracle material”.⁹ However, it is crucial to note that most of the above stated outstanding characteristics can only be attained by samples demonstrating the highest quality, such as mechanically exfoliated graphene.¹ At present, there are no graphene materials by other methods of preparation displaying equivalent characteristics. Nonetheless, the synthesis methods for graphene preparation are developing rapidly.

Figure 1. Various preparation methods of graphene.

In general, the preparation methods of graphene can be broadly classified into two key approaches, namely bottom-up or top-down (Figure 1). In the instance of the bottom-up approach, it attempts to fabricate graphene by employing small carbon sources and this approach is comprised of epitaxial growth on silicon carbide, chemical vapour deposition (CVD) and chemical solid deposition. On the other hand, in the case of the top-down approach, the basis of its working principle lies in peeling off individual sheets of graphene from a graphite source material. Techniques based on this approach include mechanical and liquid phase exfoliation of graphite, oxidation of graphite to graphite oxide (GPO) with subsequent chemical, thermal or electrochemical reduction, and unzipping of carbon nanotubes.^{9, 10}

With respect to epitaxial growth on silicon carbide, graphene can be grown on the silicon and carbon faces of hexagonal silicon carbide substrate under an annealing temperature of more than 1000 °C. This is because, at high temperature, the silicon atoms are sublimed, resulting in a graphitized surface to yield a layer of epitaxial graphene. Consequently, the properties of the grown graphene are determined by the morphology of the silicon carbide substrate used.¹¹ To date, it is possible to control the number of graphene layers grown¹² and to produce single-crystal graphene films of high quality in the size of hundreds of micrometers.¹³

CVD technique represents one of the preferred bottom-up approaches for graphene synthesis as it can generate large area of high quality graphene with controlled number of layers, and possibly at low mass production costs.¹⁴ The fabrication process generally involves supplying a gaseous carbon feedstock and hydrogen gas into a furnace and through a hot zone. At the hot zone, a metal substrate is positioned and this results in a catalytic reaction between the carbon feedstock and hydrogen gas, forming layers of

graphene upon cooling. The quality of the resulting graphene layers, in terms of defects, grain boundaries, thickness and domain size,¹⁵ can be modulated by precise control of the temperature, pressure, growth time, type of metal substrate and cooling rate. Currently, metal catalysts such as iridium, ruthenium, cobalt, platinum and iron have been successfully employed to grow graphene, with nickel and copper representing the most frequently adopted metal catalysts due to their lower costs and ease of controlling the number of graphene layers.¹⁶⁻¹⁹ Other than employing a gaseous carbon source, solid carbon sources can also be utilized and such production method is termed as chemical solid deposition. Under similar experimental conditions as CVD, chemical solid deposition has been demonstrated to produce single-layer graphene from solid carbon sources like food, waste and insects.²⁰

Among the various top-down synthesis methods, mechanical exfoliation of graphite is the most straightforward. This technique was first established by Geim and Novoselov²¹ in 2004 where an adhesive tape was used to peel the graphite crystal until single-layer, bi-layer or few-layer graphene were attained. Graphene obtained through such technique is often excellent in quality, as it is free from defects, impurities and dopants.

Apart from mechanical exfoliation, the exfoliation of graphite can also be achieved in the liquid phase. For this instance, graphite is first dispersed in aqueous solutions containing surfactants, or in non-aqueous solvents such as ionic liquids²² and N-methylpyrrolidone.²³ Thereafter, the graphite dispersion is subjected to probe ultrasonication, yielding a mixture of single-layer, bi-layer and few-layer graphene, which can subsequently be separated by centrifugation.²³

Figure 2. Illustration for the preparation of chemically modified graphene (CMG) materials with graphite as the starting material. (A) GPO is first generated via oxidation from graphite. This is followed by: (B) thermal reduction/exfoliation of GPO to yield thermally reduced graphene oxide (TRGO); (C) exfoliation by ultrasonication to produce graphene oxide (GO); (D) electrochemical reduction of GO to generate electrochemically reduced graphene oxide (ERGO); and (E) chemical reduction of GO to produce chemically reduced graphene oxide (CRGO).

Of the various routes of preparation based on the top-down approach, oxidation of graphite to GPO with subsequent chemical, thermal or electrochemical reduction, is one of the most common methods. In this technique, graphite is usually first oxidized with a mixture of strong acid and oxidizing agent to yield GPO. It is important to mention that the oxidative treatment increases the inter-layer distance between graphene sheets

from approximately 3.33 - 3.35 Å (graphite) to approximately 6 - 12 Å (GPO).^{24, 25} Simply put, GPO is the highly oxidized form of graphite, which retains the lamellar structure of graphite but has a wider inter-layer spacing due to the presence of oxygen functionalities. These oxygen functionalities include hydroxyl, epoxide, carbonyl and carboxylic groups. In general, the basal plane of GPO is populated with hydroxyls and epoxides while the edge plane is decorated with mainly carbonyl and carboxylic groups. Consequently, the wider inter-layer spacing results in a more straightforward ensuing exfoliation step as the sheets are generally held together by van der Waals' interactions.²⁶ An ultrasonication treatment of the GPO then leads to the breakdown of the lamellar structure, producing GO. GO is an important intermediate between GPO and graphene. Compared to GPO, GO is structurally different. GO exists mainly as mono-layer, bi-layer or few-layer sheets while retaining most of the oxygen functionalities in GPO. From GO, successive chemical or electrochemical treatments then remove most of the oxygen-containing functional groups to produce single-layer, double-layer or few-layer graphene. For the case of chemical treatment, various chemical reductants can be employed. On the other hand, for the case of electrochemical treatment, it is usually performed by applying a negative potential on an electrode which is coated with GO for a period of time. In addition, apart from the two above stated treatments, a thermal shock treatment of more than 1000 °C on GPO can simultaneously exfoliate and reduce GPO to graphene. The above three different methods are expected to produce graphene materials of various properties and morphologies. Nonetheless, the obtained graphene materials by the various treatments are collectively referred to as CMGs¹⁰ or they can be termed individually, based on the treatment method, as CRGO, ERGO or TRGO.

Table 1. Properties and applications of graphene derived via different preparation methods.

Method	Crystallite size (μm)	Sample size (mm)	Charge carrier mobility (at 25 $^{\circ}\text{C}$) ($\text{cm}^2\text{Vs}^{-1}$)	Applications
Mechanical exfoliation	> 1000	> 1	$> 2 \times 10^5$ and $> 10^6$ (at low temperature)	Research
Liquid phase exfoliation	≤ 0.1	Infinite as a layer of overlapping flakes	100 (for a layer of overlapping flakes)	Coatings, paint/ink, composites, transparent conductive layers, energy storage, bio-applications
Top-down approach via graphite oxide	~ 100	Infinite as a layer of overlapping flakes	1 (for a layer of overlapping flakes)	Coatings, paint/ink, composites, transparent conductive layers, energy storage, bio-applications
Chemical vapour deposition	1000	~ 1000	10000	Photonics, nanoelectronics, transparent conductive layers, sensors, bio-applications
Epitaxial growth on silicon carbide	50	100	10000	High-frequency transistors and other electronic devices

The different methods of graphene production give rise to an array of graphene materials with varying properties and hence qualities. Consequently, the quality of the resulting graphene materials plays a crucial role in determining its applications. As shown in Table 1,⁹ the different methods of graphene preparation can result in differences in terms of crystallite size, sample size, and charge carrier mobility. This in turn influences the type of applications the resulting graphene materials can have.

In recent years, there has been an expanding interest in the fabrication of electrochemical biosensors employing graphene and its analogues due to the large specific surface area, fast heterogeneous electron transfer kinetics and ease of surface functionalizations.²⁷⁻²⁹ A biosensor can be simply defined as an analytical tool which converts a biological response into a quantifiable and processable signal.³⁰ These biosensors can be further classified as immunosensors,³¹⁻³⁷ aptasensors³⁸⁻⁴¹ and genosensors.⁴²⁻⁴⁴ The main difference between these biosensors lies in the recognition element and the target biomolecule. For immunosensor, aptasensor and genosensor, their recognition elements are antibodies, aptamers and nucleic acids respectively while their target biomolecules are usually antigens, proteins and nucleic acids accordingly.

In electrochemical biosensors which employ graphene materials, the graphene materials can serve three main functions. First of all, it can be utilized as a transducing platform.⁴⁵⁻⁴⁸ For instance, by immobilizing glucose oxidase on the surface, CRGO and ERGO^{49, 50} were assessed for glucose detection through monitoring the electron transfer from glucose oxidase to the electrode surface. It was discovered that CRGO had better performance than ERGO in terms of detection range, detection limit and sensitivity. In another study, Bonanni and co-workers⁴² presented a graphene platform for the detection of DNA hybridization and single nucleotide polymorphism by employing electrochemical impedance spectroscopy as the detection technique. A highly selective hairpin DNA oligonucleotide, which was physically immobilized on the graphene surface, was employed as the recognition element. The performance of three different graphene platforms, each consisting of a different number of layers, was compared, with few-layer graphene exhibiting the best sensitivity.

Secondly, graphene materials can function as a label.⁵¹⁻⁵³ Wang and co-workers⁵¹ demonstrated a new electrochemical biosensing approach by exploiting the excellent electron transfer ability of graphene and its robust π - π interactions with single-stranded DNA. The basis of detection is that the immobilized aptamer could strongly adsorb graphene in the absence of target molecule, leading to a large decrease in the charge transfer resistance. In a related study, Park and co-workers⁵² presented an electrochemical mercury ion sensor utilizing GO as the indicator. Charge transfer resistance was shown to decrease with increasing concentration of mercury ion as GO played the role of an insulator in this context.

Last of all, graphene and its analogues can also serve as a mode of signal amplification by exploiting their large surface area.^{54, 55} Chen and co-workers⁵⁴ developed two electrochemical aptasensors for the detection of thrombin and adenosine triphosphate by utilizing methylene blue-anchored GO as a form of signal enhancement. In the absence of the target molecules, GO which was conjugated to the free bases of the immobilized aptamers, could load multiple methylene blue molecules onto its surface by electrostatic interactions, thereby amplifying the signal intensity. In another report, Hou and co-workers⁵⁵ illustrated a sandwich type immunosensor for carcinoembryonic antigen based on the use of GO nanosheets functionalized with a high ratio of horseradish peroxidase and detection antibodies. It was demonstrated that improved analytical features were attained with the employment of GO nanosheets as compared to the conventional enzyme-labelled detection antibodies.

The following chapters seek to attain a deeper understanding on the effects of DNA immobilization methods on its subsequent hybridization efficiency, and to validate the mechanism behind physical adsorption of DNA, with graphene and its analogues as the

platforms. After which, a total of four novel biosensing applications will be demonstrated with GO and CVD graphene.

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Chapter 3

Effects of DNA Configuration on its Hybridization
Efficiency based on a Graphene Platform

The results in this chapter resulted in the publication of the following:

1. **Loo, A. H.**; Bonanni, A.; Pumera, M., Soldering DNA to Graphene via 0, 1 and 2-point Contacts: Electrochemical Impedance Spectroscopic Investigation. *Electrochem. Commun.* 2013, 28, 83-86.

Motivation

Due to the wide utilization of graphene and its analogues for the application of electrochemical biosensing, specifically as a transducing platform for nucleic acids detection, various DNA immobilization approaches have since been developed to fabricate the biosensing interface. In addition, studies have demonstrated that the immobilization method has an impact on the overall orientation¹ and structure² of the immobilized DNA. Hence, for that reason, it is expected that the subsequent hybridization process will be affected as DNA hybridization exhibits different kinetics and dynamics when faced with spatial confinements and topological restrictions.³⁻⁵ In view of that, it is imperative to ascertain the effects of different DNA immobilization approaches and their influence on the degree of hybridization (i.e. hybridization efficiency).

In this study, we investigate how the attachment method of single-stranded DNA (ssDNA) to graphene can affect the subsequent hybridization process. To explore the effects of the different attachment methods, electrochemical impedance spectroscopy (EIS) will be employed.

Results and Discussions

Herein, we investigated three different attachment approaches: ssDNA physically adsorbed on the graphene surface (0-soldering contact); ssDNA chemically bonded to graphene surface at one end (1-soldering contact); and ssDNA attached at two ends to graphene surface (2-soldering contacts).⁶ EIS was chosen as the analytical method as it represents an extremely sensitive technique which is capable of detecting minor changes arising from the biorecognition event at the surface of graphene.⁷

The EIS spectra of graphene, graphene functionalized with ssDNA using 0, 1, or 2-soldering contacts, and graphene functionalized with ssDNA after hybridization with a complementary ssDNA, were recorded and shown in the right panel of Figure 1.

In the case of 0-soldering contact (Figure 1A), that was ssDNA being only physically adsorbed on the surface of graphene, the charge transfer resistance (R_{ct}) of the platform increased from 417 Ω (graphene) to 8.04 k Ω (ssDNA modified graphene). Subsequently, when the ssDNA modified graphene was incubated with a complementary ssDNA, the R_{ct} decreased by 27 % to 5.80 k Ω , which indicates the partial removal of the hybridized DNA duplex from the surface of graphene.⁸

On the other hand, a completely different situation was observed when 1-soldering contact procedure was employed. Under such circumstance, the R_{ct} of graphene surface increased to 2.12 k Ω from 675 Ω upon the attachment of ssDNA using carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry, and the observed increase in R_{ct} signified successful covalent attachment of the ssDNA strand.⁹ Consequently, when ssDNA/graphene hybrid with 1-soldering point was exposed to a complementary ssDNA,

the R_{ct} further increased by 64 % to 3.47 k Ω , signifying that the majority of the ssDNA, which was conjugated to graphene, had hybridized with the complementary ssDNA.

Figure 1. Left: Schematics showing the concept of (A) 0-soldering; (B) 1-soldering and (C) 2-soldering contacts attachment of the ssDNA to a graphene sheet (soldering points are represented by the red dots). Right: Corresponding EIS spectra of graphene (black), graphene with ssDNA before (red) and after (blue) hybridization with a complementary ssDNA.

The interesting twist arose when the hybridization process with 2-soldering contacts ssDNA/graphene hybrid was examined. While graphene surface showed R_{ct} of 665 Ω , the 2-soldering points ssDNA/graphene hybrid platform displayed a R_{ct} of 2.48 k Ω . After hybridization with a complementary ssDNA, the R_{ct} increased by only 9 % to 2.71 k Ω . As such, this implied that the hybridization on 2-soldering points attached ssDNA was significantly less efficient than that of 1-soldering point attached ssDNA strand. Such

observation could be reasonably attributed to the reason that the attached ssDNA with 2-soldering contacts was unable to rotate during the hybridization process.²

Therefore, from the results attained from this study, it could be concluded that the number of soldering points employed for the attachment of ssDNA affects the subsequent DNA hybridization to a great extent, and the number of ssDNA attachment points is one of the key factors in the efficiency of DNA hybridization.

Thereafter, in order to ascertain that the carbodiimide used for the activation of carboxylic groups in covalent immobilization of ssDNA did not contribute to the hybridization process,¹⁰ and hence the increase in R_{ct} upon incubation with the complementary ssDNA strand, an additional control experiment was conducted with the findings displayed in Figure 2.

Figure 2. Comparison of the impedimetric signals derived after hybridization with (cyan) and without (yellow) prior EDC/NHS treatment. Signal is represented as R_{ct} ratio $[(R_{ct} \text{ hybridized} - R_{ct} \text{ graphene}) / (R_{ct} \text{ ssDNA} - R_{ct} \text{ graphene})]$. Error bars correspond to triplicate experiments. Amount of ssDNA and complementary ssDNA used is 30 pmol. All measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution (pH 7.0) at room temperature.

From Figure 2, it could be understood that when there was no EDC/NHS treatment with ssDNA, similar to the case of 0-soldering contact, there was a decrease in R_{ct} upon incubation with a complementary ssDNA. In contrast, when there was EDC/NHS treatment with ssDNA, there was effectively no hybridization occurring as the R_{ct} ratio value was approximately 1 (i.e. no increase or decrease in R_{ct} upon incubation with a complementary ssDNA). Such observations could be rationalized that when there was EDC/NHS treatment with ssDNA which did not contain any NH_2 terminals, the activated carboxylic groups interacted with the amino groups of the nitrogenous bases in ssDNA instead and the ssDNA became affixed on the graphene surface. Subsequently, the remaining carboxylic groups were deactivated¹¹ before incubation with the complementary ssDNA. The ssDNA which was affixed on the graphene surface, had no rotation capability during hybridization and thus, there was no effective hybridization and this led to a R_{ct} ratio value of approximately 1. Therefore, it could be concluded that the carbodiimide used for the activation of carboxylic groups in covalent immobilization of ssDNA did not contribute to the hybridization process.

Conclusions

In summary, we have demonstrated that the topological constraints of ssDNA attached on graphene sheet exhibit dramatic influence on its ability to undergo effective hybridization with a complementary ssDNA strand. The most efficient hybridization event took place on 1-soldering point ssDNA/graphene hybrid while 2-soldering points ssDNA attachment to graphene practically inhibited its ability to successfully hybridize with a complementary ssDNA. These findings are expected to have a profound influence upon the construction of nanobioelectronic devices.

Experimental Section

Materials

N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide sodium salt (NHS), graphite microparticles ($< 20 \mu\text{m}$), sodium borohydride (NaBH_4), nitric acid (HNO_3) (conc. $> 90 \%$), sulphuric acid (H_2SO_4) (conc. $95 - 98 \%$), potassium chlorate (KClO_3), hydrochloric acid (HCl) (conc. 37%), sodium phosphate dibasic salt (Na_2HPO_4), sodium chloride (NaCl), potassium hexacyanoferrate (II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$), potassium hexacyanoferrate (III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$), sodium dodecyl sulfate (SDS) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA sequences are as follows:

ssDNA: 5' ACC AGG CGG CCG CAC ACG TCC TCC AT 3'

NH_2 -ssDNA: 5' NH_2 -ACC AGG CGG CCG CAC ACG TCC TCC AT 3'

NH_2 -ssDNA- NH_2 : 5' NH_2 -ACC AGG CGG CCG CAC ACG TCC TCC AT- NH_2 3'

Complementary ssDNA: 5' ATG GAG GAC GTG TGC GGC CGC CTG GT 3'

Trisodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) and hydroxylamine hydrochloride ($\text{HONH}_2 \cdot \text{HCl}$) were purchased from Alfa Aesar (Singapore).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of conductivity no lesser than $18.2 \text{ M}\Omega \text{ cm}$.

The following buffers were used in this study:

0.1 M PBS (0.1 M NaCl + 10 mM Na_2HPO_4 , pH 7.0)

5xSSC (0.75 M NaCl + 75 mM trisodium citrate, pH 7.0)

2xSSC (0.30 M NaCl + 30 mM trisodium citrate, pH 7.0)

Disposable electrical printed (DEP) electrodes were obtained from BioDevice Technology (Nomi, Japan).

Synthesis of chemically modified graphene (CMG) materials and detailed characterizations from transmission electron microscopy (TEM), raman spectroscopy, electrochemical impedance spectroscopy (EIS) and x-ray photoelectron spectroscopy (XPS) of the CMG materials, were reported in our previous work.¹² In brief, the D/G raman signal ratio for graphene oxide (GO), and electrochemically reduced graphene oxide (ERGO) was reported to be 1.05 and 1.08 respectively. XPS determined the C/O ratio to be 2.8 and 5.1 for GO and ERGO respectively. In addition, EIS displayed charge transfer resistance (R_{ct}) values of 11.75 and 4.71 k Ω for GO and ERGO accordingly.

Equipment

Electrochemical experiments were conducted with an Autolab potentiostat PGSTAT302 (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer and controlled by NOVA software version 1.7. Impedance measurements were carried out between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The experiments were performed at room temperature using DEP electrodes and 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1 molar ratio) in PBS buffer solution as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane.

Procedures

Preparation of ERGO modified DEP electrode

GO was modified onto each DEP electrode surface via physical adsorption. 3 μL of GO at a concentration of 1 mg mL^{-1} in ultrapure water was deposited on the electrode surface and left to dry under room temperature overnight. Excess of GO that did not get adsorbed onto the electrode surface was then removed by gentle washing with ultrapure water. Prior to casting GO onto the electrode surface, GO suspension was sonicated for 15 minutes in order to ensure maximum uniform dispersion of the material. To obtain ERGO modified electrode, GO modified electrode was electrochemically reduced at -1.2 V for 900 s in PBS buffer solution at room temperature.

Physical immobilization of ssDNA

ssDNA was immobilized onto the ERGO modified electrode surface by dry physical adsorption. 3 μL of ssDNA in PBS buffer solution at a concentration of $10 \text{ }\mu\text{M}$ was deposited onto each ERGO modified electrode surface and dried at a temperature of $60 \text{ }^{\circ}\text{C}$ for 10 minutes in an oven. Consequently, the electrode was washed in PBS buffer solution with gentle stirring at room temperature to remove the excess of ssDNA which was not immobilized onto the ERGO modified electrode surface successfully.

Covalent immobilization of NH_2 -ssDNA and NH_2 -ssDNA- NH_2

ssDNA was immobilized onto ERGO modified DEP electrode via covalent binding. 3 μL of 0.05 M EDC and 0.03 M NHS in PBS buffer solution was first deposited onto ERGO modified electrode surface for 1.5 hours in order to activate carboxylic groups. Subsequently, the electrode was rinsed with PBS buffer solution. After that, 3 μL of

ssDNA at a concentration of 10 μM in PBS buffer solution was casted onto the electrode and left to stand overnight at room temperature in a humid environment. This was then followed by washing with 0.05 % SDS and 0.04 M hydroxylamine hydrochloride in order to remove non-specific adsorbed ssDNA and to deactivate any remaining carboxylic groups¹¹ before another washing step with PBS buffer solution.

Hybridization

DEP electrode modified with ssDNA was next incubated in Eppendorf tube containing 5xSSC buffer solution (pH 7.0) with 30 pmol of the complementary ssDNA sequence (total volume: 100 μL). The incubation was conducted at 42 °C for 30 minutes with gentle stirring. After which, two washings with 2xSSC buffer solution (pH 7.0) were performed to remove the excess of non-specific adsorbed ssDNA sequences.

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Chapter 4

Validation of Physical Immobilization Mechanism on
Chemically Modified Graphenes

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The results in this chapter resulted in the publication of the following:

1. **Loo, A. H.**; Bonanni, A.; Pumera, M., An Insight into the Hybridization Mechanism of Hairpin DNA Physically Immobilized on Chemically Modified Graphenes. *Analyst* 2013, *138*, 467-471.

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Motivation

For the construction of the sensing interface of a biosensor targeted for electrochemical detection of nucleic acids, physical immobilization of the recognition elements represents the simplest and most straightforward immobilization technique. Hence, physical immobilization remains one of the commonly used immobilization methods. In addition, to achieve label-free detection of the target nucleic acids, such fabricated sensing interface is usually paired with electrochemical impedance spectroscopy (EIS) as the analytical measurement technique. The rationale behind the choice of EIS is its outstanding sensitivity for probing interfacial properties of modified electrode surfaces, thereby allowing the monitoring of every single step in the biosensing process, without the use of an electroactive label.

For the context of DNA biosensing on graphene using physically adsorbed DNA recognition element, it has been widely reported that the EIS signal increases with physical immobilization of the DNA recognition element and decreases with successful formation of double helix with a complementary DNA target. The initial increase in EIS signal is commonly explained by the steric hindrance and charge repulsion resulting from the adsorbed DNA recognition element, while the decrease in EIS signal is assumed to be due to the desorption of the formed double-stranded DNA (dsDNA) from the graphene surface. However, such assumptions should be validated by independent methods since changes in EIS signal may be owing to a combination of different factors such as charge repulsion, steric hindrance and even conformational changes.

To the aim of validating the proposed detection mechanism by EIS, this study exploits the inherent electroactivity of DNA nitrogenous bases¹ and employs differential pulse voltammetry (DPV) as the analytical technique.

Results and Discussions

In brief, the approach taken by this study² was to utilize an inosine-substituted hairpin DNA probe (INO-Hprobe), which was physically immobilized onto a graphene surface. A hairpin DNA probe refers to a DNA sequence which is capable of partial self-hybridization, thereby comprising of an unpaired loop and a double-helix stem. INO-Hprobe denotes a DNA sequence which has all its guanine bases replaced by inosine. Inosine, in contrast to guanine, is not electroactive and no oxidation signal will be produced. Therefore, when INO-Hprobe binds preferentially to the complementary DNA target, the appearance of the complementary DNA target's guanine residues oxidation signal provides a direct and convenient examination of the hybridization process.³

Figure 1. Schematic of the protocol and differential pulse voltammograms of INO-Hprobe (blue), complementary DNA target (pink), single-base mismatch mutant DNA target (green), and negative control non-complementary DNA target (black). Amount of INO-Hprobe and DNA targets used was 30 pmol. All measurements were performed in 50 mM PBS buffer solution (pH 7.4) with Ag/AgCl as the reference electrode.

Figure 1 describes the general scheme of the work, whereby INO-Hprobes were first

physically immobilized on the surface of graphene before the exposure to different DNA targets.

Upon incubation with (i) complementary single-stranded DNA (ssDNA), it is commonly assumed from EIS data^{4, 5} that majority of the hybridized dsDNA is released from the graphenic surface. Secondly, upon incubation with (ii) mutant ssDNA, due to partial hybridization, a smaller portion of the non-completely hybridized dsDNA is released from the surface and non-hybridized mutant ssDNA target may also non-specifically adsorb onto the surface. Lastly, upon incubation with (iii) non-complementary ssDNA, there is no release of INO-Hprobes from the surface and non-complementary ssDNA can also non-specifically adsorb onto the surface of graphene. To verify the above assumptions, we employed DPV and measured the oxidation signal of guanine bases in DNA target sequences (complementary, mutant and non-complementary).

Figure 1 exhibits the differential pulse voltammograms obtained in an experiment with complementary, mutant and non-complementary DNA sequences using INO-Hprobes. The guanine peak recorded at peak potential of approximately 0.875 V was examined, after each modification step in the protocol. As shown in Figure 1, INO-Hprobes modified surface did not produce an observable guanine peak as they do not contain any guanine bases. After incubation with the various target sequences (complementary, mutant and non-complementary), a significant guanine peak was observed. The peak current was greatest for the case of hybridization with a non-complementary sequence, followed by the mutant sequence and lastly the complementary target sequence. These results supported the previous ones obtained with EIS,^{4, 5} and the observed trend could thus be unambiguously attributed to the partial release of INO-Hprobes from the electrode surface upon successful hybridization. When hybridization was effective, as in

the case of the complementary DNA target, most of the hybridized INO-Hprobes were released from the surface and a relatively small guanine peak current was resulted. However, when hybridization was less effective, as in the case of the mutant target, a lower amount of the hybridized INO-Hprobe was released from the electrode surface, and thus a greater guanine peak current was recorded. For the case of the non-complementary target, poor hybridization occurred and the large guanine peak current observed was reasonably attributed to the non-specific adsorption of the non-complementary target sequence onto the INO-Hprobes modified surface.

To get a deeper understanding of the mechanism of DNA hybridization based on physical immobilization of hairpin DNA probes, we investigated and compared the behaviours of various chemically modified graphene (CMG) platforms. CMG materials such as graphene oxide (GO), thermally reduced graphene oxide (TRGO), and electrochemically reduced graphene oxide (ERGO) were studied.

First of all, differential pulse voltammograms were recorded after hybridization with each DNA target sequence for all three CMG (GO, ERGO and TRGO) surfaces. From the series of voltammograms obtained, the guanine peak currents were noted and represented in a histogram, as shown in Figure 2. From Figure 2A, it could be deduced that for all three surfaces, with the exception of ERGO, the guanine peak current was the highest for non-complementary DNA target, followed by mutant DNA target, and lastly complementary DNA target. In addition, the overall relative standard deviation percentage values for GO, ERGO and TRGO surfaces for this set of experiments were 11.6, 13.6 and 23.8 % respectively. The disparity in the percentage values is most likely due to the difference in the degree of homogeneity of the material suspensions. As GO has more oxygen-containing groups than TRGO,⁶ it is consequently more soluble in

water. Hence, GO suspension was comparatively more homogeneous than TRGO suspension, and the overall relative standard deviation value for GO was much lower than TRGO based on three replicates. With the above findings in mind, GO was deemed to have the greatest potential to be adopted as the transducing platform. Hence, GO was selected to be the employed material for further experimental studies since it displayed the best performance as compared to the other CMG materials.

Figure 2. (A) Histograms representing a comparison of guanine peak currents on GO, ERGO and TRGO modified electrodes when hybridized with complementary, single-base mismatch mutant and non-complementary DNA targets. Error bars corresponded to triplicate experiments. (B) - (D) Differential pulse voltammograms of guanine peak on GO, ERGO and TRGO modified electrodes when hybridized with complementary, single-base mismatch mutant and non-complementary DNA targets. Amount of INO-Hprobe and DNA targets used is 30 pmol. All measurements were performed in 50 mM PBS buffer solution (pH 7.4) with Ag/AgCl as the reference electrode.

Moving on, INO-Hprobe optimization was next conducted on GO surface to determine the optimum amount of INO-Hprobe to be physically immobilized onto the GO platform.

To do so, differential pulse voltammograms were recorded after hybridization with complementary and non-complementary DNA targets for a series of INO-Hprobe amounts. From the series of voltammograms obtained, the guanine peak currents were again noted and represented in a histogram, as illustrated in Figure 3.

Figure 3. Histograms representing a comparison of guanine peak currents when hybridized with complementary and non-complementary DNA targets for a range of INO-Hprobe amounts. Error bars corresponded to triplicate experiments. Amount of DNA targets used is 30 pmol. All measurements were performed in 50 mM PBS buffer solution (pH 7.4) with Ag/AgCl as the reference electrode.

As derived from Figure 3, the percentage difference between complementary and non-complementary DNA targets was 9.6, 10.2, 49.2, 26.6 and 5.4 % for 3, 15, 30, 45 and 60 pmol of INO-Hprobe accordingly. Hence, at 30 pmol of INO-Hprobe, the best distinction between the two DNA targets could be made. However, by taking the error bars into consideration, the best distinction was instead at 45 pmol of INO-Hprobe. Therefore, it was concluded that the optimum amount of INO-Hprobe to be immobilized onto GO surface to ensure maximum surface coverage was 45 pmol.

Conclusions

In summary, DPV was employed for the label-free detection of DNA hybridization and polymorphism. The results obtained confirm the previous assumptions made to explain the change of impedimetric signal in a similar protocol.^{4, 5} The lower guanine oxidation peak signals obtained for complementary and mutant DNA targets were due to the partial removal of hybridized dsDNA from the electrode surface during the hybridization event. After comparing the performance of various CMG surfaces, such as GO, ERGO and TRGO, it was established that GO exhibited comparatively the best performance in terms of results reproducibility and the ability to distinguish between the different DNA sequences. We believe that our findings are important in order to gain deeper insights into the mechanism of DNA biosensing.

Experimental Section

Materials

Graphite microparticles (< 20 μm), nitric acid (HNO_3) (conc. > 90 %), sulphuric acid (H_2SO_4) (conc. 95 – 98 %), potassium chlorate (KClO_3), hydrochloric acid (HCl) (conc. 37 %), sodium phosphate dibasic (Na_2HPO_4), sodium chloride (NaCl), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA sequences are as follows:

Inosine-substituted Hairpin probe (INO-Hprobe): 5' ATI IAI ACC AII CII CCI CAC ACI TCC TCC AT 3'

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

Single-base mismatch mutant: 5' ATG GAG GAC GTG CGC GGC CGC CTG GT 3'

Non-complementary: 5' TTT TTT TTT TTT TTT GGT TGG TGT GGT TGG 3'

Trisodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) was purchased from Alfa Aesar (Singapore).

Disposable electrical printed (DEP) electrodes were obtained from BioDevice Technology (Nomi, Japan).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of conductivity no lesser than 18.2 $\text{M}\Omega\text{ cm}$.

Synthesis of CMG materials and detailed characterizations from transmission electron microscopy (TEM), raman spectroscopy, electrochemical impedance spectroscopy (EIS) and x-ray photoelectron spectroscopy (XPS) of the CMG materials, are reported in our

previous work.⁶ In brief, the D/G raman signal ratio for GO, TRGO and ERGO was reported to be 1.05, 1.35 and 1.08 respectively. XPS determined the C/O ratio to be 2.8, 23.3 and 5.1 for GO, TRGO and ERGO respectively. In addition, EIS displayed charge transfer resistance (R_{ct}) values of 11.75, 3.05 and 4.71 k Ω for GO, TRGO and ERGO accordingly.

Equipment

DPV measurements were performed with a μ Autolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer and controlled by General Purpose Electrochemical System (GPES) software version 4.9. The parameters applied were: 60 s accumulation time at 0.2 V, 50 ms modulation time, 0.5 s interval time, 25 mV modulation amplitude and 5 mV step. The raw data produced from the DPV measurements was treated with a baseline correction of peak width 0.01 using the GPES software. All measurements were conducted at room temperature ($\sim 25^\circ\text{C}$) by utilizing DEP electrodes with Ag/AgCl as the reference electrode, and in 50 mM PBS buffer solution (pH 7.4) unless otherwise stated.

Procedures

Preparation of CMG modified DEP electrode

GO and TRGO were immobilized onto each DEP electrode surface via physical adsorption. 3 μL of CMG materials at a concentration of 1 mg mL^{-1} in ultrapure water was deposited on the electrode surface and left to dry under room temperature overnight. Excess of CMG materials that did not get adsorbed onto the electrode surface was then removed by gentle washing with ultrapure water. Prior to casting the CMG materials onto the electrode surfaces, GO and TRGO suspensions were sonicated for 15 minutes in

order to ensure maximum uniform dispersion of the materials. To obtain ERGO modified electrode, GO modified electrode was electrochemically reduced at -1.2 V for 900 s in PBS buffer solution (pH 7.4) at room temperature.

Physical immobilization of INO-Hprobe

INO-Hprobe was immobilized onto the CMG modified electrode surface by physical adsorption. 3 μL of INO-Hprobe in PBS buffer solution (pH 7.4) at a concentration of 10 μM was deposited onto the modified electrode surface and dried in the oven at 60 $^{\circ}\text{C}$ for 10 minutes. Subsequently, the electrode was washed with PBS buffer solution (pH 7.4) and ultrapure water to remove the excess INO-Hprobes which were not well adsorbed on the electrode surface.

Hybridization

DEP electrodes modified with INO-Hprobes were next incubated in Eppendorf tubes containing 5xSSC buffer solution (pH 7.0) with the desired concentrations of DNA target sequences (total volume: 100 μL). The incubation was conducted at 42 $^{\circ}\text{C}$ for 30 minutes with gentle stirring. After which, two washings with 2xSSC buffer solution (pH 7.0) were performed to remove the excess of non-specifically adsorbed DNA sequences.

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Chapter 5

Graphene Oxide as Electroactive Label for Various
Biosensing Applications

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The results in this chapter resulted in the publication of the following:

1. **Loo, A. H.**; Bonanni, A.; Pumera, M., Thrombin Aptasensing with Inherently Electroactive Graphene Oxide Nanoplatelets as Labels. *Nanoscale* 2013, 5, 4758-4762.
2. **Loo, A. H.**; Bonanni, A.; Pumera, M., Mycotoxin Aptasensing Amplification by using Inherently Electroactive Graphene Oxide Nanoplatelet Labels. *ChemElectroChem* 2015, 2, 743-747.
3. **Loo, A. H.**; Bonanni, A.; Pumera, M., Inherently Electroactive Graphene Oxide Nanoplatelets as Labels for Specific Protein-Target Recognition. *Nanoscale* 2013, 5, 7844-7848.

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Motivation

As described earlier in Chapter 2, graphene and its analogues have been widely examined for electrochemical biosensing applications since their discovery. In these studies, there are two common functions which graphene and its associated materials serve. The materials either serve as the transducing platform, owing to their high conductivity and electron transfer rate, or as a mode of signal amplification, due to their large surface area. However, in recent years, there has been a paradigm shift in the usage of graphene and its analogues in electrochemical biosensing, where these materials are employed as labels instead. In those reports, the electron transfer ability of the materials, and their different affinities towards single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) were exploited to yield different charge transfer resistance values before and after the biorecognition process.^{1, 2}

Apart from that, it is worthy to highlight that graphene oxide, the oxidized form of graphene, emitted a strong inherent reduction signal stemming from the reduction of the oxygen-containing groups present on the surface.³⁻⁵ Therefore, the inherent reduction signal can be effectively used for the quantification of the material and as an analytical signal for biosensing applications.

To that end, we explore the novel utilization of graphene oxide nanoplatelets (GONPs), as an inherently electroactive label, for multiple biosensing systems. In addition, differential pulse voltammetry (DPV) will be employed as the analytical technique in these studies.

Results and Discussions (Part I)

In the first part of this chapter, we demonstrate the employment of GONPs as an inherently electroactive label for the electrochemical aptasensing of thrombin (THR).⁶ The term “aptasensing” refers to detection with the use of aptamers. Aptamers are artificial ssDNA or RNA oligonucleotides which are in vitro selected from nucleic acid libraries through SELEX (systematic evolution of ligands by exponential enrichment).^{7, 8} In theory, it is possible to acquire all the different types of aptamers to recognize their respective target molecules with high affinity and specificity. As alternatives to antibodies, aptamers offer numerous unprecedented advantages over the former. The advantages include easy isolation, modification, labelling and storage, good reproducibility, long-term stability and wide applicability.⁹⁻¹³ THR, an omnipresent protein in mammals, is a form of serine protease. THR found in humans is comprised of two chains bound together by disulphide bridges, with an isoelectric point of 7.05 and a molecular weight of 36.7 kDa.¹⁴ It has several crucial roles to perform in various physiological and pathological processes such as blood coagulation, inflammation, tissue restoration at the walls of blood vessels and platelet activation.^{15, 16} In view of the above, the development of an aptasensor towards THR detection with good selectivity, sensitivity, high throughput and simplicity is of immense importance in both clinical and research applications.

Figure 1 illustrates the analytical protocol adopted in this study. Thrombin aptamer (THR-APT-15) was first immobilized onto the surface of the electrode by dry physical adsorption.¹⁷⁻¹⁹ Subsequently, the aptamer modified electrode was exposed to THR before it was incubated in a solution containing a fixed concentration of GONPs (A), or it was just incubated with the solution containing GONPs (B). In the case of (A), upon the

exposure of aptamer modified electrode to THR, THR bound specifically to the immobilized THR-APT-15 and resulted in the partial removal of immobilized THR-APT-15 due to the conformational change of aptamer when interacting with THR. Hence, when the modified electrode underwent further incubation with GONPs, the GONPs conjugated to the remaining immobilized THR-APT-15 due to the strong π - π interactions between GONPs and THR-APT-15. In the case of (B), the aptamer modified electrode was incubated with GONPs solution directly and similar to (A), the GONPs conjugated to the immobilized THR-APT-15 due to the unique strong π - π interactions. For both cases, (A) and (B), differential pulse voltammograms were recorded and demonstrated in Figure 2.

Figure 1. Illustration of the utilization of GONPs as an electroactive label for the detection of THR. (A) In the presence of THR, THR-APT-15 bound specifically to THR and resulted in partial removal of immobilized THR-APT-15 from the electrode surface due to conformational changes. GONPs then adsorbed onto the remaining immobilized THR-APT-15 due to the strong π - π interactions and a reduction peak originating from the inherent reduction of GONPs label was produced. (B) In the absence of THR, GONPs adsorbed onto the immobilized THR-APT-15.

Figure 2. Differential pulse voltammogram of the GONPs reduction peak in the presence (red) and absence (blue) of THR. Concentration of THR-APT-15, THR and GONPs used is $10 \mu\text{M}$, 40.5 nM and 0.2 mg mL^{-1} respectively. All measurements were conducted in PBS buffer solution (pH 7.0) at room temperature.

As displayed in Figure 2, it can be seen that when THR-APT-15 modified electrode was exposed to THR prior to incubation with GONPs (red), a reduction peak of greater magnitude was attained as compared to a THR-APT-15 modified electrode which was incubated with GONPs directly (blue). Such observation can be related to the result of the partial removal of immobilized THR-APT-15 upon specific binding with THR. While the specific binding of THR to THR-APT-15 led to the partial removal of the immobilized THR-APT-15, it also uncovered more of the electrode surface at the same time. Hence, when GONPs conjugated to the remaining immobilized THR-APT-15 subsequently, there was a larger electrode surface available for charge transfer and a greater reduction signal was resulted.

In order to determine the optimum concentration of THR-APT-15 to be immobilized onto the electrode surface to ensure maximum surface coverage, THR-APT-15 optimization was next carried out using electrochemical impedance spectroscopy (EIS), which is a very

sensitive technique for such task.²⁰ Impedance spectra obtained were analyzed and illustrated as histograms as shown in Figure 3.

Figure 3. Comparison of impedimetric responses towards the different concentrations of THR-APT-15 deposited on bare DEP electrode surface. Signal was represented as ($R_{ct \text{ aptamer}} - R_{ct \text{ blank}}$). Error bars corresponded to triplicate experiments. All measurements were conducted with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6] / \text{K}_3[\text{Fe}(\text{CN})_6]$ in Tris buffer solution (pH = 8.2) at room temperature.

From Figure 3, it was observed that with increasing concentration of THR-APT-15, a larger impedimetric signal was obtained. A larger impedimetric signal signifies that there was a greater amount of THR-APT-15 immobilized on the electrode surface. This is because, THR-APT-15 being negatively charged due to the phosphate backbone, would repel the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe which is also negatively charged. Hence, with a higher amount of THR-APT-15 immobilized on the electrode surface, it would lead to a greater repulsion of the redox probe and this in turn caused a larger impedimetric signal. In addition, it is important to note that from 10 to 20 μM of THR-APT-15, the slight increase in impedimetric signal was accompanied with a greater error bar. Therefore, 10 μM was determined to be the optimum concentration of THR-APT-15 to be immobilized on the electrode surface.

Since GONPs were used as the electroactive label in this study, it was hence crucial to determine the optimum concentration of GONPs to be employed. Figure 4 depicts the voltammetric response of the proposed sensor in relation to the concentration of GONPs. It was noted that increasing concentration of GONPs gave rise to an increasing voltammetric signal until a plateau was reached. The optimum concentration of GONPs was established to be 0.20 mgmL^{-1} as it produced the greatest peak height value with the smallest error bar.

Figure 4. Histograms illustrating the voltammetric signal towards GONPs concentration. Concentration of THR-APT-15 and THR used was 10 μM and 40.5 nM respectively. Error bars corresponded to triplicate experiments. All measurements were carried out in PBS buffer solution (pH 7.0) at room temperature.

The voltammetric response of the fabricated sensor towards different THR concentrations was next investigated to ascertain the range of detection. As depicted in Figure 5, the voltammetric peak height increased with increasing concentration of THR. For the range of 3 pM to 0.3 μM , the sensor displayed good linearity as the R^2 value achieved was 0.937.

Figure 5. Voltammetric response towards THR concentration. Concentration of THR-APT-15 and GONPs used was $10\ \mu\text{M}$ and $0.2\ \text{mgmL}^{-1}$ respectively. Error bars corresponded to triplicate experiments. All measurements were carried out in PBS buffer solution (pH 7.0) at room temperature.

Since it is of a high importance that biosensing protocol shows selectivity for the target protein and not for any other proteins, the selectivity of the sensor was assessed by conducting negative control experiments, with the acquired outcomes shown in Figure 6. As presented in Figure 6, incubation with THR yielded a voltammetric signal greater than THR-APT-15 modified electrode while incubation with bovine serum albumin (BSA), immunoglobulin G (IgG) and avidin (Av), all as negative controls, produced voltammetric signals which are smaller than THR-APT-15 modified electrode. This observation can be explained by the reason that as only THR bound to THR-APT-15, leading to the partial removal of the immobilized THR-APT-15 and consequently uncovering more electrode surface for charge transfer, a greater voltammetric signal was obtained as compared to a THR-APT-15 modified electrode which did not undergo any incubation with a protein target. For the case of negative controls (BSA, IgG and Av), they did not interact with the immobilized THR-APT-15. As a result, there was no partial removal of the immobilized THR-APT-15. In addition, non-specific binding of the negative controls to the electrode

surface might have occurred, leading to further blocking of the electrode surface for charge transfer and thus, relatively smaller voltammetric signals were acquired. Therefore, the fabricated sensor can be deduced to be selective towards THR.

Figure 6. Histograms representing the peak height values obtained before and after incubation with various protein targets. BSA, IgG and Av were used as negative controls. Concentration of THR-APT-15 and THR used was 10 μM and 50 nM respectively. Concentration of BSA, IgG and Av used was 0.25 μM . Concentration of GONPs used was 0.2 mgmL^{-1} . Error bars corresponded to triplicate experiments. All measurements were carried out in PBS buffer solution (pH 7.0) at room temperature.

Conclusions (Part I)

In conclusion, we have shown that GONPs can serve as an excellent label for the aptasensing of THR, due to their extraordinary electrochemistry. GONPs, being much smaller than the traditional graphene sheets and with higher uniformity of the sizes, can lead to a high amplification of the voltammetric signal. Such nanoplatelets are capable of serving a similar function as metal nanoparticles; as an outstanding voltammetric label. However, GONPs offer a strong advantage over metal nanoparticles as labels. The advantage of GONPs over metallic nanoparticles is that for the latter, toxic chemicals are used for obtaining the analytical signal via a wet chemistry approach. Using GONPs however, adopts solid state electrochemistry which does not involve the use of any corrosive or toxic chemicals. Furthermore, the biosensing protocol illustrated here shows that the GONPs system is both sensitive and selective; with a range of detection determined to be 3 pM - 0.3 μ M, and a good discrimination towards interferences such as IgG, avidin and BSA. The use of GONPs as labels, instead of using them as transducers, exhibits an immense potential for the widespread applications in biomedical fields.

Results and Discussions (Part II)

In the second part of this chapter, the applicability of GONPs as the inherently electroactive labels for the aptasensing of an important mycotoxin, ochratoxin A (OTA), is demonstrated.²¹ Mycotoxins are one of the common food contaminants and they are toxins synthesized from the secondary metabolism of fungi. Till date, more than 400 mycotoxins, with their individual chemical structures and toxic effects, have been discovered.²² OTA represents one of the most dangerous forms owing to its abundance and extreme toxicity,²³ having nephrotoxic,²⁴⁻²⁶ teratogenic, hepatotoxic and mutagenic effects on various mammalian species.^{27, 28} Moreover, clinical tests have also established that OTA could result in cancer in rats and mice, and is involved in the pathogenesis of tumors in the upper urinary tract of humans.²⁹ Till date, OTA has been commonly detected in a wide variety of food and beverages such as beans, spices,³⁰ cereals,^{28, 31, 32} nuts, dried fruits,³³ coffee,^{34, 35} wine³⁶ and beer. Unfortunately, it is not viable to reduce the levels of OTA in food products as it has high resistance to food preparation processes including roasting, fermenting and cooking.³⁷ Hence, it is essential to acquire selective and sensitive detection of OTA in order to ensure food safety and minimize the potential health risk to humans as well as the environment.

Figure 7 illustrates the analytical procedure utilized in this study. In brief, ochratoxin A aptamer (OTA-apt) was first immobilized onto the surface of the electrode by dry physical adsorption to serve as the selective biorecognition element. Subsequently, the OTA-apt modified electrode then underwent prior exposure to OTA before incubation with GONPs solution. In addition, control experiment in the absence of OTA was also carried out. For the latter instance, GONPs adsorbed onto the immobilized OTA-apt via π - π interactions. Whereas, for the former instance, upon exposure to OTA, the

immobilized OTA-apt bound specifically to OTA and conformational changes of the OTA-apt were induced. Consequently, the induced conformational changes led to a partial removal of the immobilized OTA-apt from the electrode surface.^{6, 38, 39} Hence, when the modified electrode underwent subsequent incubation with GONPs, the GONPs conjugated to the remaining immobilized OTA-apt due to π - π interactions between OTA-apt and GONPs. Analytical signal was drawn from the inherent electrochemical reduction of GONPs and differential pulse voltammograms, as displayed in Figure 8, were recorded for both instances.

Figure 7. Representation of the application of GONPs as electroactive labels for the aptasensing of OTA. In the absence of OTA, GONPs were adsorbed onto the immobilized OTA-apt via π - π interactions. In the presence of OTA, OTA-apt bound specifically to OTA and led to partial removal of immobilized OTA-apt from the electrode surface due to conformational changes. GONPs then adsorbed onto the remaining immobilized OTA-apt. Inset at top right hand corner: Molecular structure of OTA.

As indicated in Figure 8, a greater GONPs reduction signal was attained when the electrode modified with OTA-apt underwent prior exposure to OTA before incubation with GONPs (red line). This was in comparison with an OTA-apt modified electrode which was incubated with GONPs directly (blue line, control experiment). Such

observations may be associated with the partial removal of immobilized OTA-apt upon specific binding with OTA. While the specific binding of OTA to OTA-apt led to conformational changes and subsequent partial removal of the immobilized OTA-apt, it also uncovered more of the electrode surface simultaneously. Therefore, when GONPs conjugated to the remaining immobilized OTA-apt subsequently, there was a larger electrode surface present for charge transfer and a greater reduction signal was obtained.

Figure 8. Differential pulse voltammograms of the GONPs reduction peak in the presence (red) and absence (blue) of OTA. Concentration of OTA-apt, OTA and GONPs used was 10 μM , 0.309 μM and 0.2 mgmL^{-1} respectively. All electrochemical measurements were performed in PBS buffer solution (pH 7.0) at room temperature.

Figure 9. Histograms illustrating the R_{ct} of bare DEP electrode, OTA-apt modified DEP electrode and OTA-apt modified DEP electrode after incubation with OTA. Concentration of OTA-apt and OTA used was 10 μM and 0.309 μM respectively. Error bars corresponded to replicate experiments. All electrochemical measurements were conducted with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ / $\text{K}_3[\text{Fe}(\text{CN})_6]$ in Tris buffer solution (pH = 8.2) at room temperature.

To reinforce the proposed mechanism of partial removal of the immobilized OTA-apt upon specific binding with OTA, two sets of control experiments were conducted with the findings illustrated in Figures 9 and 10. For the first set of control experiments, EIS was utilized to measure the charge transfer resistance (R_{ct}) between the electrode surface and negatively charged $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe for the successive modification steps. As depicted in Figure 9, R_{ct} increases drastically with the immobilization of OTA-apt due to electrostatic repulsion between similarly negatively charged OTA-apt and redox probe. Subsequently, upon incubating with OTA, R_{ct} experienced a decrease in magnitude, implying a partial removal of the immobilized OTA-apt. In order to substantiate the proposed mechanism, the second set of control experiments was performed with DPV. For this set of experiments, guanine oxidation peak was exploited as the analytical signal, and the magnitude of the peak height is a measure of the amount of OTA-apt present on the electrode surface. From Figure 10, the peak height

decreased after incubation with OTA, signifying again the removal of some immobilized OTA-apt. Therefore, the proposed mechanism was reasonably established.

Figure 10. Histograms representing a comparison of guanine peak current of OTA-apt modified DEP electrode before (orange) and after (purple) incubation with OTA. Concentration of OTA-apt and OTA used was 10 μM and 0.309 μM respectively. Error bars corresponded to triplicate experiments. All electrochemical measurements were performed in PBS buffer solution (pH 7.0) at room temperature.

Subsequently, optimization of OTA-apt, which functioned as the biorecognition element, was performed by employing EIS. The aim of the optimization experiments was to determine the optimum concentration of OTA-apt to be deposited onto the electrode surface, in order to ensure maximum coverage. Impedance spectra were collected and analyzed for various concentrations of OTA-apt, with the results displayed as histograms in Figure 11.

Figure 11. Illustration of the impedimetric response towards various concentrations of OTA-apt deposited onto the surface of bare DEP electrode. Signal was represented as ($R_{\text{ct Aptamer}} / R_{\text{ct Blank}}$). Error bars corresponded to triplicate experiments. All electrochemical measurements were conducted with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6] / \text{K}_3[\text{Fe}(\text{CN})_6]$ in Tris buffer solution (pH = 8.2) at room temperature.

From Figure 11, it can be discerned that the largest impedimetric signal was achieved for the OTA-apt concentration of 10 μM . The magnitude of the impedimetric signal is a measure of the amount of OTA-apt being successfully immobilized onto the surface of the electrode. This rationale lies in OTA-apt being negatively charged due to the phosphate backbone. As such, there is electrostatic repulsion between OTA-apt and the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe which is also similarly charged. Hence, with a higher amount of OTA-apt immobilized on the electrode surface, it will lead to a greater repulsion of the redox probe and a larger impedimetric signal is expected. Therefore, 10 μM was concluded to be the optimum concentration of OTA-apt to be deposited onto the surface of the electrode so as to achieve maximum surface coverage. Furthermore, it is also worthy to highlight that the error bar was comparatively the smallest at 10 μM of OTA-apt.

Figure 12. Comparison of voltammetric signals with different concentrations of OTA. Concentration of OTA-apt and GONPs utilized was 10 μM and 0.2 mgmL^{-1} respectively. Error bars corresponded to triplicate experiments. All electrochemical measurements were carried out in PBS buffer solution (pH 7.0) at room temperature.

The voltammetric response of the fabricated aptasensor towards various concentrations of OTA was next examined in order to define the range of detection. As demonstrated in Figure 12, the voltammetric response increased with increasing concentration of OTA until a plateau is reached at around 310 pM. Beyond that concentration point, further increment of OTA concentration did not lead to any significant changes in the magnitude of the voltammetric signal. Therefore, the range of OTA detection was concurred to be from 310 fM to 310 pM. Furthermore, it should be noted that our acquired detection range was comparatively more sensitive than other proposed electrochemical OTA aptasensors,⁴⁰⁻⁴² and our detection assay was also relatively more straightforward.⁴³

To establish the selectivity performance of the fabricated aptasensor towards OTA, ascorbic acid, which is a common component in several foodstuffs, was employed as the negative control. As presented in Figure 13, the voltammetric signal derived for ascorbic acid was considerably lower than OTA. Furthermore, a t-test was conducted at 95%

confidence interval and four degrees of freedom, with $t_{\text{cal}} = 3.719 > t_{\text{table}} = 2.776$. Hence, the voltammetric signals for ascorbic acid and OTA can be considered to be significantly different. Such observations can be justified based on the grounds that since OTA-apt can only bind to OTA, stimulating conformational changes of its structure and leading to partial removal of the immobilized OTA-apt, a relatively greater voltammetric response was attained for OTA. Therefore, the aptasensor was deduced to be selective for OTA. Lastly, it is also worthy to highlight that the average relative standard deviation percentage value derived for this study was 11.8%, suggesting good reproducibility.

Figure 13. Histograms demonstrating the results of the negative control experiment. Concentration of OTA-apt, ascorbic acid and OTA used was 10 μM , 0.312 nM and 0.309 nM respectively. Concentration of GONPs employed was 0.2 mgmL^{-1} . Error bars corresponded to triplicate experiments. All electrochemical measurements were performed in PBS buffer solution (pH 7.0) at room temperature.

Conclusions (Part II)

In summary, this study has demonstrated the potential of employing GONPs as electroactive labels for the aptasensing of OTA. GONPs are proven to be an effective electroactive label due to their inherent electrochemistry and small, uniform sizes. The proposed analytical procedure exhibited that the GONPs approach was sensitive and selective, with an established detection range of 310 fM to 310 pM, and a good discrimination against ascorbic acid. These findings are expected to have a profound impact on the fabrication of future biosensors related to food safety analysis.

Results and Discussions (Part III)

In the last part of this chapter, the proof-of-concept of employing GONPs as an inherently electroactive label for the detection of protein-target interactions is illustrated.⁴⁴

Figure 14. Schematic diagram depicting the employment of GONPs as a possible electroactive label for the detection of avidin. (A) Upon incubation with avidin, biotin-probe interacted specifically with avidin and partial removal of immobilized biotin-probe from the electrode surface was resulted. GONPs subsequently adsorbed onto the residual immobilized biotin-probe owing to robust π - π interactions. (B) Without incubation with avidin, GONPs were adsorbed directly onto the immobilized biotin-probe.

As a model system, a biotinylated DNA probe (biotin-probe) was used for the detection of avidin target molecule.⁴⁵ The system is based on the use of a biotin-probe to be immobilized on the electrode surface as the biorecognition element, instead of simply just the biotin molecule itself. This ensures both a strong and stable adsorption of the biorecognition element onto the electrode surface,^{46, 47} and a higher degree of freedom for the biotin label which will not be involved in any interactions with the electrode

surface.

Figure 14 depicts the analytical protocol followed in this work. Briefly, the biotin-probe was immobilized onto the surface of electrode via dry physical adsorption method.¹⁷⁻¹⁹ After which, the probe modified electrode was either incubated in a solution containing a fixed concentration of avidin before it was next incubated with GONPs solution (A) or it was just directly incubated with GONPs solution (B). In the instance of (A), during the incubation with avidin, the biotin moiety on the immobilized biotin-probe bound specifically to avidin and led to the partial removal of immobilized biotin-probe from the electrode surface.^{38, 48, 49} Therefore, as subsequent incubation with GONPs solution followed, GONPs attached to the residual immobilized biotin-probe owing to the robust π - π interactions between GONPs and nitrogenous bases of biotin-probe. On the other hand, in the instance of (B) where probe modified electrode was directly incubated in GONPs solution, the GONPs attached to the immobilized biotin-probe as a result of the π - π interactions, similar to (A). For both instances, DPV measurements were conducted and their respective voltammograms are exhibited in Figure 15.

From Figure 15, it was observed that when probe modified electrode was incubated with avidin prior to subsequent incubation with GONPs (blue), a reduction peak of higher intensity was achieved as contrasted to a probe modified electrode which underwent direct incubation with GONPs (red). These observations made can be attributed to the partial removal of immobilized biotin-probe upon the specific binding of the biotin moiety to avidin. Owing to the partial removal of the immobilized biotin-probe from the electrode surface, more of the electrode surface was uncovered and thus there was a greater electrode surface available for the charge transfer between GONPs and electrode when GONPs subsequently attached onto the residual immobilized biotin-

probe. Hence, a reduction signal of higher intensity was attained.

Figure 15. Differential pulse voltammograms of GONPs reduction peak upon incubation (blue) and no incubation (red) with avidin. The concentration of biotin-probe, avidin and GONPs used was $10\ \mu\text{M}$, $0.25\ \mu\text{M}$ and $0.2\ \text{mg mL}^{-1}$ respectively. All measurements were performed with PBS buffer solution (pH 7.0) at room temperature.

To affirm the optimum concentration of biotin-probe to be immobilized onto the electrode surface in order to achieve maximum coverage of the surface, biotin-probe optimization was next performed employing EIS, which is an exceptionally sensitive and appropriate technique for such task.^{18, 50} EIS measurements were conducted with a range of different biotin-probe concentrations and the spectra obtained were examined and displayed as histograms in Figure 16.

Figure 16. Histograms illustrating the different impedimetric responses towards the different concentrations of biotin-probe immobilized on bare DEP electrode surface. Signal was represented as $R_{\text{ct}} (\text{Probe} - \text{Blank})$. Error bars corresponded to triplicate experiments. All measurements were performed with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6] / \text{K}_3[\text{Fe}(\text{CN})_6]$ in Tris buffer solution (pH = 8.2) at room temperature.

As presented in Figure 16, a larger impedimetric signal was achieved with increasing concentration of biotin-probe. Biotin-probe, consisting of a phosphate backbone, is negatively charged and will repel the redox probe which is also negatively charged. As such, with a greater amount of biotin-probe successfully immobilized on the electrode surface, it will give rise to a larger repulsion of the redox probe. This in turn brings about a greater impedimetric signal. Therefore, a greater impedimetric signal implies that a higher amount of biotin-probe was immobilized onto the electrode surface and more biotin-probe was immobilized on the electrode surface with increasing concentration of biotin-probe. Furthermore, it is interesting to mention that the impedimetric signal increases rapidly for the initial concentrations before starting to plateau off from 10 μM . Therefore, 20 μM was determined as the optimum concentration of biotin-probe to be immobilized on the surface of electrode to ensure maximal surface coverage.

Following which, the selectivity performance of the analytical protocol was assessed by

performing negative control experiments and the results are depicted in Figure 17.

Figure 17. Histograms representing the peak height values obtained before and after incubation with various protein targets. BSA and IgG were used as negative controls. The concentration of avidin, BSA and IgG used was $0.25 \mu\text{M}$. Concentration of GONPs used was 0.2 mg mL^{-1} . Error bars corresponded to triplicate experiments. All measurements were performed with PBS buffer solution (pH 7.0) at room temperature.

As depicted in Figure 17, a voltammetric signal greater than probe modified electrode was only acquired when incubation was performed with avidin. Incubation performed with BSA and IgG, as negative controls, yielded voltammetric signals which were lower than probe modified electrode. Such observations can be related to the rationale that as only avidin was capable of specifically interacting with the biotin moiety in biotin-probe, leading to partial removal of the immobilized biotin-probe and exposing more electrode surface for charge transfer, a larger voltammetric signal was achieved as contrasted to a probe modified electrode which did not undergo incubation with a protein target. For the instance of the two negative controls (BSA and IgG), they were incapable of resulting in partial removal of the immobilized biotin-probe as they do not interact with the immobilized biotin-probe. Moreover, non-specific binding of the negative controls could have taken place, giving rise to additional blocking of the electrode surface and

comparatively lower voltammetric signals were obtained. Hence, the proposed analytical protocol was shown to be selective for avidin. To further confirm our observations from a statistical point of view, a t-test was performed in order to compare the results obtained for positive and negative controls. The difference between the mean values of avidin and BSA was significant at 80% confidence level and 4 degrees of freedom ($t_{\text{calc}} = 1.97 > t_{\text{tab}} = 1.53$). Moreover, the difference between the mean values of avidin and IgG was significant at 95% confidence level and 4 degrees of freedom ($t_{\text{calc}} = 3.10 > t_{\text{tab}} = 2.78$).

Figure 18. Comparison of voltammetric signal towards GONPs concentration. The concentration of biotin-probe and avidin used was 20 μM and 0.25 μM respectively. Error bars corresponded to triplicate experiments. All measurements were performed with PBS buffer solution (pH 7.0) at room temperature.

Lastly, as GONPs were employed as an electroactive label in this work, we then carried out optimization experiments to determine the optimum concentration of GONPs to be used. In Figure 18, the relationship between voltammetric response and concentration of GONPs is illustrated. From 0.2 to 0.3 mgmL^{-1} of GONPs, there was a steady increment in voltammetric signals and it was also noted that these concentrations produced

relatively high peak height values with small error bars. Therefore, the optimum concentration of GONPs was concluded to be 0.25 mgmL^{-1} , which was the intermediate concentration for that range. In addition, it was also noted that when GONPs concentration was increased to 0.4 mgmL^{-1} , a decrease in peak height was obtained. This may be due to a lower efficiency of the conjugation process of GONPs onto the modified electrode because of the increased concentration. A similar behaviour was previously observed for gold nanoparticles used for bioconjugation purpose.⁵¹⁻⁵³

We like to point out that the strong avidin-biotin interactions are actually beneficial for the sensitivity of the proposed system. This is because, as a result of the strong interactions between avidin and biotin, the strongest known non-covalent interaction, partial removal of the immobilized biotin-probe bound to avidin could occur. This in turn uncovered more of the electrode surface and there was a greater electrode surface available for the charge transfer between GONPs and electrode when GONPs were subsequently conjugated onto the residual immobilized biotin-probe. Hence, a reduction signal of higher intensity was attained compared to the case of no avidin, thereby conferring sensitivity on the system.

Conclusions (Part III)

In conclusion, we have validated the proof-of-concept of using GONPs as an inherently electroactive label for the monitoring of protein-target interactions due to their exceptional electrochemical behaviour. The proposed analytical concept may also be extended to the detection of avidin associated biomolecules such as streptavidin. In this report, the GONPs served effectively as a voltammetric label owing to their small dimensions and high consistency in sizes. In addition, the analytical protocol employed in this study has also proven to be selective, with a distinctive discrimination towards other proteins such as BSA and IgG at concentration levels similar to real samples.⁵⁴ It is expected in the future that the electroactive GONPs labels will play the same role as gold nanoparticle labels that were developed more than a decade ago for the same purposes,⁵⁵ albeit with safer and simpler protocols. The application of presented concept is not confined only to the given system, but can be extended to the detection of microbes such as bacteria or fungi.⁵⁶

Experimental Section

Materials

Stacked graphite nanofibers (GNFs) were purchased from Strem Chemicals (Newburyport, MA, USA).

Thrombin from human plasma (THR), immunoglobulin G (IgG) from rabbit serum, albumin from bovine serum (BSA), avidin (Av), Ochratoxin A from *Petromyces albertensis* (OTA), L-ascorbic acid, sulfuric acid (H₂SO₄) (95 – 98%), hydrochloric acid (HCl) (conc. 37%), potassium permanganate (KMnO₄), sodium nitrate (NaNO₃), hydrogen peroxide (H₂O₂), sodium phosphate dibasic salt (Na₂HPO₄), sodium chloride (NaCl), tris(hydroxymethyl)aminomethane (NH₂C(CH₂OH)₃) (Tris), potassium hexacyanoferrate (II) trihydrate (K₄[Fe(CN)₆].3H₂O), potassium hexacyanoferrate (III) (K₃[Fe(CN)₆]) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA sequences are as follow:

Thrombin aptamer (THR-APT-15): 5' GGT TGG TGT GGT TGG 3'

Ochratoxin A aptamer (OTA-apt): 5' GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA 3'

Biotinylated DNA probe (Biotin-probe): 5' Biotin – TTT TTT TTT TTT TTT GGT TGG TGT GGT TGG 3'

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of resistivity of 18.2 MΩ cm.

The following buffers were used in these studies:

PBS (0.01 M Na_2HPO_4 + 0.1 M NaCl, pH 7.0)

Tris (0.025 M Tris + 0.3 M NaCl, pH 8.2)

Graphene oxide nanoplatelets (GONPs) were prepared with detailed characterization according to our previous report.⁵⁷ Briefly, GNFs were used as the starting material for the synthesis of GONPs. A mixture of NaNO_3 , H_2SO_4 and KMnO_4 was used for the oxidation of GNFs to yield graphite oxide powder. Consequently, the obtained graphite oxide powder was then dispersed in ultrapure water and ultrasonicated for 1 hour in order to acquire GONPs.

Disposable electrical printed (DEP) carbon electrodes were obtained from BioDevice Technology (Nomi, Japan). A three-electrode system was employed in this study which included a carbon-based working and counter electrode, and a Ag/AgCl reference electrode.

Equipment

All electrochemical measurements were performed with a μ Autolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer. Differential pulse voltammetry (DPV) measurements were controlled by General Purpose Electrochemical System (GPES) software version 4.9 and the parameters applied were: 50 ms modulation time, 0.5 s interval time, 10 mV step potential, 50 mV modulation amplitude and 20 mVs^{-1} scan rate. DPV measurements were conducted in PBS buffer solution and the raw data produced were treated with a baseline correction of peak width 0.01 using the GPES software. Impedance measurements were controlled by NOVA software version 1.7 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The

measurements were performed with 10 mM $K_4[Fe(CN)_6]$ / $K_3[Fe(CN)_6]$ (1:1 molar ratio) in Tris buffer solution as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane. All electrochemical measurements were conducted at room temperature ($\sim 25^\circ\text{C}$) by utilizing DEP carbon electrodes unless otherwise stated.

Procedures

THR-APT-15 / OTA-apt / biotin-probe was immobilized onto the surface of a DEP electrode by dry physical adsorption. 3 μL of THR-APT-15 / OTA-apt / biotin-probe in PBS buffer solution at the optimum concentration was casted onto the clean bare electrode surface and dry in the oven at 60°C for 10 minutes. Subsequently, the electrode was washed in PBS buffer solution for 5 minutes at 25°C with gentle stirring to remove the excess aptamer / probe which was not adsorbed on the electrode surface.

DEP electrodes modified with THR-APT-15 / OTA-apt / biotin-probe were next incubated in Eppendorf tubes with Tris buffer solution containing desired concentrations of target biomolecules (total volume: 100 μL). The incubation was performed for 1 hour at 37°C with gentle stirring. Two washing steps were then conducted in Tris buffer solution at 37°C .

Incubation with GONPs was performed in Eppendorf tubes with PBS buffer solution containing desired concentrations of GONPs (total volume: 100 μL). The incubation was carried out for 20 minutes at 25°C with gentle stirring. This was then followed by two washing steps in PBS buffer solution at 25°C .

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Chapter 6

Chemical Vapour Deposition Fabricated Graphene
for Immunosensing

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The results in this chapter resulted in the publication of the following:

1. **Loo, A. H.**; Ambrosi, A.; Bonanni, A.; Pumera, M., CVD Graphene based Immunosensor. *RSC Adv.* 2014, 4, 23952-23956.

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Motivation

The methods for the preparation of graphene can be broadly categorized into two main approaches; bottom-up and top-down. For the instance of top-down approach, the working principle is based on stripping individual sheets of graphene from a graphite source material. This approach includes methods such as, oxidation of graphite to graphite oxide with subsequent thermal, chemical or electrochemical reduction, and mechanical or liquid-phase exfoliation of graphite.¹⁻³ In contrast, for the case of bottom-up approach, the working principle is based on employing small carbon sources to fabricate graphene. For the bottom-up approach, it includes methods such as, epitaxial growth on silicon carbide,⁴ and chemical vapour deposition (CVD).⁵

In recent times, tremendous research interest has been directed to the synthesis of graphene by CVD, owing to its potential to generate graphene of superior quality, with control over the number of layers.⁶ However, the disadvantages are its low yield and high costs, resulting in a huge challenge for large-scale production. While the quest is still on for the large-scale production of CVD graphene, we examine the utilization of CVD graphene for biosensing applications, specifically immunosensing. For this study, electrochemical impedance spectroscopy (EIS) will be employed as the detection technique.

Results and Discussions

Herein, we investigate the analytical proof-of-concept of performing immunosensing of immunoglobulin G (IgG) on CVD graphene.⁷ IgG is the most abundant type of antibody which is present in all body fluids. Upon specific binding to its receptor, it activates a reaction cascade which serves as a protection mechanism against bacterial and viral infections. Based on the level of IgG present in the body, medical practitioners are capable of drawing conclusions on the state of the immune system and thus relate it to infections or autoimmune diseases.⁸

For a start, in order to gain a better understanding of the material employed as the sensing platform, characterizations by scanning electron microscopy (SEM), atomic force microscopy (AFM) and raman spectroscopy were conducted with CVD graphene.

Figure 1. SEM images of commercial CVD graphene. Magnification of (A) 370 × and (B) 10 000 ×. Scale bars of (A) 10 μm and (B) 1 μm.

Characterization of CVD graphene was first performed with SEM to acquire information regarding its surface morphology. The SEM images obtained are shown in Figure 1. Imaging of CVD graphene was carried out at two different magnifications of 370 X (Figure 1A) and 10 000 X (Figure 1B). From the SEM images, it can be observed that the CVD graphene, which was grown on nickel foil, demonstrates several grain boundaries between continuous islands of graphene. In addition, different colours ranging from

white to dark grey, indicating regions with different number of graphene layers, can also be seen. Such observation is a common occurrence in samples grown on thin nickel films.⁹ The homogeneous colour distribution, however, suggested a homogeneous graphene film thickness. Figure 1B clearly shows the edges of the graphene islands grown during the catalytic process. The edges were indicated by white lines which also accounted for folded graphene. On the other hand, the darker lines seen in Figure 1B might indicate structural discontinuity or cracks.

Figure 2. AFM characterization of commercial CVD graphene. (A) Two-dimensional height and amplitude profile, (B) Three-dimensional height profile and (C) Cross-sectional analysis.

In order to gain more insights about the surface of the CVD graphene employed, AFM analysis was next conducted. Figure 2 presents the AFM characterization of CVD graphene. The two-dimensional and three-dimensional profiles of CVD graphene are depicted in Figure 2A and B respectively. From the two figures, it can be confirmed that several of the boundaries visible in SEM as white lines, resembled folded graphene sections which emerged from the film surface at a height of approximately 60 nm (Figure 2C).

Lastly, raman spectroscopy was carried out to attain further structural information such as the presence of defects and the number of layers in CVD graphene. Figure 3 displays the raman spectrum obtained in this study and it was noted that there was an absence

of the D band ($\sim 1350\text{ cm}^{-1}$) in the spectrum. This suggested that there was no significant presence of structural defects in the sp^2 lattice of CVD graphene. The average ratio of the intensity of G band ($\sim 1560\text{ cm}^{-1}$) to 2D band ($\sim 2700\text{ cm}^{-1}$) was calculated to be approximately 2.21. This implied that the CVD graphene was of multilayer structure.¹⁰ Moreover, another piece of evidence which indicated the multilayer property was the slight shoulder observed at 2D band as single layer graphene would exhibit a symmetrical 2D band.¹¹

Figure 3. Raman spectrum of commercial CVD graphene.

Following the various characterizations, CVD graphene was employed as the transducing platform for the immunosensing of rabbit IgG by using EIS as the detection technique.¹² Figure 4 illustrates the analytical protocol adopted. In summary, anti-rabbit IgG probes were first immobilized onto the surface of CVD graphene by physical adsorption. The successful attachment of anti-rabbit IgG probes onto CVD graphene was characterized by the appearance of a N 1s peak in X-ray photoelectron spectroscopy (XPS) study after the anti-rabbit IgG immobilization process (see Figure 5). After which, bovine serum albumin (BSA) blocking was performed by dropcast method in order to block off the

remaining CVD graphene surface and deter non-specific binding. Lastly, incubation with rabbit IgG target was carried out. For each stage of the analytical procedure, impedance measurement was conducted and the respective Nyquist plots are shown in Figure 4.

Figure 4. Schematic illustration of the protocol and Nyquist plots ($-Z''$ vs. Z') of CVD graphene (black box), CVD graphene with immobilized anti-rabbit IgG (red circle), anti-rabbit IgG modified CVD graphene after BSA blocking (blue triangle) and anti-rabbit IgG modified CVD graphene after BSA blocking and incubation with rabbit IgG (pink pentagon). All measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution (pH 7.4) with Ag/AgCl as a reference electrode at room temperature.

Briefly, bare CVD graphene demonstrated relatively the lowest charge transfer resistance (R_{ct}), corresponding to the smallest diameter of the semi-circle, as it was entirely accessible to the redox probe. Subsequently, anti-rabbit IgG probes were immobilized onto the surface of CVD graphene and the magnitude of R_{ct} increased owing to the decrease in accessibility of CVD graphene by the redox probe. After which, blocking of the remaining CVD graphene surface with BSA was performed and this further lowered the accessibility by redox probe and R_{ct} was further enhanced. Last of all, incubation with rabbit IgG, which binds specifically to anti-rabbit IgG, was carried out and it led to another increase in R_{ct} . This can be either attributed to the additional steric

hindrance caused by the bulky rabbit IgG protein molecule, or the electrostatic interactions between rabbit IgG and redox probe.^{13, 14} It should be noted that in our previous study, we have compared physical adsorption, chemical linker and biotin/avidin linker for aptasensing on graphenes, and we found that the highest sensitivity was exhibited by physical adsorption.¹⁵

Figure 5. XPS characterization of commercial CVD graphene before and after anti-rabbit IgG immobilization by physical adsorption technique. (A) Wide-scan spectrum of CVD graphene, (B) Wide-scan spectrum of CVD graphene after anti-rabbit IgG immobilization, (C) High-resolution N 1s core-level spectrum of CVD graphene and (D) High-resolution N 1s core-level spectrum of CVD graphene after anti-rabbit IgG immobilization.

Selectivity is a fundamental requirement of a functional sensor. Hence, the selectivity performance of the proposed immunosensing platform was investigated by conducting negative control experiments with BSA, hemoglobin and avidin. The conclusions from the experiments are exemplified in Figure 6.

From Figure 6, the impedimetric signal ($R_{ct \text{ Target-Probe}}$) of rabbit IgG was much greater than that of the negative control proteins (BSA, hemoglobin and avidin). This indicated that the negative control proteins have negligible interactions with the immobilized anti-rabbit IgG probes. As such, the increase in R_{ct} after incubation with the negative control proteins was small, resulting in the low impedimetric signals. Therefore, the proposed immunosensing platform was deduced to be selective for rabbit IgG.

Figure 6. Illustration of the impedimetric response towards different proteins. BSA, hemoglobin and avidin were employed as negative controls. Signal was represented as $R_{ct \text{ Target-Probe}}$. The error bars corresponded to triplicate experiments. All measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution (pH 7.4) with Ag/AgCl as a reference electrode at room temperature.

Subsequently, to assess the sensitivity of the proposed sensing system and to evaluate the range of detection, the variation of impedimetric signal with rabbit IgG concentration was examined. As depicted in Figure 7, the impedimetric signal increased with increasing concentration of rabbit IgG and the linear range of detection was determined to be from 0.1 to 100 $\mu\text{g mL}^{-1}$ with the limit of detection assessed to be 0.136 $\mu\text{g mL}^{-1}$. It should be noted that the concentration of IgG in human serum and plasma

samples is $4 - 16 \text{ mg mL}^{-1}$ which is fully compatible with the linear range of calibration presented here.

Figure 7. Impedimetric response towards different concentration of rabbit IgG. Signal was represented as $R_{\text{ct Target - Probe}}$. The error bars corresponded to triplicate experiments. All measurements were performed with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in PBS buffer solution (pH 7.4) with Ag/AgCl as a reference electrode at room temperature.

With the selectivity and sensitivity aspects of the proposed sensing platform studied, we next move on to the optimization of the analytical protocol employed.

Anti-rabbit IgG optimization was carried out to establish the optimum concentration of anti-rabbit IgG probe to be deposited onto the surface of CVD graphene in order to achieve the best surface coverage. Impedance measurements were performed for a series of anti-rabbit IgG concentrations and the spectra acquired were analysed and displayed as histograms shown in Figure 8.

Figure 8. Comparison of impedimetric response towards different concentration of anti-rabbit IgG immobilized. Signal was represented as $R_{\text{ct Probe / Blank}}$. The error bars corresponded to triplicate experiments. All measurements were performed with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in PBS buffer solution (pH 7.4) with Ag/AgCl as a reference electrode at room temperature.

As exhibited in Figure 8, the impedimetric signal ($R_{\text{ct Probe/Blank}}$) increased progressively as the concentration of anti-rabbit IgG increased from 0.01 to 10 $\mu\text{g mL}^{-1}$. However, when the concentration of anti-rabbit IgG further increased to 100 $\mu\text{g mL}^{-1}$, a decrease in the signal could be observed. A higher impedimetric signal correlated to a greater amount of anti-rabbit IgG being successfully immobilized onto the surface of CVD graphene. For that reason, 10 $\mu\text{g mL}^{-1}$ was determined to be the optimum concentration of anti-rabbit IgG to be deposited onto the surface of CVD graphene as it ensured the greatest coverage of the surface.

Conclusions

In summary, we have demonstrated in this study the proof-of-principle of performing immunosensing of IgG on CVD graphene. The proposed sensing concept was shown to be selective for IgG, with good discrimination from hemoglobin, avidin and BSA. Furthermore, the linear range of detection was determined to be from 0.1 to 100 $\mu\text{g mL}^{-1}$, thereby conferring reasonable sensitivity to the proposed system. Probe optimization study concluded that 10 $\mu\text{g mL}^{-1}$ was the optimum concentration of anti-rabbit IgG to be deposited on the surface of CVD graphene. CVD graphene represents a quality nanomaterial with vast potential to be employed for biomedical applications in the near future.

Experimental Section

Materials

Immunoglobulin G from rabbit serum (rabbit IgG), anti-rabbit immunoglobulin G produced in goat (anti-rabbit IgG), albumin from bovine serum (BSA), avidin, human hemoglobin, hydrochloric acid (HCl) (conc. 37%), sodium phosphate dibasic salt (Na_2HPO_4), sodium chloride (NaCl), Tween[®] 20, potassium hexacyanoferrate(II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$) and potassium hexacyanoferrate(III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$) were purchased from Sigma-Aldrich (Singapore).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of resistivity 18.2 M Ω cm.

Buffer solutions used in this study are as follows:

PBS (0.01 M Na_2HPO_4 + 0.135 M NaCl, pH 7.4)

PBS-B (0.01 M Na_2HPO_4 + 0.135 M NaCl + 1% BSA, pH 7.4)

PBS-T (0.01 M Na_2HPO_4 + 0.135 M NaCl + 0.05% Tween[®]20, pH 7.4)

Multilayer graphene (105 nm thick on average) on nickel foil (CVD graphene) was purchased from Graphene Laboratories Inc (Calverton, New York).

Equipment

All electrochemical measurements were conducted with a μ Autolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer. Impedance measurements were controlled by NOVA software version 1.8 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The obtained impedance spectra, presented as

Nyquist plots in the complex plane, underwent electrochemical circle fitting. All electrochemical measurements were performed at room temperature with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1 : 1 molar ratio) in PBS buffer solution as the redox probe, and Ag/AgCl as the reference electrode.

Raman spectra were acquired by using a confocal microRaman LabRam HR instrument (Horiba Scientific) in backscattering geometry with a CCD detector. A 514.5 nm Ar laser and a 100x objective lens mounted on a Olympus optical microscope were employed for the focusing of the samples. The initial calibration was conducted at 0 and 520 cm^{-1} with a silicon wafer as the reference to give a peak position resolution of less than 1 cm^{-1} .

Scanning electron microscope images were attained by utilizing a JSM-7600F Schottky Field Emission Scanning Electron Microscope (JEOL, Japan) at 5 kV accelerating voltage.

Atomic force microscopy was performed with a MultiMode 8 atomic force microscope (Bruker, Singapore) controlled by Nanoscope 8.15 software and using the ScanAsyst mode.

X-ray photoelectron spectroscopy data were obtained with a Phoibos 100 spectrometer and an Mg X-ray radiation source (SPECS, Germany) for the measurement of wide-scan survey spectra and high-resolution N 1s spectra.

Procedures

Commercial CVD graphene was washed gently with ultrapure water before use.

Anti-rabbit IgG was immobilized onto the surface of CVD graphene by dry physical adsorption. 50 μl of anti-rabbit IgG in PBS buffer solution at a concentration of 100 $\mu\text{g mL}^{-1}$ was deposited onto CVD graphene and left to dry under the lamp for 30 minutes. Subsequently, the modified surface underwent gentle washings with PBS-T buffer solution, PBS buffer solution and ultrapure water to remove the excess anti-rabbit IgG that was not well adsorbed on the surface.

50 μL of PBS-B buffer solution was next dropcast on the anti-rabbit IgG modified CVD graphene surface and placed under the lamp for 10 minutes for drying. After which, gentle washings with PBS-T buffer solution, PBS buffer solution and ultrapure water were performed to remove the excess BSA which was not well adsorbed on the surface.

CVD graphene modified with anti-rabbit IgG and BSA then underwent incubation with rabbit IgG in PBS-T buffer solution. The incubation was performed at 37 °C for 1 hour in the oven. Finally, gentle washings with PBS-T buffer solution, PBS buffer solution and ultrapure water were performed to remove the excess of non-specifically adsorbed species. For selectivity study, negative controls were conducted using BSA, hemoglobin and avidin.

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Section B

Transition Metal Dichalcogenides

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Chapter 7

Introduction to Transition Metal Dichalcogenides

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For a long time, layered materials have existed and they have been extensively studied for more than a century.^{1, 2} Graphene is the most successful example of layered materials till date and following the success of graphene, other two-dimensional layered materials are attracting immense interest from researchers. In particular, transition metal dichalcogenides (TMDs) represent a widely explored research field. TMDs are characterized by a general formula of MX_2 , where M is a transition metal of groups 4 to 10 and X is a chalcogen. Similar to graphene, TMDs comprise of layers stacked on top of one another, with each layer typically having a thickness of approximately 6 Å. Within a layer, it is made up of a hexagonally arranged layer of transition metal atoms sandwiched between two layers of chalcogen atoms, and conjugated via covalent bonds. On the other hand, between individual layers, they are coupled by weak van der Waals interactions, thus allowing the stacked structure to cleave readily along the layer surface.³ Figure 1 shows an illustration of the structure of bulk TMDs.

Figure 1. Schematic representation of the structure of bulk TMDs.

Interesting to note, despite belonging to the same class of nanomaterials, bulk TMDs can exhibit diverse properties, ranging from insulators, semi-conductors, semi-metals, to true metals.⁴⁻⁶ For their mono-layer and few-layer counterparts, although the bulk properties are largely preserved, they can also display additional characteristics due to confinement effects.⁷⁻⁹ In general, mono-layer and few-layer TMDs have been found to display enhanced optical, electronic, chemical, mechanical and thermal properties as compared to their bulk form. As a result, the synthesis of mono-layer and few-layer TMDs is critical for various applications. To date, various synthetic methods have been developed to achieve mono-layer and few-layer TMDs, and these methods can be categorized into two broad sections, top-down and bottom-up.¹⁰

Top-down synthetic routes denote those that are based on exfoliating bulk TMDs into atomically thin layers. As mentioned earlier, the interaction between adjacent layers in bulk TMDs is dominated by weak van der Waals forces of attractions. Hence, few-layer or even mono-layer TMDs can be attained by using physical (mechanical exfoliation¹¹⁻¹⁴) or chemical (solvent exfoliation,¹⁵⁻¹⁸ lithium-intercalation¹⁹⁻²¹) methods to overcome the van der Waals forces in bulk TMDs.

Mechanical exfoliation, also commonly referred to as the scotch-tape method, has been used in 1966 by Frindt²² to peel bulk molybdenum disulfide (MoS_2) into thin sheets of several molecular layers. In addition, it is also viable to obtain single-layer TMDs by simply rubbing the bulk crystals against another solid surface.¹³ However, the disadvantage of mechanical exfoliation is that most of its products are multi-layer TMD sheets instead of mono-layer. Therefore, additional thinning techniques²³ are required to further thin multi-layer TMDs into mono-layer TMDs. Furthermore, mechanical

exfoliation is also not suitable for large-scale production. Nonetheless, mechanical exfoliation allows the preservation of the inherent properties of the materials.

In order to exfoliate bulk TMDs into few-layer or single-layer sheets on a large scale, we look to chemical exfoliation. Chemical exfoliation encompasses two techniques, solvent exfoliation and lithium intercalation which is then followed with exfoliation. For solvent exfoliation, an effective strategy for exfoliating bulk TMDs involves dispersing the bulk materials in a suitable solvent and exposing the suspension to ultrasonic waves.¹⁵ To gain deeper insights into that strategy, theoretical calculations were conducted and the results show that if the surface energy of the employed solvent is similar to the TMD material, a stable suspension can be formed. This is because the driving force for re-aggregation of exfoliated sheets can be overcome.²⁴ Based on the above method, several different layered compounds have been exfoliated into few-layer sheets with success. In addition, N-methyl-pyrrolidone was demonstrated to be an ideal solvent for efficient exfoliation of MoS₂ and tungsten disulfide in the same work.¹⁵ It is evident that solvent exfoliation seems to be the perfect solution for the scalable and economical production of few-layer TMD sheets. In the context of lithium intercalation, it consists of using violent reactions between water and lithium intercalants to exfoliate bulk TMDs. The principle behind such exfoliation method lies in lithium reacting violently with water to evolve hydrogen gas, which can then be utilized to separate bulk TMDs into few-layer or single-layer sheets with the aid of ultrasonication. Using the above concept, Morrison and colleagues managed to exfoliate MoS₂ into single-layer sheets.²⁵ Unfortunately, in the same study, it was also demonstrated that the intercalation of lithium into TMD layers was slow, requiring at least 48 hours, and random. To circumvent that, an alternative method proposed the usage of an electrochemical cell, with lithium foil as

the anode and TMDs as the cathode. This method enables the lithium intercalation process to be completed in several hours, and the process can also be monitored and well-controlled.²⁰ Subsequently, mono-layer or few-layer TMD sheets can be obtained after reacting with water. In summary, although chemical exfoliation has the potential to mass-produce few-layer TMD nanosheets, the sonication process commonly used in chemical exfoliation is detrimental when it comes to obtaining few-layer TMD sheets with large lateral size. Crucial to highlight, the electrical properties and degree of crystallinity of the exfoliated TMD sheets are different from their bulk counterparts.^{5, 16}

Moving on to bottom-up synthetic routes, these methods are based on the directed or the self-assembly of precursor molecules to form few-layer or single-layer TMD sheets. The bottom-up approach includes chemical vapour deposition (CVD),²⁶⁻³³ physical vapour transport (PVT)³⁴ and hydrothermal synthesis.³⁵

CVD represents the most popular bottom-up route for the fabrication of TMD sheets. For a typical CVD process for growing mono-layer TMDs, it involves a substrate which is coated with a thin layer of transition metal or metal oxide by using physical vapour deposition. Thereafter, the coated substrate is exposed to a chalcogen atmosphere^{27, 36} under high temperature, forming a thin TMD layer.

PVT is an alternative to CVD and the difference lies in PVT uses TMD powders as the source material.^{34, 37} In a regular run, TMD powder is placed inside an alumina container and positioned at the center of a horizontal quartz tube furnace. A clean substrate is then placed in a downstream location, in the low-temperature region. After which, the quartz tube is heated up and a pressure is maintained under argon flow. Finally, mono-layers of TMD are achieved on the substrate after 15 minutes of growth.

Hydrothermal synthesis is another method to produce TMD nanosheets via the bottom-up approach. To synthesize TMD nanosheets, transition metal and chalcogen precursors^{35, 38} are first added into water in a Teflon autoclave before subjecting the autoclave to a certain temperature for various durations. Subsequently, the product is filtered, washed and dried. Based on the above procedure, thin MoS₂ nanosheets have been successfully synthesized. The hydrothermal reaction suggests an alternative route for the mass production of few-layer TMDs. However, wet chemistry synthesis of TMD nanosheets is still at the early stages and more studies have to be done to understand the properties of the synthesized products and overcome challenges in the synthesis process.

Having discussed the various methods to obtain mono-layer and few-layer TMDs which display enhanced optical, electronic, chemical, mechanical and thermal properties, the applications of these materials are next examined. Till date, there have been reports on the utilization of these materials for several applications, with hydrogen evolution reaction (HER) and energy storage receiving the greatest attention.

Currently, expensive and rare platinum is being used as the catalyst for HER owing to its high HER activity. Hence, the search for suitable replacement catalysts which are electrochemically stable, inexpensive and environmentally friendly is imperative. The identification of TMD materials as promising electrocatalysts for HER has uncovered new paths in this field.^{39, 40} In recent years, several reports have demonstrated that MoS₂ has the potential to exhibit comparable HER activity.⁴¹ In addition, the performance may be improved by increasing the concentration of reactive edge sites. This is because, from density functional theory calculations and experimental investigations,⁴² it is proven that the edges of TMD nanosheets are active for HER.^{41, 43, 44} On the same note, this also

explains the outstanding performance which single-layer and few-layer TMDs have over their bulk form, as the latter has a lower concentration of catalytically active sites. In addition, by combining MoS_2 with other materials, it can also lead to superior electrocatalytic performance as a result of synergistic interactions.³

Owing to the spacing between the layers in TMDs, a convenient environment for the accommodation of various chemical species, such as lithium ions, is resulted. In view of that, earlier studies have examined bulk TMD materials as electrode materials for lithium ion batteries as the ions can be readily intercalated or removed from these layered materials.⁴⁵ However, the applicability of bulk TMD materials as cathode materials was found to be adversely limited by their structural instability upon lithiation, and their low average voltage and energy density. On the other hand, studies on exfoliated TMD nanosheets demonstrated that they can be employed as anodes for lithium ion batteries as they are structurally stable. The structural stability is attributable to the nanosheets being loosely stacked and allowing for structural changes.³ Nonetheless, other factors such as electrical conductivity, cycling stability, morphology and particle size are vital parameters in the performance of TMDs as anodes in lithium ion batteries. Currently, only composites made with MoS_2 and graphene have displayed notable electrochemical performance as anodes in lithium ion batteries, and such performance is a result of the synergistic effects and robust composite structure.⁴⁶⁻⁴⁹

In the following chapters, the inherent resistance of various TMDs was first characterized before using them to investigate their interactions with DNA. Thereafter, the influence of different transition metals, in TMDs, on their performance as a fluorescence quencher was studied. Last of all, a new approach of using MoS_2 for DNA detection was established.

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Chapter 8

Electrochemical Impedance Spectroscopic
Characterization of Transition Metal Dichalcogenides

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The results in this chapter resulted in the publication of the following:

1. **Loo, A. H.**; Bonanni, A.; Sofer, Z.; Pumera, M., Exfoliated Transition Metal Dichalcogenides (MoS₂, MoSe₂, WS₂, WSe₂): An Electrochemical Impedance Spectroscopic Investigation. *Electrochem. Commun.* 2015, 50, 39-42.

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Motivation

Following the success of graphene, other forms of two-dimensional layered materials such as transition metal dichalcogenides (TMDs) are experiencing a surge of interest.^{1, 2} Similar to graphene, individual layers of TMDs can be isolated from the bulk materials through a wide array of methods such as, mechanical cleavage,³⁻⁵ liquid exfoliation,^{6, 7} and ion intercalation.⁸⁻¹⁰ In particular, ion intercalation has been demonstrated to be more efficient than the other separation techniques.⁹

Till date, TMD materials have been found to display a broad range of optical, electronic, chemical, mechanical and thermal properties.^{2, 11} Nonetheless, it is extremely vital to highlight that the exfoliated forms of TMD materials often present properties which are greatly different from the bulk forms.⁹

In view of the above and the emerging trend of employing TMDs for electrochemical biosensing applications,¹² it is crucial to garner more insights on the inherent electrochemical characteristics of the materials. Specifically, a fundamental study on the effects of exfoliation on the inherent electrochemical properties of TMDs is imperative.¹³ To this end, electrochemical impedance spectroscopy (EIS) is adopted to monitor the changes in charge transfer resistance (R_{ct}) of the TMD materials after undergoing exfoliation by lithium ion intercalation. In addition, to achieve a deeper understanding, lithium intercalants of different strengths are also studied.¹⁴

Results and Discussions

Figure 1. Nyquist plots ($-Z''$ vs. Z') of the different TMD materials: (A) MoS₂; (B) MoSe₂; (C) WS₂; and (D) WSe₂, in their bulk (black) and various exfoliated forms (red, blue and pink). All measurements were conducted under ambient conditions with 10 mM K₄[Fe(CN)₆]/K₃[Fe(CN)₆] in PBS buffer solution as the redox probe.

EIS represents a powerful, sensitive and non-destructive tool which is widely employed for investigating interfacial properties of a broad range of materials.^{15, 16} Specifically, EIS is capable of assessing the ability of the material to transfer and exchange charges with molecules in proximity. Hence, EIS was performed to evaluate the resistance of the various TMD materials, in bulk and exfoliated forms, towards heterogeneous charge transfer. Figure 1 displays the Nyquist plots attained from the EIS measurements. In brief, four types of TMDs, molybdenum disulfide (MoS₂), molybdenum diselenide (MoSe₂), tungsten disulfide (WS₂) and tungsten diselenide (WSe₂) were investigated. Furthermore, for each TMD material, the bulk and different exfoliated forms were examined. The diameter of the semi-circle in a Nyquist plot corresponds to the R_{ct} value,

which is inversely proportional to the rate of heterogeneous charge transfer. From the acquired Nyquist plots in Figure 1, the respective R_{ct} values were inferred and analysed, with the results of the analysis demonstrated as histograms, as represented in Figure 2.

Figure 2. Histograms illustrating the impedimetric signal of the different TMD materials in their bulk and various exfoliated forms. Impedimetric signal was represented as R_{ct} (TMD – Bare). Error bars corresponded to triplicate experiments. All measurements were conducted under ambient conditions with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution as the redox probe.

As illustrated in Figure 2, the different TMD materials in their bulk and various exfoliated forms, produced impedimetric signals of varying intensities. From the histograms plotted, a few conclusions could be drawn. First of all, selenide TMDs (MoSe₂ and WSe₂) presented faster heterogeneous electron transfer (HET), while sulfide TMDs (MoS₂ and WS₂) showed a slower rate of HET towards the $[Fe(CN)_6]^{3-/4-}$ redox probe. Such conclusion was inferred from the overall lower impedimetric signals demonstrated by MoSe₂ and WSe₂, as compared to MoS₂ and WS₂. Another conclusion which could be derived from Figure 2 was that, while the impedimetric signals of the various exfoliated forms for MoSe₂ and WS₂ did not seem to follow any particular trend with lithium

intercalants of increasing strength ($\text{MeLi} < \text{BuLi} < t\text{-BuLi}$), a general decreasing trend and a slight increasing trend could be observed for the case of MoS_2 and WSe_2 respectively. In addition, it is also worthy to highlight that the average relative standard deviation percentage value obtained for the series of experiments illustrated in Figure 2 was 7.2%.

Subsequently, in order to assess the potential impact which electrochemical activation, via the cycling of potential, has on the R_{ct} of the TMD materials, comparison experiments were conducted with $t\text{-BuLi}$ exfoliated TMD materials. Electrochemical activation was achieved by using cyclic voltammetry over a potential range of 1 to -1 V, at a scan rate of 0.1 Vs^{-1} , and for two scans. Figure 3 depicts the obtained results from the comparison study.

From Figure 3, it is apparent that electrochemical activation of the TMD materials led to a lower impedimetric signal and hence weaker resistance towards charge transfer with the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe. Such observations could be elucidated by changes in the surface chemistry of the TMD materials upon electrochemical activation, hence resulting in lesser repulsion of the redox probe and facilitating HET. On another note, the average relative standard deviation percentage value determined for this set of experiments was 18.3%.

Figure 3. Histograms depicting the impedimetric signals of the different *t*-BuLi exfoliated TMD materials with (yellow) and without (pink) prior activation step. Impedimetric signal was represented as $R_{ct} \text{ (TMD / Bare)}$. Error bars corresponded to replicate experiments. All measurements were conducted under ambient conditions with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution as the redox probe.

Conclusions

In summary, we have evaluated the resistance of various TMD materials towards heterogeneous charge transfer by utilizing EIS. These TMD materials (MoS_2 , MoSe_2 , WS_2 and WSe_2) included both the bulk form and the exfoliated forms, which were attained by lithium ion intercalation with lithium intercalants of differing strengths. From the study, it was demonstrated that selenide TMD materials generally displayed a faster rate of HET as compared to the sulfide counterparts. In addition, it was also discovered that electrochemical activation of the TMD materials, via the cycling of potential, could lead to an enhancement in the rate of HET. These conclusions are expected to provide constructive insights on the implications of the exfoliation process on the inherent electrochemistry of TMD materials.

Experimental Section

Materials

Hydrochloric acid (HCl) (conc. 37%), sodium phosphate dibasic salt (Na_2HPO_4), sodium chloride (NaCl), potassium hexacyanoferrate (II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$) and potassium hexacyanoferrate (III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$) were purchased from Sigma-Aldrich (Singapore).

Molybdenum disulfide (MoS_2), methyl lithium (CH_3Li) (1.6 M in diethyl ether), n-butyl lithium ($n\text{-C}_4\text{H}_9\text{Li}$) (1.6 M in hexane) and t-butyl lithium ($(\text{CH}_3)_3\text{CLi}$) (1.7 M in pentane) were obtained from Sigma-Aldrich (Czech Republic).

Hexane was obtained from Lach-ner (Czech Republic).

Molybdenum diselenide (MoSe_2), tungsten disulfide (WS_2) and tungsten diselenide (WSe_2) were obtained from Alfa Aesar (Germany).

Argon (99.9999% purity) was obtained from SIAD (Czech Republic).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of resistivity 18.2 $\text{M}\Omega \text{ cm}$.

Disposable electrical printed (DEP) carbon chips were obtained from BioDevice Technology (Nomi, Japan). A three-electrode system was employed in this study which included a carbon-based working and counter electrode, and a Ag/AgCl reference electrode.

The following TMD materials were employed in this study:

MoS₂ (bulk, methyl lithium exfoliated, n-butyl lithium exfoliated and *t*-butyl lithium exfoliated forms)

MoSe₂ (bulk, methyl lithium exfoliated, n-butyl lithium exfoliated and *t*-butyl lithium exfoliated forms)

WS₂ (bulk, methyl lithium exfoliated, n-butyl lithium exfoliated and *t*-butyl lithium exfoliated forms)

WSe₂ (bulk, methyl lithium exfoliated, n-butyl lithium exfoliated and *t*-butyl lithium exfoliated forms)

Equipment

All electrochemical measurements were performed with a μ Autolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer.

Impedance measurements were controlled by NOVA software version 1.9 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The measurements were performed with 10 mM K₄[Fe(CN)₆]/K₃[Fe(CN)₆] (1:1 molar ratio) in 0.1 M PBS buffer solution (0.1 M NaCl + 10 mM Na₂HPO₄, pH 7.0) as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane.

All electrochemical measurements were conducted under ambient conditions by utilizing DEP carbon chips unless otherwise stated.

Procedures

Exfoliation of TMD materials (MoS_2 , MoSe_2 , WS_2 and WSe_2) were carried out by suspending 3 g of bulk TMD powder in 20 mL of 1.6 M methyl lithium in diethyl ether, 20 mL of 1.6 M n-butyl lithium in hexane, or 20 mL of 1.7 M t-butyl lithium in pentane. The solution was then stirred for 72 hours at 25 °C under argon atmosphere. The Li-intercalated material was next separated by suction filtration under argon atmosphere and the intercalated compound was washed several times with hexane (dried over Na). Subsequently, the exfoliated TMD with intercalated Li was placed in water (100 mL) and repeatedly centrifuged (18 000 g). Finally, the material was dried in a vacuum oven at 50 °C for 48 hours prior to further use.

DEP carbon chip surface was modified with TMD materials by physical adsorption. 3 μL of TMD materials at a concentration of 1 mg mL^{-1} in ultrapure water was deposited onto each carbon chip surface and left to dry under ambient conditions overnight. The excess of TMD materials which was not well adsorbed on the chip surface was then removed by gentle rinsing with ultrapure water. Prior to depositing the materials onto the surface of the chips, TMD suspensions were sonicated for 15 minutes to achieve maximum dispersion of the materials.

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Chapter 9

Assessing the Interactions between Hairpin DNA and
Transition Metal Dichalcogenides

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The results in this chapter resulted in the publication of the following:

1. **Loo, A. H.**; Bonanni, A.; Sofer, Z.; Pumera, M., Transitional Metal/Chalcogen
Dependant Interactions of Hairpin DNA with Transition Metal Dichalcogenides,
MX₂. *ChemPhysChem* 2015, 16, 2304-2306.

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Motivation

In view of the attractive properties which transition metal dichalcogenides (TMDs) display, they have found recent applications in the fabrication of biosensing devices.¹⁻³ For the development of these devices, it usually involves the immobilization of a biorecognition element such as DNA onto the surface of TMDs. The immobilization of a DNA sequence onto the surface of TMD materials can be simply achieved by van der Waals forces between the DNA nucleobases and the basal plane of TMD materials.⁴⁻⁶ Hence, it is vital to examine the interactions between TMDs and DNA.

Herein, we explore the interactions between hairpin DNA (HpDNA) and the surface of various TMDs. In particular, the influence of different transition metals and chalcogens on their interactions with HpDNA is assessed. HpDNA refers to an unpaired loop of DNA that is created when the DNA strand folds and forms base pairs with another section of the same strand. In recent times, there has been increasing studies performed with HpDNA and this is because HpDNA offers the advantage of higher selectivity over linear DNA. To examine the extent of interactions between HpDNA and the various TMD materials, we utilized electrochemical impedance spectroscopy (EIS) in this work.

Results and Discussions

Figure 1. Schematics illustrating the physical immobilization of HpDNA molecules onto TMD materials (MoS_2 , MoSe_2 , WS_2 and WSe_2).

In this work,⁷ the interactions of HpDNA molecules with the surface of different TMD materials are investigated (Figure 1).⁸⁻¹⁰ In order to acquire insights on how the different transition metals and chalcogens present in TMD materials can affect the resulting interactions with HpDNA, molybdenum disulfide (MoS_2), molybdenum diselenide (MoSe_2), tungsten disulfide (WS_2) and tungsten diselenide (WSe_2) were studied. In addition, both bulk (non-exfoliated) form as well as exfoliated (*t*-BuLi exfoliated) form of the layered TMDs were also examined to gain a deeper understanding.⁸ To these aims, EIS was utilized to study the interactions between HpDNA and various TMD materials, by evaluating the resistance of a surface towards heterogeneous charge transfer.¹¹⁻¹⁴

EIS measurements were conducted before and after the physical immobilization of HpDNA molecules onto the surface of TMD materials to assess the change in charge transfer resistance (R_{ct}) upon the HpDNA immobilization process.

Figure 2. Histograms demonstrating the impedimetric signals of different TMD materials in their bulk (purple) and *t*-BuLi exfoliated (cyan) forms after the physical immobilization of HpDNA molecules. Impedimetric signal was represented as $R_{ct}(\text{HpDNA} - \text{TMD})$. Error bars related to replicate experiments. All measurements were performed under ambient conditions with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in PBS buffer solution as the redox probe.

With the immobilization of HpDNA molecules onto the surface of TMD materials, an enhancement in the R_{ct} was observed. This is because HpDNA molecule contains a negatively charged phosphate backbone and this will in turn lead to repulsion with the similarly negatively charged redox probe and further hinder charge transfer. Hence, an increase in R_{ct} was resulted. The increase in R_{ct} upon the physical immobilization of HpDNA molecules ($R_{ct}(\text{HpDNA} - \text{TMD})$) was then utilized as the analytical impedimetric signal, as demonstrated in Figure 2.

As illustrated in Figure 2, the interactions of HpDNA with two types of TMD materials (bulk and *t*-BuLi exfoliated) were studied. From the plotted histograms which were reflecting the increase in R_{ct} and thus the amount of HpDNA at the solid/liquid interface, a few important conclusions could be inferred. First of all, for both Mo and W, the difference in impedimetric signals between their sulfides and selenides was more

pronounced in the bulk form (Figure 2, purple colour) as compared to the *t*-BuLi exfoliated form (Figure 2, cyan colour). This implied that in the bulk form, the selenide TMDs (MoSe_2 and WSe_2) have much stronger interactions with HpDNA molecules than their sulfide counterparts (MoS_2 and WS_2). However, upon undergoing exfoliation with *t*-BuLi, the difference in the strength of interactions was much smaller.

Secondly, for both bulk and *t*-BuLi exfoliated forms, selenide TMDs consistently displayed relatively greater impedimetric signals as compared to sulfide TMDs. Such observation suggests that the increase in R_{ct} after the physical immobilization of HpDNA molecules was higher for selenide TMDs than sulfide TMDs. Hence, the physical immobilization of HpDNA molecules was more favourable on the surface of selenide TMDs than sulfide TMDs and selenide TMDs have stronger interactions with HpDNA than sulfide TMDs.

On another note, by assessing molybdenum and tungsten, it could be noticed that molybdenum TMDs (MoS_2 and MoSe_2) also constantly exhibited larger impedimetric signals than that of their tungsten associates (WS_2 and WSe_2) for both bulk and *t*-BuLi exfoliated forms. Therefore, this signified that molybdenum TMDs have greater interactions with HpDNA in comparison with tungsten TMDs.

Conclusions

To summarise, we have assessed the extent of interactions between HpDNA and TMD materials. The influence of three factors on the interactions was studied in this work and these factors were, the type of transition metal (Mo and W), the type of chalcogen (S and Se), and the exfoliation of TMDs. From the study, it was revealed that selenide and molybdenum TMD materials presented stronger interactions with HpDNA in contrast to sulfide and tungsten TMD materials respectively. In addition, it was also noted that selenide TMDs have much greater interactions with HpDNA molecules than their sulfide counterparts in the bulk form. However, upon undergoing exfoliation, the difference in the degree of interactions became less significant. These findings are anticipated to contribute useful insights on the fabrication of any DNA based architectures on TMD platforms, including the ones for biosensing devices.

Experimental Section

Materials

Hydrochloric acid (HCl) (conc. 37%), sodium phosphate dibasic salt (Na_2HPO_4), sodium chloride (NaCl), potassium hexacyanoferrate (II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$), potassium hexacyanoferrate (III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$) and hairpin DNA (HpDNA) were purchased from Sigma-Aldrich (Singapore).

Molybdenum disulfide (MoS_2) and t-butyl lithium ($(\text{CH}_3)_3\text{CLi}$) (1.7 M in pentane) were obtained from Sigma-Aldrich (Czech Republic).

Molybdenum diselenide (MoSe_2), tungsten disulfide (WS_2) and tungsten diselenide (WSe_2) were obtained from Alfa Aesar (Germany).

Argon (99.9999% purity) was obtained from SIAD (Czech Republic).

Hexane was obtained from Lach-ner (Czech Republic).

The sequence of HpDNA is as follows:

5' ATG GAG ACC AGG CGG CCG CAC ACG TCC TCC AT 3'

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of resistivity 18.2 M Ω cm.

Disposable electrical printed (DEP) carbon chips were obtained from BioDevice Technology (Nomi, Japan). A three-electrode system was employed in this study which included a carbon-based working and counter electrodes, and a Ag/AgCl reference electrode.

The following TMD materials were employed in this study:

MoS₂ (bulk and *t*-butyl lithium exfoliated forms)

MoSe₂ (bulk and *t*-butyl lithium exfoliated forms)

WS₂ (bulk and *t*-butyl lithium exfoliated forms)

WSe₂ (bulk and *t*-butyl lithium exfoliated forms)

Equipment

All electrochemical measurements were performed with a μ Autolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer.

Impedance measurements were controlled by NOVA software version 1.9 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The measurements were performed with 10 mM K₄[Fe(CN)₆]/K₃[Fe(CN)₆] (1:1 molar ratio) in 0.1 M PBS buffer solution (0.1 M NaCl + 10 mM Na₂HPO₄, pH 7.0) as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane.

All electrochemical measurements were conducted under ambient conditions by utilizing DEP carbon chips unless otherwise stated.

Procedures

Exfoliation of TMD materials (MoS₂, MoSe₂, WS₂, WSe₂) were carried out by suspending 3 g of bulk TMD powder in 20 mL of 1.7 M *t*-butyl lithium in pentane. The solution was then stirred for 72 hours at 25 °C under argon atmosphere. The Li-intercalated material was next separated by suction filtration under argon atmosphere and the intercalated

compound was washed several times with hexane (dried over Na). Subsequently, the separated TMD with intercalated Li was placed in water (100 mL) and repeatedly centrifuged (18 000 g). Finally, the material was dried in a vacuum oven at 50 °C for 48 hours prior to further use.

DEP carbon chip surface was modified with TMD materials by physical adsorption. 3 μL of TMD materials at a concentration of 1 mg mL^{-1} in ultrapure water was deposited onto each carbon chip surface and left to dry under ambient conditions overnight. The excess of TMD materials which was not well adsorbed on the chip surface was then removed by gentle rinsing with ultrapure water. Prior to depositing the materials onto the surface of the chips, TMD suspensions were sonicated for 15 minutes to achieve maximum dispersion of the materials.

To physically immobilize HpDNA molecules, a 3 μL aliquot of HpDNA molecules at a concentration of 10 μM was deposited onto TMDs modified electrodes and dried in the oven at 60 °C for 10 minutes. Subsequently, two washing steps were conducted in PBS buffer solution to remove HpDNA molecules which were not well adsorbed.

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Chapter 10

Comparing Molybdenum Disulfide and Tungsten Disulfide as Fluorescence Platforms for DNA Detection

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The results in this chapter resulted in the publication of the following:

1. **Loo, A. H.**; Bonanni, A.; Pumera, M., Strong Dependence of Fluorescence Quenching on the Transition Metal in Layered Transition Metal Dichalcogenide Nanoflakes for Nucleic Acid Detection. *Analyst* 2016, *141*, 4654-4658.

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Motivation

In recent times, the key focus of the research on transition metal dichalcogenides (TMDs) has been shifting towards the development of sensors and biosensors.¹ To date, there have been a number of studies performed on the employment of TMDs for the fabrication of biosensors. In some of these studies, the fluorescence quenching ability of the TMD materials is exploited. Different TMD materials such as single-layer tungsten disulfide (WS_2), molybdenum disulfide (MoS_2), tantalum disulfide (TaS_2) and titanium disulfide (TiS_2) nanosheets have been utilized as the fluorescence quenching platform thus far, and a fluorophore-labelled single-stranded DNA (ssDNA) acts as the biorecognition element for the detection of vital target biomolecules.²⁻⁵ These target biomolecules comprise of DNA, microRNA, thrombin and adenosine triphosphate. The principle of the sensing strategy lies in the different affinities of TMD materials towards the biorecognition element and biorecognition element-target biomolecule complex. Hence, the difference in fluorescence signal, before and after the biorecognition process, can be monitored for detection.

Nevertheless, while different TMDs have been employed for the fabrication of fluorescence biosensors with superior performance, no research has been conducted to draw comparisons across materials containing different transition metals to investigate how the transition metal present in the TMD materials can affect their potential to be utilized as a fluorescence quenching platform for the biosensing of nucleic acids. Such research is critical as it can shed light on how the performance of fluorescence biosensor can be affected by the transition metal present and aid in the selection of suitable TMD materials for the development of biosensors.

To this aim, we examine the performance of two types of TMD nanoflakes, namely MoS₂ and WS₂, for the fluorescence detection of nucleic acids in this work. It is worth highlighting that MoS₂ and WS₂ nanoflakes are chosen for comparison as the transition metals (Mo and W) belong to the same periodic group and would have similar chemical properties.

On another note, this work can also serve as a reference to assess how fluorescence detection method fares against electrochemical detection method, which will be demonstrated in Chapter 11 where MoS₂ nanoflakes will be applied as the electroactive labels.⁶

Results and Discussions

Herein, we investigate the effect of different transition metals in layered TMD nanoflakes on their potential as the fluorescence sensing platform for nucleic acid detection. To this aim, the performance of two types of TMD nanoflakes, specifically MoS₂ and WS₂, was evaluated.⁷ The general principle behind the utilization of TMD nanoflakes as a fluorescence nucleic acid sensing platform, as depicted in Figure 1, is based on the adsorption of the fluorescently labelled ssDNA probe, FAM-Lprobe, onto the surface of the TMD nanoflakes, resulting in fluorescence quenching. On the other hand, when the FAM-Lprobe underwent prior hybridization with its complementary DNA target to form double-stranded DNA (dsDNA), the extent of interactions between dsDNA and TMD nanoflakes was so low such that the dsDNA was distant from the nanoflakes, thereby leading to retention of the fluorescence from the FAM-Lprobe.

The different affinities of TMD nanoflakes towards the FAM-Lprobe and dsDNA could be elucidated by the following. The FAM-Lprobe could be readily adsorbed onto TMD nanoflakes due to van der Waals forces of attraction between the basal plane of nanoflakes and the exposed nitrogenous bases of the FAM-Lprobe, giving rise to significant fluorescence quenching. On the contrary, upon hybridization with its complementary DNA target to form dsDNA, the nitrogenous bases became effectively shielded by the negatively charged phosphodiester backbone of dsDNA, leading to weak interactions between dsDNA and TMD nanoflakes. Hence, dsDNA was far away from the nanoflakes, resulting in low fluorescence quenching.

Figure 1. Schematic representation of TMD nanoflakes as a fluorescence sensing platform for the detection of DNA.

With the aim of substantiating the adsorption mechanism illustrated in Figure 1 and to examine the effect of different transition metals in layered TMD nanoflakes on their potential of being employed as a nucleic acid fluorescence sensing platform, the FAM-Lprobe was subjected to different experimental conditions with MoS_2 and WS_2 nanoflakes accordingly.

Figure 2. Fluorescence emission spectra of FAM-Lprobe (40 nM) under various experimental conditions for (A) MoS₂ nanoflakes and (B) WS₂ nanoflakes. Black: Tris-HCl buffer solution; red: TMD nanoflakes; blue: FAM-Lprobe; pink: FAM-Lprobe + TMD nanoflakes and green: FAM-Lprobe + DNA target + TMD nanoflakes.

From Figure 2, it is observed that in the absence of TMD nanoflakes, the FAM-Lprobe exhibits intense fluorescence emission (Figure 2, blue line). However, upon the introduction of MoS₂ and WS₂ nanoflakes, about 75 % and 71 % quenching of the FAM-Lprobe fluorescence emission were resulted, respectively (Figure 2, pink line). Hence, this suggested that both TMD nanoflakes have similar quenching efficiencies and could adsorb the FAM-Lprobe to a comparable extent. In contrast, when the FAM-Lprobe undergoes prior hybridization with its complementary DNA target to form dsDNA, the fluorescence emission was significantly retained in the presence of TMD nanoflakes

(Figure 2, green line). Interesting to note, for the same amount of complementary DNA target, there was lower quenching of the fluorescence signal by WS₂ nanoflakes (22 %) than MoS₂ nanoflakes (59 %). This indicated that the extent of interactions between DNA and WS₂ nanoflakes was much weaker than that of MoS₂ nanoflakes. Therefore, this indicates that the type of transition metal in layered TMD nanoflakes has a strong influence on the strength of interactions between DNA and TMD nanoflakes, and this would in turn affect their potential for being utilized for nucleic acid detection. In addition, it is also crucial to point out that both TMD nanoflakes did not display any fluorescence emission in the region of interest, and therefore did not lead to any interference.

Moving on, since TMD nanoflakes served as the nanoquenchers in this study, it was imperative to ascertain the optimum amount of MoS₂ and WS₂ nanoflakes to be adopted for the detection assay. As such, the fluorescence emission spectra of the FAM-Lprobe upon exposure to various volumes of MoS₂ and WS₂ nanoflakes were measured and demonstrated in Figure 3.

Figure 3. Fluorescence emission spectra of FAM-Lprobe (40 nM) upon the introduction of increasing volumes of (A) MoS₂ nanoflakes and (B) WS₂ nanoflakes.

As depicted in Figure 3, it is evident that with increasing volume of TMD nanoflakes introduced, the fluorescence intensity of the FAM-Lprobe decreased, signifying an increase in the degree of quenching. Saturation in quenching was achieved at 60 μL for MoS₂ nanoflakes (Figure 3A) and 200 μL for WS₂ nanoflakes (Figure 3B). Hence, the optimum volume of MoS₂ and WS₂ nanoflakes to be employed was established to be 60 μL and 200 μL respectively.

Figure 4. Calibration curves for the detection of DNA hybridization with (A) MoS₂ nanoflakes and (B) WS₂ nanoflakes.

Next, the calibration experiment with different concentrations of the complementary DNA target was conducted to assess the dynamic range of detection for MoS₂ and WS₂ nanoflakes. As displayed in Figure 4, at the region of low complementary DNA target concentrations, there were no significant variations in the fluorescence signal. Subsequently, as the complementary DNA target approached higher concentrations, substantial enhancement in the fluorescence intensity was detected before saturation was attained. Drawing conclusions from Figure 4, it could be inferred that MoS₂ nanoflakes imparted a wider linear range of detection as compared to WS₂ nanoflakes. It was estimated that MoS₂ nanoflakes exhibited a detection range of 9.60 – 366 nM while

WS₂ nanoflakes displayed a detection range of 13.3 – 143 nM. Hence, the transition metal present in TMD nanoflakes has an impact on the detection range and could affect the sensitivity of the fluorescence sensing platform.

Figure 5. Fluorescence emission peak intensity of FAM-Lprobe (40 nM) upon incubation with complementary and non-complementary DNA targets, in the presence of (A) MoS₂ nanoflakes and (B) WS₂ nanoflakes.

Lastly, in order to assess the selectivity performance of the two types of TMD nanoflakes, a hybridization experiment was performed with complementary and non-complementary DNA targets, and the findings are demonstrated in Figure 5. For both MoS₂ and WS₂ nanoflakes, it was noted that the non-complementary DNA target gave

rise to lower fluorescence intensity than the complementary DNA target. This is because the non-complementary DNA target underwent inefficient hybridization with the FAM-Lprobe, leading to a large amount of remaining FAM-Lprobe which was subsequently adsorbed onto TMD nanoflakes and has its fluorescence quenched. On the contrary, complementary DNA target hybridized with the FAM-Lprobe effectively to yield dsDNA which has weak affinity with TMD nanoflakes and thus has its fluorescence retained. Furthermore, it is crucial to highlight that the disparity in signal between complementary and non-complementary DNA targets was more pronounced for MoS₂ nanoflakes (97.8 % difference) than for WS₂ nanoflakes (44.3 % difference). In addition, for the case of MoS₂ nanoflakes, the fluorescence of the non-complementary DNA target was more comparable to the blank control than WS₂ nanoflakes. Hence, MoS₂ nanoflakes displayed enhanced selectivity over WS₂ nanoflakes.

Conclusions

In summary, the influence of the transition metal in layered TMDs on their application as a fluorescence sensing platform for nucleic acid detection was examined by comparing the performance of MoS₂ and WS₂ nanoflakes. From the findings in this study, it was deduced that WS₂ nanoflakes have weaker interactions with nucleic acids than MoS₂ nanoflakes. This could then be related to the narrower range of detection and lower selectivity demonstrated by WS₂ nanoflakes. Specifically, it was reported that MoS₂ and WS₂ nanoflakes exhibited a detection range of 9.60 – 366 nM and 13.3 – 143 nM, respectively. In addition, MoS₂ nanoflakes showed 97.8 % disparity with negative control while WS₂ nanoflakes only exhibited 44.3 %. Hence, the transition metal in layered TMD nanoflakes has a strong effect on their fluorescence quenching efficiency. The conclusions derived from this investigation are anticipated to provide invaluable insights on the selection of TMD materials for the future fabrication of biosensors.

Experimental Section

Materials

Molybdenum disulfide (MoS_2) and tungsten disulfide (WS_2) nanoflakes solution was purchased from Graphene Laboratories Inc. (Calverton, NY, USA).

Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) was purchased from Quality Reagent Chemical (Auckland, New Zealand).

Tris(hydroxymethyl)aminomethane ($\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$), potassium chloride (KCl), sodium chloride (NaCl), hydrochloric acid (HCl) (conc. 37 %) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA oligonucleotide sequences, relating to Alzheimer's disease, are as follow:

FAM-Lprobe: 5' [6-FAM (6-Carboxyfluorescein)] ACC AGG CGG CCG CAC ACG TCC TCC AT 3'

DNA target (complementary): 5' ATG GAG GAC GTG TGC GGC CGC CTG GT 3'

Negative control DNA (non-complementary): 5' AAA AAA AAA AAA AAA AAA AAA AAA AA 3'

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) with a resistivity of 18.2 $\text{M}\Omega \text{ cm}$.

The following buffer was used in this study:

20 mM Tris-HCl (pH 7.4, 100 mM NaCl + 5 mM KCl + 15 mM MgCl_2)

Equipment

Fluorescence measurements were performed at room temperature on a Varian Cary Eclipse fluorescence spectrophotometer. The excitation wavelength was fixed at 490 nm, with the emission spectra recorded over the range of 500 to 800 nm.

Procedures

For fluorescence quenching, the working solution of the fluorescent DNA oligonucleotide (FAM-Lprobe) was prepared by diluting the stock solution to about 40 nM with Tris-HCl buffer solution. An aliquot at the optimum volume of TMD nanoflakes was then added to the Tris-HCl buffer solution containing FAM-Lprobe, and allowed to incubate for 20 minutes at room temperature.

For the detection of DNA hybridization, DNA target was first added to 40 nM of FAM-Lprobe with incubation at 50 °C for 30 minutes. After the incubation, TMD nanoflakes at the optimum volume was introduced and left for 20 minutes at room temperature.⁶ After which, fluorescence measurement of the mixture was conducted. The final concentration of the DNA target in the mixture ranged from 0.004 nM to 1000 nM.

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Chapter 11

Molybdenum Disulfide as Electroactive Labels for
DNA Detection

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The results in this chapter resulted in the publication of the following:

1. **Loo, A. H.**; Bonanni, A.; Ambrosi, A.; Pumera, M., Molybdenum Disulfide (MoS₂) Nanoflakes as Inherently Electroactive Labels for DNA Hybridization Detection. *Nanoscale* 2014, 6, 11971-11975.

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Motivation

As highlighted in the previous chapters, research on transition metal dichalcogenides (TMDs) is experiencing a surge of interest in recent years.^{1, 2} As one of the more studied TMDs, molybdenum disulfide (MoS_2) nanosheet has garnered considerable attention from researchers due to its large surface specific area and its excellent optical, mechanical, and electronic properties.³⁻⁸ From a structural point of view, MoS_2 nanosheet is constituted of three atomic layers: one Mo metal layer sandwiched between two S layers by covalent bonds. Such three-layer stacks are then held together by weak van der Waals interactions.⁹ As a result of this layered structure, MoS_2 presents different interesting electronic properties at the edge and basal planes. Furthermore, significant electrochemical and catalytic differences can be found between the basal plane portion of the material and its edges.¹⁰ Such observations can be explained by the enhancement of planar electric transportation properties,¹¹ as assisted by the two dimensional electron-electron correlations among Mo atoms. In view of these exceptional features, MoS_2 nanosheet has been employed in various areas such as sensors,^{12, 13} fuel cells¹⁰ and transistors¹⁴ thus far. In particular, the application of MoS_2 nanosheet in sensing and biosensing devices represents an upcoming research direction.

Till date, a number of studies have been performed on the utilization of MoS_2 nanosheet in the research field of sensing and biosensing. In most of these studies, MoS_2 nanosheet is either involved as a fluorescence quencher^{15, 16} or as the electrochemical transducing platform. For the latter, MoS_2 nanosheet can either perform the function by itself,^{13, 17} or in the form of a composite with other components such as nanoparticles^{18, 19} and polymers.^{20, 21} Herein, a novel approach of employing MoS_2 in the development of DNA biosensors is proposed. The detection of specific DNA sequences plays a critical role in

the areas of medical diagnostics, environmental monitoring, drug discovery and food safety. This has therefore become a strong driving force behind the ever-increasing demand for simple, cost-effective, highly sensitive and selective DNA biosensors. In this work, MoS₂, in the form of nanoflakes, is utilized as an electroactive label for the voltammetric detection of DNA hybridization, correlating to the diagnosis of Alzheimer's disease. The rationale behind the proposed approach lies in the inherent electroactivity of MoS₂ nanoflakes, originating from the oxidation of the nanoflakes, as well as the differential affinity of the nanoflakes towards single and double-stranded DNA.^{15, 16} To monitor the inherent oxidation peak of MoS₂ nanoflakes, differential pulse voltammetry (DPV) was chosen as the analytical technique.

Results and Discussions

Herein, we investigated the interactions between MoS₂ nanoflakes and DNA strands for the detection of DNA hybridization, correlating to the diagnosis of Alzheimer's disease. It was previously demonstrated that MoS₂ nanoflakes have a different degree of interactions with single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA).¹⁵ Hence, by utilizing the different degree of interactions which MoS₂ nanoflakes exhibit with DNA strands, as well as the inherent electrochemical behaviour of MoS₂ nanoflakes, we propose a sensing strategy for DNA hybridization, with MoS₂ nanoflakes functioning as electroactive labels.

Figure 1. Inherent electrochemical behaviour of MoS₂ nanoflakes. Cyclic voltammograms depicting the electrochemical behaviour of MoS₂ nanoflakes in 0.1 M PBS buffer solution (pH 7.0) and scan rate of 0.1 Vs⁻¹ under ambient conditions.

The electrochemical behaviour of MoS₂ nanoflakes was investigated by performing cyclic voltammetry (CV) between 2.0 to - 2.0 V. As demonstrated in Figure 1, a series of oxidation waves were observed in the first scan only. This suggested that the oxidation of MoS₂ nanoflakes was chemically irreversible. Subsequently, the oxidation wave at

approximately 0.62 V was employed as the analytical signal for the aim of DNA sensing, as oxidation waves at other potentials faced interferences. In addition, DPV technique was then adopted over CV due to its improved sensitivity.

Figure 2 illustrates the sensing strategy employed in this study. First of all, ssDNA probe was immobilized onto the surface of bare disposable electrical printed (DEP) carbon chip via physical adsorption. Upon the addition of complementary DNA target (Path B), the ssDNA probe hybridized with the complementary DNA target to form a dsDNA duplex. Subsequently, the modified DEP chips (Path A and B) were exposed to MoS₂ nanoflakes for conjugation. As demonstrated in Figure 2, MoS₂ nanoflakes conjugated to the modified DEP chips surfaces to different extent depending on the presence of complementary DNA target. As such, a different amount of MoS₂ nanoflakes was affixed onto the modified DEP chips surfaces and voltammetric signals of varying intensities were attained.

In detail, for the case of Path A, MoS₂ nanoflakes could effectively adsorb onto the immobilized ssDNA probe via van der Waals forces of attractions between the nucleobases and the basal plane of MoS₂ nanoflakes. In contrast, when the ssDNA probe underwent hybridization with its complementary DNA target (Path B), the nucleobases involved in the formation of the double helix became buried deep within the densely negatively charged helical phosphate backbone, and the degree of interactions between the resulting dsDNA duplex and MoS₂ nanoflakes was severely reduced. Hence, ssDNA has a higher affinity with MoS₂ nanoflakes as compared to dsDNA and a significantly higher amount of MoS₂ nanoflakes was conjugated to the ssDNA probe modified DEP chip surface in relative to dsDNA duplex modified DEP chip surface and thus, a greater

voltammetric signal, stemming from the oxidation of MoS₂ nanoflakes, was achieved for the former case.

Figure 2. Schematic illustration of the experimental approach adopted.

In order to validate that MoS₂ nanoflakes indeed have a different degree of interactions with ssDNA and dsDNA, DNA hybridization experiments were conducted with three different DNA sequences, complementary, single-base mismatch and non-complementary, with the attained results displayed in Figure 3.

Specifically, when the hybridization experiment was conducted using the complementary sequence, a comparatively lowest oxidation voltammetric signal was produced (black line). For the instance of single-base mismatch sequence, a higher voltammetric signal was achieved (red line) and finally for the instance of non-complementary sequence, the highest voltammetric signal was obtained (blue line). This signifies that the smallest amount of MoS₂ nanoflakes was conjugated to the modified DEP chip surface when hybridization was performed with complementary sequence, followed by single-base mismatch sequence and lastly non-complementary sequence.

Figure 3. Differential pulse voltammograms of the MoS₂ nanoflakes oxidation peak when the hybridization step was performed with complementary sequence (black), single-base mismatch sequence (red) and non-complementary sequence (blue). The concentration of DNA probe, DNA targets and MoS₂ nanoflakes employed was 10 μM, 300 nM and 0.018 mgmL⁻¹ respectively. All electrochemical measurements were conducted in 0.1 M PBS buffer solution (pH 7.0) under ambient conditions.

The observed trend could be attributed to the different degrees of hybridization efficiency demonstrated by the three DNA sequences. Explicitly, the complementary sequence demonstrated the highest hybridization efficiency and the modified DEP chip surface consisted mainly of dsDNA strands upon the hybridization step. In contrast, for the case of non-complementary sequence, the modified DEP chip surface consisted primarily of non-hybridized ssDNA probe strands due to its poor hybridization efficiency. As described earlier, ssDNA possessed higher affinity with MoS₂ nanoflakes as compared to dsDNA, hence, the highest amount of MoS₂ nanoflakes would be affixed onto modified DEP chip which had undergone incubation with non-complementary sequence. Conversely, for the instance of complementary sequence, the amount of conjugated MoS₂ nanoflakes would be the lowest. Therefore, the largest voltammetric signal was displayed after incubation with non-complementary sequence. In the case of single-base

mismatch sequence, its voltammetric signal was greater than the complementary sequence due to the occurrence of partial hybridization. Hence, through these control experiments, it was validated that MoS₂ nanoflakes have a different extent of interactions with ssDNA and dsDNA, with ssDNA having a higher affinity for the nanoflakes than dsDNA, and the intensity of the resulting voltammetric signal reflected the amount of MoS₂ nanoflakes which were conjugated to the modified DEP chips surfaces.

To determine the optimum concentration of DNA probe to be deposited onto the DEP chip surface in order to achieve maximum surface coverage and to reduce the effects of non-specific binding subsequently, probe optimization experiments were performed using electrochemical impedance spectroscopy (EIS).²²⁻²⁴

A series of DNA probe concentrations were employed for the optimization study and their impedimetric responses were recorded and represented as histograms in Figure 4. As depicted in Figure 4, it could be observed that the intensity of impedimetric signal increased from 0.001 μ M to 10 μ M before experiencing a slight decrease at 20 μ M. The impedimetric signal arose from the charge transfer resistance (R_{ct}) between the Fe(CN)₆^{3-/4-} redox probe and the DEP chip surface. With the immobilization of DNA probe onto the surface of the DEP chip, enhancement of the R_{ct} was expected. This is because DNA probe contains negatively charged phosphate backbone which will in turn lead to repulsion with the similarly negatively charged redox probe, and further hinder charge transfer, resulting in the R_{ct} to increase and thus a larger impedimetric signal. Therefore, with increasing concentration of DNA probe being immobilized onto the DEP chip surface, there would be greater repulsion of the redox probe and the magnitude of impedimetric signal would increase. As such, at 10 μ M of DNA probe which produced

the greatest impedimetric signal, it has the highest amount of DNA probe immobilized on the DEP chip surface and it was thereby capable of providing the maximum coverage of DEP chip surface. Hence, the optimum concentration of DNA probe to be deposited onto the DEP chip surface was established to be 10 μM .

Figure 4. Impedimetric responses towards different concentrations of DNA probe deposited on the surface of bare DEP chips. Signal was displayed as $R_{\text{ct}} (\text{Probe} - \text{Blank})$. Error bars corresponded to the standard deviations obtained from triplicate experiments. All electrochemical measurements were performed with 10 mM $\text{K}_4\text{Fe}(\text{CN})_6/\text{K}_3\text{Fe}(\text{CN})_6$ in 0.1 M PBS buffer solution (pH 7.0) under ambient conditions.

Since the voltammetric signal stemming from the oxidation of MoS_2 nanoflakes was utilized as the analytical signal in this report, it was hence crucial to determine the proper concentration of MoS_2 nanoflakes to be employed for the final labelling step.

To do so, a calibration with various concentrations of MoS_2 nanoflakes, obtained by serial dilution from the stock solution, was carried out. The histograms illustrated in Figure 5 represent the voltammetric peak height of MoS_2 nanoflakes oxidation in relation to the different concentrations of MoS_2 nanoflakes. For each individual concentration, DNA hybridization was performed with the complementary (yellow),

single-base mismatch (light blue) and non-complementary (pink) sequences. From Figure 5, it was noticed that the voltammetric peak height was the greatest at 0.018 mgmL^{-1} . In addition, at 0.018 mgmL^{-1} , there was also maximum discrimination among the three different sequences. Henceforth, this concentration of MoS_2 nanoflakes was used for subsequent experimental studies.

Figure 5. Histograms representing the voltammetric responses towards different concentrations of MoS_2 nanoflakes employed. Error bars corresponded to the standard deviations obtained from triplicate experiments. The concentration of DNA probe and DNA targets used was $10 \text{ } \mu\text{M}$ and 300 nM respectively. All electrochemical measurements were conducted in 0.1 M PBS buffer solution ($\text{pH } 7.0$) under ambient conditions.

The voltammetric response of the proposed biosensor towards various concentrations of the complementary DNA sequence was next assessed with the aim of defining the range of detection. As displayed in Figure 6, the voltammetric peak height decreased with increasing concentration of complementary DNA sequence for the range of 0.03 nM to 300 nM with good linearity. Furthermore, it should also be highlighted that the percentage relative standard deviation (% RSD) values exhibited for the calibration plot were lower than 10% , suggesting superior reproducibility. Therefore, the range of

detection was determined to be from 0.03 nM to 300 nM, which was of four orders of magnitude.

Figure 6. Differential pulse voltammograms of MoS₂ nanoflakes oxidation peak when DNA hybridization was conducted with 0.03 (black), 0.3 (red), 3 (blue), 30 (pink) and 300 (green) nM of complementary DNA sequence. The inset demonstrated the calibration curve of the voltammetric response to the concentration of complementary DNA sequence. Error bars corresponded to the standard deviations obtained from triplicate experiments. The concentration of DNA probe and MoS₂ nanoflakes utilized was 10 μM and 0.018 mgmL^{-1} respectively. All electrochemical measurements were conducted in 0.1 M PBS buffer solution (pH 7.0) under ambient conditions.

Conclusions

In summary, we have shown that the differential affinity of MoS₂ nanoflakes towards ssDNA and dsDNA, coupled with its inherent electrochemical behaviour, could be exploited for the detection of DNA hybridization, with the nanoflakes playing the role of an electroactive label. The electrochemical behaviour of MoS₂ nanoflakes, specifically the oxidation peak, was utilized as the analytical signal in the proposed biosensing assay. Based on the proposed biosensing assay, the optimum concentration of DNA probe to be immobilized onto the DEP chip surface was determined to be 10 μM while the optimum concentration of MoS₂ nanoflakes to be employed as electroactive labels was verified to be 0.018 mg mL^{-1} . In addition, the range of detection was established to be from 0.03 nM to 300 nM, which is of four orders of magnitude.

A novel approach for the utilization of MoS₂ in the field of biosensing was demonstrated in this study, whereby MoS₂ nanoflakes were employed as electroactive labels. The use of TMDs such as MoS₂ for sensing and biosensing purposes is a recent research field which holds great potential. Hence, the findings presented in this report are expected to have an immense influence on future works.

Experimental Section

Materials

Molybdenum disulfide (MoS_2) nanoflakes solution was purchased from Graphene Laboratories Inc. (Calverton, New York), with lateral size ranging from 100 to 400 nm and thickness of 1 to 8 layers.

Hydrochloric acid (HCl) (conc. 37%), sodium phosphate dibasic salt (Na_2HPO_4), sodium chloride (NaCl), potassium hexacyanoferrate (II) trihydrate ($\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$), potassium hexacyanoferrate (III) ($\text{K}_3\text{Fe}(\text{CN})_6$) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA sequences are as follow:

DNA probe: 5' ACC AGG CGG CCG CAC ACG TCC TCC AT 3'

Complementary target: 5' ATG GAG GAC GTG TGC GGC CGC CTG GT 3'

Single-base mismatch target: 5' ATG GAG GAC GTG CGC GGC CGC CTG GT 3'

Non-complementary target: 5' AAA AAA AAA AAA AAA AAA AAA AAA AA 3'

Trisodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) was purchased from Alfa Aesar (Singapore).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of resistivity of 18.2 $\text{M}\Omega \text{ cm}$.

The following buffers were used in this study:

0.1 M PBS (0.1 M NaCl + 10 mM Na_2HPO_4 , pH 7.0)

TSC 1 (0.75 M NaCl + 75 mM trisodium citrate, pH 7.0)

TSC 2 (0.30 M NaCl + 30 mM trisodium citrate, pH 7.0)

Disposable electrical printed (DEP) carbon chips were obtained from BioDevice Technology (Nomi, Japan). A three-electrode system was employed in this study which included carbon-based working and counter electrodes, and a Ag/AgCl reference electrode.

Equipment

All electrochemical measurements were performed with a μ Autolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer.

Differential pulse voltammetry (DPV) measurements were controlled by General Purpose Electrochemical System (GPES) software version 4.9 and the parameters applied were: 50 ms modulation time, 0.5 s interval time, 10 mV step potential, 50 mV modulation amplitude and 20 mVs⁻¹ scan rate. DPV measurements were conducted in 0.1 M PBS buffer solution (pH 7.0) and the raw data produced were treated with a baseline correction of peak width 0.01 using the GPES software.

Impedance measurements were controlled by NOVA software version 1.9 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The measurements were performed with 10 mM K₄Fe(CN)₆/K₃Fe(CN)₆ (1:1 molar ratio) in 0.1 M PBS buffer solution (pH 7.0) as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane.

All electrochemical measurements were performed under ambient conditions by utilizing DEP carbon chips unless otherwise stated.

Procedures

DNA probe was immobilized on the surface of DEP carbon chip by dry physical adsorption. A 3 μL aliquot of DNA probe solution in PBS buffer at the optimum concentration of 10 μM was deposited onto the DEP chip surface and left in the oven for 10 minutes at 60 $^{\circ}\text{C}$ for drying. After which, the DNA probe modified DEP chip was washed twice in PBS buffer solution with gentle stirring at 25 $^{\circ}\text{C}$ in order to remove the excess DNA probe which was not well adsorbed.

The DNA probe modified DEP chip then underwent incubation with hybridization solution (TSC 1 buffer) containing desired concentrations of DNA targets, with the total volume fixed at 100 μL . The incubation step was performed at 42 $^{\circ}\text{C}$ for 30 minutes with gentle stirring. Subsequently, two washing steps were conducted in TSC 2 buffer.

Conjugation with MoS_2 nanoflakes was next carried out by incubating the modified DEP chip with 100 μL of MoS_2 nanoflakes solution at the desired concentration in PBS buffer. The incubation was performed for 20 minutes at 25 $^{\circ}\text{C}$ with gentle stirring. After which, two washing steps in PBS buffer was conducted.

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Section C

Carbon Quantum Dots

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Chapter 12

Introduction to Carbon Quantum Dots

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Moving away from two-dimensional layered materials, we explore carbon quantum dots (CQDs) which are zero-dimensional. Together with graphene quantum dots, CQDs belong to a new class of carbon nanomaterials with sizes below 10 nm.¹ CQDs can be simply described as quasi-spherical nanoparticles which contain amorphous to nanocrystalline cores with predominantly graphitic or sp^2 carbon or graphene and graphene oxide sheets fused by sp^3 hybridized carbon insertions (Figure 1).²⁻⁵ CQDs were first discovered by chance in 2004 during the purification and separation of single-walled carbon nanotubes.⁶ Since the discovery, CQDs have gradually become a rising star as a new member in the family of carbon nanomaterials owing to their fascinating properties.

Figure 1. Chemical structure of a CQD. (Reproduced from reference 2)

Traditionally, research in the field of quantum dots has been dominated by semiconductor quantum dots which have been extensively studied for their strong and tunable fluorescence emission properties. These studies have enabled their applications in bioimaging and biosensing. However, semiconductor quantum dots exhibit certain limitations such as high toxicity due to the usage of heavy metals in their synthesis.⁷⁻⁹ It is commonly known that heavy metals are extremely toxic even at relatively low levels.^{10, 11} This has therefore prompted the search of alternative nanomaterials and the

discovery of CQDs in 2004 came as a timely solution to replace semiconductor quantum dots. Apart from displaying similar fluorescence properties, CQDs also demonstrate low toxicity, high resistance to photobleaching, good solubility and biocompatibility, facile modification, low cost, robust chemical inertness, and outstanding electronic properties.^{1, 12}

However, despite of the favourable position which CQDs are in for attaining unprecedented performance, CQDs have its share of challenges to overcome. Some of these obstacles include their generally low quantum yields, complex procedures for their separation, purification and functionalization, and ambiguity in their geometry, structure and composition. The above issues have to be tackled before CQDs can truly replace their semiconductor quantum dots counterparts.¹²

To attain CQDs, there are several synthetic routes available. These synthetic approaches can be generally grouped into two categories – top-down and bottom-up. For top-down synthetic approach, it involves the breaking down of larger carbon structures, such as graphite,¹³ graphite oxide,¹⁴ carbon soot,¹⁵ carbon nanotubes¹⁶ and activated carbon¹⁷ via methods like laser ablation, arc discharge and electrochemical oxidation. An example of synthesizing CQDs by laser ablation was first reported by Sun and co-workers.¹³ In the work, CQDs were produced by laser ablation of a carbon material in the presence of water vapour with argon as the carrier gas. The starting carbon material was formed from a mixture of graphite powder and cement by hot-pressing and followed with stepwise baking, curing, and annealing in argon atmosphere. After laser ablation of the carbon material under controlled temperature and pressure, nanoscale carbon particles in aggregates of different sizes were obtained. On another note, the employment of electrochemical oxidation as the synthesis route was revealed by Ding's and Kang's

groups.^{16, 18} In Ding's study, multi-walled carbon nanotubes (MWCNT) were used as the starting material and they were first grown on a carbon paper by chemical vapour deposition. Thereafter, the MWCNT-modified carbon paper was introduced into an electrochemical cell which contained degassed acetonitrile solution with tetrabutylammonium perchlorate as the supporting electrolyte. Potential cycling was then conducted on the MWCNT-modified carbon paper working electrode with cyclic voltammetry to acquire CQDs. With the aim of increasing the yield of CQDs and to lower the production cost, Kang and colleagues proposed a single-step electrochemical method to prepare CQDs, utilizing cheap and abundant graphite rod as the carbon source. To attain CQDs, two graphite rods were simply inserted into ultrapure water and static potentials were applied to the system with a direct current power supply. Lastly, for the case of arc discharge, it was demonstrated in the study by Scrivens and co-workers.⁶ In their work, CQDs were an accidental discovery in their attempt to purify single-walled carbon nanotubes (SWCNT) derived from arc-discharged soot. The arc-discharged soot was first oxidized with nitric acid, before undergoing extraction with sodium hydroxide to yield a black suspension which contained SWCNT and CQDs. Subsequently, gel electrophoresis was performed to separate out the CQDs.

For the context of bottom-up approach, it involves the synthesis of CQDs from molecular precursors such as carbohydrates^{19, 20} and citrates,²¹ through thermal treatments and microwave irradiation. For instance, Giannelis's group described a straightforward, single-step thermal decomposition method of producing CQDs from ammonium citrate.²¹ In addition, in a study conducted by Yang and co-workers,²² it showed that CQDs were readily synthesized by heating a solution of poly(ethylene glycol) and saccharide in a microwave oven for a short time. In recent years however, green

synthetic routes were introduced and with these methods, CQDs could be formed in a single step without the use of expensive materials and complex experimental conditions. One of the green routes involved a one-step electrochemical treatment of ethanol with sodium hydroxide.²³ Apart from ethanol, other inexpensive and biocompatible starting materials such as ascorbic acid,^{24, 25} chitosan,^{26, 27} gelatine,²⁸ fruit peels^{29, 30} and juices³¹⁻³³ have also been examined.

Figure 2. Schematic representation of CQDs synthesis by top-down and bottom-up approaches. Modification of CQDs can be achieved by functionalization and doping. (Reproduced from reference 34)

Following the synthesis of CQDs, surface modifications of the CQDs can also be achieved to modulate the properties. As seen from Figure 2, the modification process can be performed either during or after the preparation of CQDs and it includes functionalization and doping. For functionalization of the surface of CQDs, it can be accomplished through surface chemistry or interactions like coordination,³⁵ covalent bonding,^{26, 36-38} and π - π interactions.³⁹ Covalent bonding is a highly feasible approach owing to the majority of CQDs being rich in oxygen-containing groups.¹ An example of surface functionalization via covalent bonding was demonstrated in Chen's work⁴⁰

whereby they modified the surface of CQDs with spiropyrans through the formation of amide linkages, in order to tune the photoluminescence properties. On the other hand, for doping of CQDs, various methods and elements such as nitrogen,^{26, 41-46} sulphur,^{47, 48} and phosphorous⁴⁹ may be adopted.

As a newly emerged carbon nanomaterial, CQDs have shown outstanding promise as a versatile nanomaterial, suitable for a broad range of applications, such as bioimaging, nanomedicine, electrocatalysis and photocatalysis.

CQDs have immense potential to be employed for bioimaging due to their good chemical and photochemical stability. Most importantly, since carbon is mainly non-toxic and environmentally friendly, CQDs can be readily used to visualise biological systems in vitro and in vivo.³³ Generally, as the carbon cores of CQDs are non-toxic, any cytotoxicity behaviour exhibited by the CQDs can be largely attributed to the chemical species present on their surface.⁵⁰ Moreover, it has been revealed that CQDs containing surface functionalities of low cytotoxicity can be utilized safely at high concentrations for in vivo bioimaging.¹² For instance, CQDs functionalized with poly(ethylene glycol) displayed no observable toxic effects for 28 days in mice. In addition, the physiological indicators for mice exposed to various dosages of CQDs and control were at similar levels, suggesting the non-toxic nature of CQDs. It is also worth highlighting that for CQDs modified with chemical species of high cytotoxicity, they can still be utilized for in vivo bioimaging applications as long as the chemical species are fixed at low concentrations and the incubation time is kept short.¹² Another major advantage of having CQDs for bioimaging, is their ability to display multi-colour emissions, and such characteristic sets them apart from most of the other labelling agents. Owing to the above, researchers have the flexibility to control and select the excitation and emission wavelengths.⁵¹ Such property

of tunable emissions is evident from the studies conducted by Chang's and Liu's groups, where light at different wavelengths was emitted upon excitation at different wavelengths.^{52, 53}

Similar to bioimaging, CQDs are employed for nanomedicine applications due to their high biocompatibility and low toxicity.^{52, 54} For the context of nanomedicine, it can be divided into two parts, photodynamic therapy and drug delivery system. Photodynamic therapy is a clinical treatment which targets primarily superficial tumours.⁵⁵ The treatment involves localization and accumulation of photosensitizers in the tumour tissue, before being subjected to irradiation, resulting in the formation of singlet oxygen species that lead to cell death. It has been validated that CQDs have high inhibition effects on two types of cancer cells⁵² due to their ability to generate more reactive oxygen species, making them effective photosensitizers. In addition, the circulation and uptake of CQDs in the body were discovered to be dependent on the route of administration and surface coating.⁵⁶ On another note, for the case of drug delivery system, CQDs make an excellent choice as a result of the wide array of modifications that can be performed on their surface. This in turn enables many possibilities for conjugation with drug molecules, therefore expanding the drug choices for delivery.⁵⁷ In a study conducted by Sun and colleagues, they loaded an anti-cancer pro-drug onto the surface of CQDs via chemical coupling.³⁷ The modified CQDs were then taken up by the cancer cells through endocytosis and the drug was released from CQDs upon the reduction of the pro-drug, as catalysed by the highly reducing environment in cancer cells. Moreover, by monitoring the fluorescence signal of CQDs, the distribution of the modified CQDs can be closely traced.

Apart from biological applications, CQDs can also function as electrocatalysts which are commonly used to improve the sluggish kinetics of oxygen reduction reaction (ORR).⁵⁸

The promising potential of CQDs as electrocatalysts is largely credited to their ultra-small sizes, high stability and superior electrical conductivity.¹² Most importantly, the production cost of CQDs is also much lower as compared to the current platinum-based electrocatalysts. One of the earlier reports on the usage of CQDs as electrocatalysts for ORR demonstrated that nitrogen-doped CQDs, which are rich in oxygen-containing functional groups on the surface, are electrocatalytically active towards electrochemical reduction of oxygen. Interestingly, the onset potential of ORR displayed by the CQDs were determined to be -0.16 V (vs. Ag/AgCl), close to that of commercial platinum-based electrocatalysts.⁵⁹ Subsequently, similar findings were also reported by two other groups which utilized different procedures to prepare their CQDs.^{60, 61}

Recently, photocatalytic processes are gaining fast momentum as green alternatives to organic synthesis due to the realisation that sunlight is an inexhaustible energy source.⁶²⁻

⁶⁵ However, the intense energy of ultra-violet and short wavelength visible light can cause organic compounds to be severely damaged.⁶² In view of their ability to harness long wavelength light and perform energy exchange, CQDs show great potential to be used as photocatalysts in organic synthesis. To that aim, a recent investigation has proven that small CQDs (1 – 4 nm) are superior photocatalysts for selective oxidation of alcohols to benzaldehydes with high conversion efficiency (92 %) and selectivity (100 %), owing to their excellent catalytic activity for hydrogen peroxide decomposition and light-driven electron transfer property.⁶⁶ Furthermore, additional studies suggested that the photocatalytic activity of CQDs can be effectively regulated by tailoring the surface functional groups^{67, 68} and doping.⁶⁹ For larger CQDs (5 – 10 nm), they displayed light-

driven proton properties in solution and can be exploited as acid catalysts to initiate a series of organic transformations under visible light.⁷⁰

In the following chapter, the application of CQDs for the detection of nucleic acids will be addressed in detail.

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Chapter 13

Carboxylic Carbon Quantum Dots for DNA Detection
with Fluorescence Spectroscopy

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The results in this chapter resulted in the publication of the following:

1. **Loo, A. H.**; Sofer, Z.; Bousa, D.; Ulbrich, P.; Bonanni, A.; Pumera, M., Carboxylic Carbon Quantum Dots as a Fluorescence Sensing Platform for DNA Detection. *ACS Appl. Mater. Interfaces* 2016, 8, 1951-1957.

Motivation

Carbon quantum dots (CQDs) belonging to a new class of carbon nanomaterials with sizes below 10 nm, are gradually becoming a rising star in the research of nanomaterials.^{1, 2} The rationale behind the much acquired interest can be attributed to the superior and unique properties which CQDs present. Till date, many studies on their synthesis, properties and potential applications have been reported, and some of the reported potential applications include bioimaging^{3, 4} and biomedicine delivery systems,^{5, 6} solar cells⁷ and supercapacitors,⁸ and catalysts.⁹ However, CQDs have yet to be examined for biosensing applications, specifically for the aim of nucleic acids detection.

The intensive development of sensitive, selective, cost-effective and rapid systems for the detection of nucleic acids is imperative due to their widespread applications in various fields such as clinical disease diagnostics and treatments, genetic profiling, food safety and forensic studies.¹⁰⁻¹² In recent years, considerable effort has been channelled into fabricating fluorescence biosensors for nucleic acids detection owing to the inherent advantages which they exhibit. These advantages include ease of operation, high sensitivity and in-situ imaging properties.^{13, 14}

Interesting to note, biosensors belonging to this category are often based on fluorescence resonance energy transfer or quenching mechanisms for detection,¹⁵⁻¹⁷ and they usually comprise of a fluorophore and a quencher molecule attached to the opposite ends of a DNA probe molecule. In the absence of the target molecule, the fluorophore and quencher molecules are in close proximity and fluorescence is suppressed. Upon the occurrence of hybridization with the target molecule, the

quencher molecule becomes physically separated from the fluorophore molecule and fluorescence is released. However, despite the extensive utilization of fluorescence sensors in a wide array of applications,¹⁸⁻²⁰ biosensors of such design still suffer from one major shortcoming, which is they require careful selection of fluorophore-quencher pair in order to ensure optimum efficiency.¹⁶

To circumvent such drawback, nanomaterials have been employed as quenchers in recent years as they are capable of quenching various fluorophores of different emission wavelengths and improving the signal-to-noise ratio.^{11, 21} Till date, gold nanoparticles,^{22, 23} transition metal dichalcogenides,²⁴ and metal-organic frameworks²⁵ have been adopted as efficient nanoquenchers to develop fluorescence biosensors. Nevertheless, although these nanomaterials have been successfully implemented as nanoquenchers for the detection of nucleic acids, the preparation of some of these nanomaterials is often sophisticated and strict, with difficulty in ensuring uniform size dispersion. In addition, some of these nanomaterials also display cytotoxicity behaviours.²⁶⁻²⁸ Therefore, the search for new nanomaterials as replacements is ever important.

Carbon nanoparticles represent one of the potential replacement materials, and some works on their application for the detection of nucleic acids by fluorescence spectroscopy have been conducted thus far.^{10, 17, 29} However, despite of their similarities to CQDs, these nanoparticles are larger than CQDs and more challenging to synthesize.¹⁷

In view of the above, it is prudent to investigate the potential of CQDs as a fluorescence sensing platform for nucleic acids detection. To that end, we will demonstrate the proof-of-concept on employing carboxylic CQDs (cCQDs), functioning as a nanoquencher, as a fluorescence sensing platform for nucleic acid detection in this chapter. In addition, the

performance of two variations of cQDs, derived from citric acid and malic acid precursors specifically, will also be assessed.³⁰

Results and Discussions

The general principle behind the proposed fluorescence nucleic acid sensing platform, as illustrated in Figure 1, is based on the adsorption of a fluorescently labelled single-stranded DNA (ssDNA) probe, FAM-Lprobe, onto the cCQDs, leading to fluorescence quenching. In contrast, when FAM-Lprobe undergoes prior hybridization with its complementary DNA target to form double-stranded DNA (dsDNA), the interactions between dsDNA and cCQDs are so weak that the dsDNA is away from the surface of cCQDs, leading to retention of the fluorescence of FAM-Lprobe.

Figure 1. Schematic illustration of the cCQDs based fluorescence detection of DNA.

Interesting to note, the interactions between DNA and cCQDs represent a constant competition of electrostatic repulsion and hydrophobic π - π interactions. The hydrophobic π - π interactions originate from the π - π stackings between DNA

nucleobases and the hydrophobic regions of the cCQDs surfaces, while electrostatic repulsion arises from the repulsion between the carboxylic groups of cCQDs and the negative phosphodiester backbone of DNA. Therefore, the difference in electrostatic/hydrophobic properties of the FAM-Lprobe and dsDNA will result in a different propensity to adsorb onto the surface of cCQDs. Theoretically, such effect will in turn impart sensitivity and selectivity to the detection assay.²⁹ Under the conditions that the hydrophobic interactions are greater than the electrostatic repulsion, the DNA will be adsorbed onto the cCQDs.

To understand the properties of the synthesized cCQDs, material characterizations were performed with X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FT-IR), atomic force microscopy (AFM) and transmission electron microscopy (TEM). In addition, the optical properties were also investigated by photoluminescence measurement.

XPS, being a very sensitive and semi-quantitative analysis method for probing the surface of a material, is capable of providing surface elemental composition and bonding arrangements of the synthesized cCQDs. Hence, survey scans from a binding energy of 1200 to 0 eV, were recorded for both citric acid QDs and malic acid QDs in order to find out their overall elemental composition. As illustrated in Figure 2, carbon-to-oxygen (C/O) ratios of 2.12 and 1.39 for citric acid QDs and malic acid QDs respectively were obtained. This suggested that citric acid QDs had undergone a greater degree of carbonization as compared to malic acid QDs. Subsequently, high resolution scans were performed and it was uncovered that citric acid QDs produced C=C, C-C, C-O, C=O and O-C=O peaks of intensity 13.0, 42.5, 16.9, 12.8 and 14.9 respectively while malic acid QDs yielded 3.49, 34.0, 27.2, 8.19 and 27.2 comparatively. These values highlighted the

presence of carboxylic moieties and hydrophobic regions on the surface of the synthesized cCQDs. Specifically, malic acid QDs were deduced to contain more carboxylic moieties than citric acid QDs while citric acid QDs have more hydrophobic regions as compared to malic acid QDs.

Figure 2. XPS spectra for survey and high resolution C 1s core-level scans of (A) citric acid QDs and (B) malic acid QDs.

Figure 3. FT-IR spectra on malic acid QDs and citric acid QDs.

From the FT-IR spectra in Figure 3, it could be observed that both cCQDs demonstrated peaks corresponding to the C=O (1720 cm^{-1}) and –OH (3380 cm^{-1}) stretching modes of the –COOH groups, and the C=C (1598 cm^{-1}) graphitic stretching mode. Hence, the presence of hydrophobic regions and carboxylic groups on the surfaces of both cCQDs was further established.

Figure 4. TEM images of (A) malic acid QDs and (B) citric acid QDs.

Figure 5. AFM measurements of (A) citric acid QDs and (B) malic acid QDs.

To gain insights on the morphology of cCQDs, TEM and AFM were utilized with the results demonstrated in Figures 4 and 5. In both cases, intensive aggregation of cCQDs was observed after drying for measurement. From the TEM images in Figure 4, the round shape of cCQDs with typical dimensions in the range of 10 – 20 nm could be seen. Moreover, the round shaped cCQDs have a platelet profile which could be spotted on the images of aggregated cCQDs by AFM (Figure 5). The thickness of these platelets was determined to be in the range of 5 – 10 nm.

Moving on, the optical properties of the synthesized cCQDs were subsequently assessed with photoluminescence measurement. In Figure 6, the excitation wavelength dependent photoluminescence spectra defined the maximum photoluminescence to be

430 nm for malic acid QDs and 465 nm for citric acid QDs. Furthermore, for both cCQDs, it could be noticed that the maximum photoluminescence was obtained with an excitation wavelength of approximately 370 nm. Interesting to note, the photoluminescence was significantly narrower for malic acid QDs as compared to citric acid QDs.

Figure 6. Excitation wavelength dependent photoluminescence spectra of (A) citric acid QDs and (B) malic acid QDs.

With the aim of validating the adsorption mechanism as described in Figure 1, the fluorescence behaviour of FAM-Lprobe under different experimental conditions was next investigated. The performance of two types of cCQDs, citric acid QDs and malic acid QDs, was also evaluated, with the findings displayed in Figure 7.

From Figure 7, it is demonstrated that in the absence of cCQDs, FAM-Lprobe exhibited strong fluorescence emission (Figure 7, blue line). However, the presence of citric acid QDs and malic acid QDs led to about 23 % and 39 % quenching of the FAM-Lprobe fluorescence emission respectively (Figure 7, pink line). This indicated that malic acid QDs could adsorb FAM-Lprobe better and quenched the fluorescence dye more effectively. However, when FAM-Lprobe was hybridized with its complementary DNA target to form dsDNA, its fluorescence was significantly retained in the presence of cCQDs (Figure 7, purple line). In addition, it is also worth noting that cCQDs (Figure 7, red

line) did not display any fluorescence emission in the region of interest and thus did not cause any interferences.

Figure 7. Fluorescence emission spectra of FAM-Lprobe (40 nM) under different experimental conditions for (A) citric acid QDs and (B) malic acid QDs. Black: Tris-HCl buffer solution; red: cCQDs; blue: FAM-Lprobe; pink: FAM-Lprobe + cCQDs and purple: FAM-Lprobe + 4 nM DNA target + cCQDs.

Since the cCQDs function as the nanoquenchers in this work, it was therefore vital to determine the optimum amount of citric acid QDs and malic acid QDs to be adopted for the sensing platform. To this aim, the fluorescence intensity of FAM-Lprobe towards various volumes of citric acid QDs and malic acid QDs introduced was measured and plotted in Figure 8.

Figure 8. Fluorescence intensity of FAM-Lprobe (40 nM) upon the introduction of different volumes of (A) citric acid QDs and (B) malic acid QDs.

As illustrated in Figure 8, with a greater volume of cCQDs introduced, there was an increase in the extent of quenching of FAM-Lprobe fluorescence emission until saturation was attained. Drawing conclusions from Figures 8A and 8B, the optimum volume of citric acid QDs and malic acid QDs to be utilized was determined to be 30 μL and 40 μL accordingly.

Subsequently, a calibration experiment with different concentrations of complementary DNA target was performed to evaluate the range of detection for citric acid QDs and malic acid QDs. As seen in both Figures 9A and 10A, with increasing concentrations of DNA target, enhancement in fluorescence intensity was observed. This can be attributed to the reason that with more complementary DNA target added, a higher degree of DNA hybridization occurs and more dsDNA is formed. Consequently, this gives rise to a lesser extent of adsorption onto the surface of cCQDs and thus lesser quenching, resulting in a higher fluorescence signal being acquired. The range of detection was established to be 0.4 to 400 nM for citric acid QDs while 0.04 to 400 nM for malic acid QDs. In addition, the limit of detection was calculated to be 45.6 nM for citric acid QDs and 17.4 nM for malic acid QDs. The wider range of detection and lower detection limit for malic acid QDs as compared to citric acid QDs may be due to the higher amount of carboxylic groups they contained, hence imparting enhanced sensitivity. Interesting to note, in two separate studies by Li and co-workers, whereby carbon nanoparticles and carbon nanospheres were employed instead, the range of detection was determined to be 33 to 300 nM and 0.1 to 300 nM respectively.^{10, 17}

Figure 9. (A) Calibration curve for the detection of DNA hybridization based on citric acid QDs. (B) Corresponding fluorescence emission spectra in the presence of different concentrations of DNA target (0.4, 2, 4, 20, 40, 200, and 400 nM).

Figure 10. (A) Calibration curve for the detection of DNA hybridization based on malic acid QDs. (B) Corresponding fluorescence emission spectra in the presence of different concentrations of DNA target (0.04, 0.4, 2, 20, 40, 200, and 400 nM).

In order to confirm that the complementary DNA target did not have any inherent quenching or fluorescence effect on the fluorescence emission by FAM-Lprobe, and to further substantiate the proposed sensing mechanism, a control study was conducted with the outcomes depicted in Figure 11. As shown in Figure 11, the addition of solely DNA target has negligible effect on the fluorescence emission by FAM-Lprobe. On the other hand, when cCQDs were present together with DNA target, quenching in FAM-Lprobe fluorescence emission was reflected. Hence, it could be concluded that DNA

target itself did not have any effect on the fluorescence signal derived and cCQDs indeed function as the nanoquencher in this work.

Figure 11. Fluorescence emission spectra of FAM-Lprobe upon DNA target hybridization in the presence and absence of (A) citric acid QDs and (B) malic acid QDs.

Last of all, to investigate the selectivity performance of the proposed detection assay, single nucleotide polymorphism analysis was performed for both citric acid QDs and malic acid QDs. For the case of citric acid QDs, the enhancement in fluorescence signal, relative to when no DNA target was introduced, was 17.0 a.u. when complementary DNA target was added while just 12.6 a.u. when single-base mismatch DNA was added. For the case of malic acid QDs, the enhancement in fluorescence signal was 27.3 a.u. for complementary DNA target while 23.2 a.u. for single-base mismatch DNA. This suggests that the proposed detection assay has the potential to distinguish single-base mismatch DNA from complementary DNA target. However, further studies have to be conducted in order to optimize the selectivity performance.

Conclusions

In summary, we have demonstrated the proof-of-concept of a novel application of cCQDs. In this work, cCQDs, functioning as a nanoquencher, were employed as a fluorescence sensing platform for the detection of nucleic acid. In addition, the performance of two variations of cCQDs, citric acid QDs and malic acid QDs, was assessed. For both cCQDs, the proposed sensing platform demonstrates superior range of detection and potential selectivity down to single-base mismatch. The linear dynamic range of detection was determined to be 0.4 – 400 nM for citric acid QDs and 0.04 – 400 nM for malic acid QDs, with detection limit of 45.6 nM and 17.4 nM for citric acid QDs and malic acid QDs respectively. Moreover, the presence of carboxylic groups on the surface of cCQDs presents versatile anchoring points which could be exploited via numerous approaches.^{31, 32} The conclusions from this study act as the foundation for future works on utilizing cCQDs for fluorescence detection of essential biomolecules and disease diagnostics.

Experimental Section

Materials

Citric acid ($\text{HOC}(\text{COOH})(\text{CH}_2\text{COOH})_2$), malic acid ($\text{HO}_2\text{CCH}_2\text{CH}(\text{OH})\text{CO}_2\text{H}$), ethylenediaminetetraacetic acid ($((\text{HO}_2\text{CCH}_2)_2\text{NCH}_2\text{CH}_2\text{N}(\text{CH}_2\text{CO}_2\text{H})_2$) and sodium hydroxide (NaOH) were obtained from Lach-Ner (Czech Republic).

Dialysis membranes (1 kDa cut-off) were obtained from P-LAB (Czech Republic).

Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) was purchased from Quality Reagent Chemical (New Zealand).

Tris(hydroxymethyl)aminomethane ($\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$), potassium chloride (KCl), hydrochloric acid (HCl) (conc. 37 %) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA oligonucleotides sequences are as follow:

FAM-Lprobe: 5' [6-FAM] ACC AGG CGG CCG CAC ACG TCC TCC AT 3'

DNA target: 5' ATG GAG GAC GTG TGC GGC CGC CTG GT 3'

Single-base mismatch DNA: 5' ATG GAG GAC GTG CGC GGC CGC CTG GT 3'

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) with a resistivity of 18.2 M Ω cm.

The following buffer was used in this study:

0.1 M Tris-HCl (pH 7.2, 50 mM KCl + 10 mM MgCl_2)

Equipment

High resolution X-ray photoelectron spectroscopy (XPS) was performed on an ESCAProbeP (Omicron Nanotechnology Ltd., Taunusstein, Germany) spectrometer equipped with a monochromatic aluminium X-ray radiation source (1486.7 eV). Survey and high resolution C 1s core-level scans were performed. The relative sensitivity factors were used in the evaluation of the carbon-to-oxygen (C/O) ratios from the survey spectra. Samples were prepared by dropcasting of cCQDs solutions onto gold coated silicon wafer.

Fourier transform infrared spectroscopy (FT-IR) spectra were recorded using a Nicolet iS50R spectrometer (Thermo Scientific, Waltham, MA, USA) with Smart SAGA specular reflectance accessory. Samples were prepared by dropcasting of cCQDs solutions onto gold coated silicon wafer.

Dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, England). The samples were measured at 20 °C using disposable PMMA cuvettes.

Fluorescence measurements were performed at room temperature on a Varian Cary Eclipse fluorescence spectrophotometer. The excitation wavelength was fixed at 490 nm, with the emission spectra recorded over the range of 500 to 800 nm.

Transmission electron microscopy (TEM) was performed with a JEOL JEM-1010 instrument at accelerating voltage of 80 kV. Images were taken by SIS MegaView III digital camera (Soft Imaging Systems) and analysed with AnalySIS v2.0 software.

Concentration of cCQDs was determined with liquiTOC II (Elementar Analysensysteme, Hanau, Germany) by measurement of the total carbon concentration.

For atomic force microscopy (AFM) images, the dialyzed cCQDs sample was ultrasonicated for 5 minutes before the suspension was dropcasted onto a freshly cleaved mica substrate. The measurements were carried out on an Ntegra Spectra instrument from NT-MDT, and surface scans were performed in tapping (semicontact) mode. A cantilever with a strain constant of 1.5 kNm^{-1} equipped with a standard silicon tip with curvature radius lower than 10 nm was used for all measurements. The measurements were conducted under ambient condition with a scan rate of 1 Hz and a scan line of 512.

Procedures

Preparation of cCQDs

A 6 g amount of citric acid / malic acid, in a glass beaker covered with a lid, was heated at 205 °C until the solid melted completely, and the temperature was held for another 15 minutes. After cooling to room temperature, 50 mL of 0.25 M NaOH was added to the reaction mixture. The resulting solution was then adjusted to pH 6.0 with 0.05 M NaOH, followed by dialysis using a 1 kDa cutoff membrane for 3 days before subsequent use. The colloidal solution of cCQDs was next evaporated to 100 mL by vacuum evaporator at 40 °C.

The size of the synthesized cCQDs was subsequently measured with DLS and determined to be 11.3 nm (full width at half-maximum (fwhm) of size distribution, 3.7 nm) for citric acid QDs and 24.7 nm (fwhm of size distribution, 7.1 nm) for malic acid QDs.

To determine the concentration of cCQDs, TOC analyser was used to measure the carbon concentration before utilizing XPS composition analysis to recalculate to cCQDs concentration. The concentration of cCQDs obtained from citric acid QDs is 81.9 mgL^{-1} and the concentration of cCQDs obtained from malic acid QDs is 352 mgL^{-1} .

Fluorescence Quenching and Detection of DNA Hybridization

For fluorescence quenching, the working solution of the fluorescent DNA oligonucleotide (FAM-Lprobe) was prepared by diluting the stock solution to about 40 nM with Tris-HCl buffer solution. An aliquot at the optimum volume of cCQDs was then added to the Tris-HCl buffer solution containing FAM-Lprobe and allowed to incubate for 20 minutes at room temperature.

For the detection of DNA hybridization, DNA target was first added to 40 nM of FAM-Lprobe with incubation at 50°C for 30 minutes. After the incubation, cCQDs at the optimum volume was introduced and left for 20 minutes at room temperature. After which, fluorescence of the mixture was measured. The final concentration of DNA target in the mixture ranged from 0.04 to 400 nM.

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Chapter 14

Conclusions and Epilogue

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Conclusions

Using three different classes of nanomaterials, this thesis sets out to acquire deeper understanding on the fundamental working principles of biosensors, and to explore novel biosensing systems with great potential.

With the first class of nanomaterials, graphene and its analogues, it was understood that the adopted immobilization technique, and thus the resulting topological constraints of single-stranded DNA (ssDNA) attached onto graphene sheet, can exert a dramatic influence on its ability to undergo hybridization with a complementary strand subsequently. The most efficient hybridization event was observed for ssDNA which was covalently attached onto graphene on one end while for ssDNA which was covalently attached onto graphene on both ends, the hybridization efficiency was close to zero. After which, using chemically modified graphenes (CMGs) as the platforms, the physical immobilization mechanism was validated with differential pulse voltammetry. It was confirmed that for physically immobilized DNA probes, they would be partially removed from the platform upon successful hybridization, parallel to the proposed mechanism based on electrochemical impedance spectroscopy (EIS). Moreover, among the different CMGs studied, graphene oxide was found to have the best performance in terms of reproducibility and the ability to distinguish between complementary and non-complementary DNA sequences.

With a better comprehension of the basic principles, novel biosensing systems were then designed. First of all, graphene oxide nanoplatelets (GONPs) were examined as an electroactive label for thrombin and ochratoxin A aptasensing, and for the monitoring of protein-target interactions. GONPs were able to effectively serve as electroactive labels owing to their extraordinary inherent electrochemistry, small dimensions and uniformity

of the sizes. For the case of thrombin aptasensing, the range of detection was determined to be 3 pM - 0.3 μ M, with a superior discrimination towards interferences such as immunoglobulin G (IgG), avidin and bovine serum albumin (BSA). On the other hand, for ochratoxin A aptasensing, a detection range of 310 fM - 310 pM was established, with a good discrimination against ascorbic acid. For the instance of monitoring protein-target interactions, the biosensing protocol was proven to be selective, with a distinctive discrimination towards other proteins such as BSA and IgG, at concentration levels similar to real samples. From the above findings, it is evident that GONPs are a promising candidate to replace metal nanoparticles, which were developed decades ago for the same purposes, as GONPs do not involve toxic or corrosive chemicals for their production. Apart from GONPs, the proof-of-concept of performing immunosensing on graphene obtained by chemical vapour deposition (CVD) was demonstrated with IgG. The fabricated biosensing platform exhibited good selectivity for IgG, against haemoglobin, avidin and BSA. In addition, the linear range of detection was revealed to be 0.1 - 100 μ g mL⁻¹.

Transition metal dichalcogenides (TMDs) are the second class of nanomaterials studied in this thesis. Similar to the approach taken for graphene and its analogues, fundamental investigations were conducted on TMDs before employing them for biosensing applications. To begin, the charge transfer resistance of various TMD materials, inclusive of molybdenum disulfide (MoS₂), tungsten disulfide (WS₂), molybdenum diselenide (MoSe₂) and tungsten diselenide (WSe₂), in their respective bulk and exfoliated forms, was evaluated with EIS. From the study, it was observed that selenide TMD materials displayed a faster rate of heterogeneous electron transfer (HET) as compared to sulfide TMD materials. Furthermore, the HET rate of these TMD materials could be enhanced

through electrochemical activation. Thereafter, the extent of interactions between hairpin DNA (HpDNA) and TMD materials was assessed with the influence of three factors in mind. These factors are the type of transition metal (Mo and W), the type of chalcogen (S and Se), and the exfoliation of TMDs (bulk and exfoliated). It was revealed that selenide and molybdenum TMDs present stronger interactions with HpDNA in contrast to sulfide and tungsten TMDs respectively. Moreover, selenide TMDs were noted to have much greater interactions with HpDNA than their sulfide counterparts in the bulk form. Hence, upon undergoing exfoliation, the difference in the degree of interactions became less significant.

Subsequently, the focus of TMDs was directed to their biosensing applications. In view of the current trend of using TMD materials as fluorescence quenchers, the influence of different transition metals present in TMDs on their performance as a fluorescence quencher for nucleic acid detection, was examined by comparing MoS₂ and WS₂ nanoflakes. Based on the results, it was deduced that WS₂ nanoflakes had weaker interactions with nucleic acids than MoS₂ nanoflakes. As such, WS₂ nanoflakes demonstrated narrower range of detection and lower selectivity. Specifically, the detection range was reported to be 9.60 - 366 nM and 13.3 - 143 nM for MoS₂ and WS₂ nanoflakes respectively. In addition, MoS₂ nanoflakes showed 97.8 % disparity with negative control while WS₂ nanoflakes only exhibited 44.3 %. Hence, it was concluded that the transition metals in TMDs could have a strong effect on their fluorescence quenching efficiency. On another note, a fresh approach for the utilization of MoS₂ nanoflakes in the field of biosensing was proposed, where MoS₂ nanoflakes functioned as electroactive labels. The differential affinity of MoS₂ nanoflakes towards ssDNA and double-stranded DNA, coupled with the inherent oxidation peak, were exploited for the

detection of DNA hybridization. With this approach, the detection range was determined to be 0.03 - 300 nM, spanning across four orders of magnitude.

The final class of nanomaterials is denoted by carbon quantum dots (CQDs) and with CQDs, a new application was evaluated with carboxylic CQDs (cCQDs) acting as a fluorescence quencher for nucleic acid detection. At the same time, the performance of two variations of cCQDs, specifically citric acid QDs and malic acid QDs, was also examined. It was discovered that both cCQDs displayed superior range of detection and potential selectivity down to single-base mismatch. The linear dynamic range of detection was shown to be 0.4 - 400 nM for citric acid QDs and 0.04 - 400 nM for malic acid QDs, with detection limit of 45.6 nM and 17.4 nM for citric acid QDs and malic acid QDs respectively.

The findings acquired from the above works are anticipated to have provided a deeper insight on the fundamental principles of biosensors. These insights will prove to be beneficial in the development of future biosensors. In addition, the various novel biosensing approaches and systems which were introduced here, hold great promise and further accentuate the immense potential which nanomaterials have for biosensing applications. Nonetheless, before the true application of nanomaterials in biosensing devices, one key challenge remains to be overcome, and that is the synthesis of uniform nanomaterials, with superior batch-to-batch reproducibility. Hence, continuous work has to be conducted in order to improve current synthetic processes or to design new methodologies.

Epilogue

Nanotechnology represents one of the most fascinating fields in analytical chemistry in recent times and nanomaterials are the key building block of nanotechnology. Till date, after extensive research, a wide variety of nanomaterials of different shapes, sizes and compositions are obtainable. The huge interest in these nanomaterials can be attributed to the wide array of desirable properties which they exhibit. Furthermore, owing to these favourable characteristics, nanomaterials possess vast potential for designing novel biosensing systems and enhancing the performance of current bioanalytical assays.

Over the past decade, several studies on the utilization of nanomaterials for the development of biosensors have been reported, with close to 19600 manuscripts being published in the past 5 years. The driving force behind such intensive research lies in the constant strong market demand for sensitive, selective, cost-effective and rapid biosensors which can be employed in various fields such as clinical disease diagnostics and treatments, genetic profiling, food safety and forensic studies. In addition, a market value of more than US\$13 billion has also been estimated for the biosensing field.

Inspiring to note, in most of the published works, enhancements in sensitivity, selectivity and stability of the fabricated biosensors are often reported. However, despite of the encouraging results achieved thus far, there are still challenges to be addressed before the actual commercialization of the proposed biosensors. Some of these challenges are, improving response time in sensitive biosensing systems, making biosensors compatible with biological matrices, and detecting multiple analytes on one biosensing device.

Hence, it is with high anticipation that the biosensing field will continue to grow in the years to come, with the emphasis on nanomaterials.

Appendices

Soldering DNA to graphene *via* 0, 1 and 2-point contacts: Electrochemical impedance spectroscopic investigation

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ABSTRACT

DNA is one of the most programmable molecules available till date and it is immensely important for nanoarchitectonic works. Herein, we investigate the influence of topological constraints of single stranded DNA, which is attached to a graphene sheet, on its ability to hybridize with a complementary strand. These findings are expected to have a profound impact on nanoarchitectonic, bioelectronic and biosensing applications of graphene.

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1. Introduction

There is an immense interest in the fabrication of graphene/DNA conjugates with a wide spectra of potential applications, ranging from biosensing and nanoarchitectonics to nanoelectronics [1–4]. This is due to the fact that single stranded DNA (ssDNA) is one of the most programmable molecules in nature, hence permitting stable storage of code. Furthermore, it allows unambiguous interpretation *via* hybridization with a complementary ssDNA strand. Such capacity of programming enables highly parallel ssDNA directed assembly of nanoarchitected elements, leading to advanced bioelectronic and biosensing devices [5].

Graphene, comprising of a single layer of carbon atoms closely arranged in a honeycomb two-dimensional lattice, has attracted much interest in recent times [6] due to the great promise it holds for biosensing. Graphene displays remarkable characteristics such as low cost, high electron conductivity, large specific surface area, fast heterogeneous electron transfer rate and simple synthetic pathways [7,8]. Therefore, it is also a highly important building block for nanoelectronic and sensing devices [9,10].

For the construction of such functional devices, the attachment of ssDNA strand to graphene is of great significance. It has been shown that the method of attachment has an impact on the overall orientation [11] and structure [12] of the immobilized ssDNA probe and consequently the hybridization process. Furthermore, it is also well known that DNA hybridization exhibits different kinetics and dynamics when faced with spatial confinements and topological restrictions [13–15]. In this work, we aim to address the issue on whether the way which

DNA is attached (“soldered”) to graphene influences its ability to hybridize with the complementary DNA sequence. As depicted in Fig. 1, we employed three different approaches: ssDNA physisorbed on the graphene sheet (0-soldering contact); ssDNA chemically bonded to graphene sheet at one end (1-soldering contact); and ssDNA attached by two ends to graphene sheet (2-soldering contacts). In order to explore to what extent the number of soldering points of ssDNA to graphene influences its biorecognition ability, which is a fundamental issue to be addressed before the utilization of these bionanostructures, we employed electrochemical impedance spectroscopy (EIS).

2. Experimental section

2.1. Materials

N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide sodium salt (NHS), graphite microparticles (<20 μm), sodium borohydride (NaBH₄), nitric acid (HNO₃) (conc. > 90%), sulphuric acid (H₂SO₄) (conc. 95–98%), potassium chlorate (KClO₃), hydrochloric acid (HCl) (conc. 37%), sodium phosphate dibasic salt (Na₂HPO₄), sodium chloride (NaCl), potassium hexacyanoferrate (II) trihydrate (K₄[Fe(CN)₆].3H₂O), potassium hexacyanoferrate (III) (K₃[Fe(CN)₆]), sodium dodecyl sulfate (SDS) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA sequences are as follows:

ssDNA: 5' ACC AGG CGG CCG CAC ACG TCC TCC AT 3'

NH₂-ssDNA: 5' NH₂-ACC AGG CGG CCG CAC ACG TCC TCC AT 3'

NH₂-ssDNA-NH₂: 5' NH₂-ACC AGG CGG CCG CAC ACG TCC TCC AT-NH₂ 3'

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Fig. 1. Left: Schematics showing the concept of (A) 0-soldering; (B) 1-soldering and (C) 2-soldering contacts attachment of the ssDNA to graphene sheet ("soldering" points are represented by the red dots). Right: Corresponding EIS spectra of graphene (black), graphene with ssDNA before (red) and after (blue) hybridization with complementary ssDNA.

Complementary ssDNA: 5' ATG GAG GAC GTG TGC GGC CGC CTG GT 3'

Trisodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) and hydroxylamine hydrochloride were purchased from Alfa Aesar (Singapore).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of conductivity no lesser than 18.2 M Ω cm.

The following buffers were used in this study:

0.1 M PBS (0.1 M NaCl + 10 mM Na_2HPO_4 , pH7.0)

5xSSC (0.75 M NaCl + 75 mM trisodium citrate, pH7.0)

2xSSC (0.30 M NaCl + 30 mM trisodium citrate, pH7.0)

Disposable electrical printed (DEP) electrodes were obtained from BioDevice Technology (Nomi, Japan).

Synthesis of chemically modified graphene (CMG) materials and detailed characterizations from Transmission electron microscopy (TEM), Raman spectroscopy, Electrochemical impedance spectroscopy (EIS) and X-ray photoelectron spectroscopy (XPS) of the CMG materials, are reported in our previous work [16]. In brief, the D/G Raman signal ratio for graphene oxide (GO), and electrochemically reduced graphene oxide (ERGO) was reported to be 1.05 and 1.08 respectively. XPS determined the C:O ratio to be 2.8 and 5.1 for GO and ERGO respectively.

2.2. Apparatus

Electrochemical experiments were conducted with an Autolab potentiostat PGSTAT302 (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer and controlled by NOVA software version 1.7. Impedance measurements were carried out between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The experiments were performed at room temperature using DEP electrodes and 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ (1:1 molar ratio) in PBS buffer solution as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane.

2.3. Procedures

2.3.1. Preparation of ERGO-modified DEP electrode

GO was immobilized onto each DEP electrode surface via physical adsorption. 3 μL of GO at a concentration of 1 mg/ml in ultrapure water was deposited on the electrode surface and left to dry under room temperature overnight. Excess of GO that did not get adsorbed onto the electrode surface was then removed by gentle washing with ultrapure water. Prior to casting GO onto the electrode surfaces, GO suspension was sonicated for 15 min in order to ensure maximum uniform dispersion of the material. To obtain ERGO modified electrode, GO modified electrode was electrochemically reduced at -1.2 V for 900 s in PBS buffer solution at room temperature.

2.3.2. Physical immobilization of ssDNA

ssDNA was then immobilized onto the ERGO-modified electrode surfaces by dry physical absorption. 3 μL of ssDNA in PBS buffer solution at a concentration of 10 pmol/ μL was deposited onto each ERGO-modified electrode surface and dried at a temperature of 60 $^{\circ}\text{C}$ for 10 min in an oven. Consequently, the electrodes were washed in PBS buffer solution with gentle stirring at room temperature to remove the surplus of ssDNA which was not immobilized onto the ERGO-modified electrode surfaces.

2.3.3. Covalent immobilization of ssDNA

ssDNA was immobilized onto ERGO-modified DEP electrodes via covalent binding. 3 μL of 0.05 M EDC and 0.03 M NHS in PBS buffer solution was deposited onto ERGO-modified electrode surfaces for 1.5 h in order to activate carboxylic acid groups. Subsequently, the electrodes were rinsed with PBS buffer solution. After that, 3 μL of ssDNA at a concentration of 10 pmol/ μL in PBS was casted onto the electrodes and left to stand overnight at room temperature under a wet environment. This was then followed by washing with 0.05% SDS and 0.04 M hydroxylamine hydrochloride in order to remove non-specific adsorbed ssDNA and to deactivate any remaining carboxylic acid groups [17] before another washing step with PBS buffer solution.

2.3.4. Hybridization

DEP electrodes modified with ssDNA were next incubated in Eppendorf tubes containing 5xSSC buffer solution (pH 7.0) with 30 pmol of the complementary ssDNA sequence (total volume: 100 μL). The incubation was conducted at 42 $^{\circ}\text{C}$ for 30 min with gentle stirring. After which, two washings with 2xSSC buffer solution (pH 7.0) were performed to remove the excess of non-specific adsorbed ssDNA sequences.

3. Results and discussion

Electrochemical impedance spectroscopy (EIS) is an extremely sensitive technique which is capable of detecting minor changes resulting from the biorecognition event on the surface of graphene [18]. The EIS spectra of graphene, graphene functionalized with ssDNA using 0, 1 or 2-soldering contacts, and graphene functionalized with ssDNA after hybridization with complementary ssDNA were recorded and are shown in the right panel of Fig. 1.

In the case of 0-soldering contact (Fig. 1A), that is ssDNA being only physisorbed on the surface of graphene, the charge transfer resistance (R_{ct}) of the platform increases from 417 Ω (graphene) to 8.04 k Ω (ssDNA modified graphene). Subsequently, when the ssDNA modified graphene was incubated with complementary ssDNA, the R_{ct} decreases by 27% to 5.80 k Ω , which indicates the partial removal of the DNA hybrid from the surface [2].

On the other hand, a completely different situation was observed when a 1-soldering contact ssDNA/graphene hybrid procedure was employed. Under such circumstance, the R_{ct} of graphene surface increases to 2.12 k Ω from 675 Ω upon the attachment of ssDNA using EDC/NHS chemistry and the observed increase in R_{ct} signifies successful covalent attachment of ssDNA strand [4]. Consequently, when ssDNA/graphene hybrid with 1-soldering point was exposed to complementary ssDNA, the R_{ct} further increases by 64% to 3.47 k Ω , signifying that the majority of the ssDNA, which was conjugated to graphene, had hybridized with the complementary ssDNA.

The interesting twist arises when the hybridization process with 2-soldering contacts ssDNA/graphene hybrid was examined. While graphene surface shows R_{ct} of 665 Ω , the 2-soldering points ssDNA/graphene platform displays a R_{ct} of 2.48 k Ω . After hybridization with complementary ssDNA, the R_{ct} increases by only 9% to 2.71 k Ω . As such, this implies that the hybridization on 2-soldering points attached ssDNA is significantly less efficient than that of 1-soldering

Fig. 2. Comparison of the impedimetric signals derived after hybridization with and without prior EDC/NHS treatment. Signal is represented as R_{ct} ratio $[(R_{\text{ct}}^{\text{hybridized}} - R_{\text{ct}}^{\text{graphene}})/(R_{\text{ct}}^{\text{ssDNA}} - R_{\text{ct}}^{\text{graphene}})]$. Error bars correspond to triplicate experiments. Amount of ssDNA and complementary ssDNA used is 30 pmol. All measurements were performed with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in PBS buffer solution (pH 7.0) at room temperature.

point attached ssDNA strand and the observation can be attributed to the reason that the attached ssDNA with 2-soldering contacts is unable to rotate during the hybridization process [12].

Therefore, from the results attained from this study, it can be concluded that the number of soldering points employed for the attachment of DNA affects DNA hybridization to a great extent and the number of DNA attachment points is one of the key factors in the efficiency of DNA hybridization.

Subsequently, in order to ascertain that the carbodiimide used for the activation of carboxylic groups in covalent immobilization of ssDNA did not affect the hybridization efficiency [19] and hence the increase in R_{ct} upon incubation with the complementary strand, an additional control experiment was conducted using unlabelled ssDNA probes with the findings displayed in Fig. 2.

From Fig. 2, it can be understood that when there was no EDC/NHS treatment with ssDNA, there is a decrease in R_{ct} upon incubation with complementary ssDNA, similar to the case of 0-soldering contact. On the other hand, when there was EDC/NHS treatment with ssDNA, there was effectively no hybridization occurring as R_{ct} ratio is approximately 1 (no increase or decrease in R_{ct} upon incubation with complementary ssDNA). Such observations can be rationalized that when there was EDC/NHS treatment with ssDNA which does not contain any NH_2 terminals, the activated carboxylic groups interacted with part of the amino groups of the nitrogenous bases in ssDNA instead, and the ssDNA became randomly affixed on the graphene surface. Subsequently, the remaining carboxylic groups were deactivated [17] before incubation with the complementary ssDNA. The ssDNA being affixed on the graphene surface, has highly reduced rotation capability during hybridization and thus, there was no effective hybridization and this led to a R_{ct} ratio of approximately 1. The good hybridization efficiency, previously observed in the case of 1-soldering contact, results in the conclusion that the carbodiimide used for the activation of carboxylic groups in covalent immobilization of ssDNA did not contribute to the hybridization process.

4. Conclusions

In summary, we have demonstrated that the topological constraints of ssDNA attached on graphene sheet exhibit dramatic influence on its ability to undergo hybridization with a complementary strand. The most efficient hybridization event took place on 1-soldering point ssDNA/graphene hybrid while 2-soldering points ssDNA attachment to graphene practically inhibits its ability to successfully hybridize with complementary ssDNA. These findings are expected to have a profound influence upon the construction of nanobioelectronic devices as well as nanobiosensors.

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An insight into the hybridization mechanism of hairpin DNA physically immobilized on chemically modified graphenes

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There is an emerging interest in developing electrochemical DNA biosensors which rely on label-free protocols for the detection of DNA hybridization and polymorphism. Lately, many of them have been using DNA probes which were physically adsorbed onto different graphene platforms. In these works, the biorecognition event is monitored by electrochemical impedance spectroscopy and the detection mechanism proposed needs verification by orthogonal methods. Here, we aim to provide an insight into the mechanism behind the impedimetric signal change upon the hybridization event on graphene platforms. For this aim, we used an orthogonal electrochemical method, differential pulse voltammetry, to examine the oxidation of guanine on target DNA molecules hybridized with an inosine-substituted hairpin DNA probe. We show that the successful biorecognition event leads to desorption of dsDNA from graphenic surfaces on a wide range of graphenic surfaces, such as graphene oxide, electrochemically reduced graphene oxide and thermally reduced graphene oxide. These results confirm the previous hypothesis based on electrochemical impedance spectroscopy data. In addition, these findings also have a profound impact on the understanding of both the interactions between DNA and graphene platforms and the DNA recognition event on graphene platforms for the construction of biosensors.

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Introduction

DNA biosensors based on nucleic acid recognition methods are being rapidly fabricated in order to achieve the goal of affordable and rapid testing of genetic and infectious diseases.¹ Electrochemical transducers are commonly being employed for the sensing of DNA hybridization processes as a result of their low cost, small dimensions, high sensitivity and compatibility with microfabrication techniques.² Such kind of devices often depend on the immobilization of a single stranded DNA probe onto the electrode surface to act as the recognition element for complementary target DNA strands *via* base pair matching. The resulting duplex structure formed is usually being detected with the use of a suitable electroactive label.^{3–5}

However, in recent years, there has been an emerging interest in developing electrochemical DNA biosensors that do not rely on an external electroactive label. One common method employed to achieve that aim is to rely on electrochemical impedance spectroscopy (EIS). This is mainly due to the potential and advantages EIS possesses for interfacial investigation.^{6–8} Another method is to exploit the electrochemical

signal of DNA bases which can be electroactive on different materials.⁹

Graphene, which comprises of a single layer of carbon atoms packed densely in a honeycomb two-dimensional lattice, has gathered enormous interest from researchers in recent times.¹⁰ It is widely considered to have huge potential for biosensing^{11–13} as a result of its exceptional characteristics such as low cost, high electron conductivity, large specific surface area, fast heterogeneous electron transfer rate and simple synthetic routes.^{14–16}

To date, there have been several reports on the detection of the DNA hybridization process based on the use of EIS and graphene.^{17–20} However, little has been done to illustrate the mechanism behind the sensing process using this approach.

It is well known that EIS shows an outstanding sensitivity for probing interfacial properties of modified electrode surfaces, which in turn allows the monitoring of every single step in the biosensing protocol.⁶ However, since the total impedance change is the result of a combination of different factors such as the presence of charged species on the electrode surface, the steric hindrance generated by the biomolecule layer, and the conformational changes upon the biorecognition event, it may thus be difficult to figure out the mechanism involved in the biosensing in some cases. For example, a decrease in the impedimetric signal upon a biorecognition event can be either

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due to the partial removal of the probe biomolecule from the electrode,^{18,21} which results in a lower charge on the surface, or due to a conformational change of the biomolecule layer, which results in a lower steric hindrance and promotes electron transfer.²² Hence, to gain more insights into the mechanism behind the impedimetric change, it is common to complement EIS experiments with other techniques like fluorescence, chronocoulometry and scanning electron microscopy characterization.^{17,23}

Generally, for DNA biorecognition event on graphene using physically adsorbed DNA probes, the EIS signal decreases with successful formation of double helix with a complementary target. The decrease in the EIS signal is commonly assumed to be due to the desorption of dsDNA from the graphenic surface.^{17,18} However, as EIS provides only information on impedimetric changes of the surface, an independent method which would enable the determination of the occurrence of this event with certainty is needed. Herein, we aim to achieve this knowledge by employing an established label-free detection method based on the intrinsic electroactivity of the nuclei acid bases.²⁴ To investigate the phenomenon of dsDNA desorption from the graphenic surface, we employed the use of an immobilized inosine-substituted (containing no guanine) hairpin DNA probe. Inosine, in contrast to guanine, is not electroactive. Therefore, when the inosine-substituted probe binds preferentially to the complementary target cytosine residues,²⁵ the appearance of the target guanine residues oxidation signal provides a direct and convenient examination of the DNA hybridization process.

On this note, we investigate here for the first time the mechanism behind physically immobilized hairpin DNA hybridization on a variety of chemically modified graphene (CMG) materials such as graphene oxide, electrochemically reduced graphene oxide and thermally reduced graphene oxide.

Experimental section

Materials

Graphite microparticles (<20 μm), nitric acid (HNO_3) (conc. > 90%), sulphuric acid (H_2SO_4) (conc. 95–98%), potassium chlorate (KClO_3), hydrochloric acid (HCl) (conc. 37%), sodium phosphate dibasic (Na_2HPO_4), sodium chloride (NaCl), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA sequences are as follows:

Inosine-substituted Hairpin probe (INO-Hprobe): 5'-ATI IAI ACC AII CII CCI CAC ACI TCC TCC AT-3'

Wild: 5'-ATG GAG GAC GTG TGC GGC CGC CTG GT-3'

Mutant: 5'-ATG GAG GAC GTG CGC GGC CGC CTG GT-3'

Non-complementary: 5'-TTT TTT TTT TTT TTT GGT TGG TGT GGT TGG-3'

Trisodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) was purchased from Alfa Aesar (Singapore).

Disposable electrochemical printed (DEP) electrodes were obtained from BioDevice Technology (Nomi, Japan).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of conductivity no lesser than 18.2 $\text{M}\Omega\text{ cm}$.

Synthesis of chemically modified graphene (CMG) materials and detailed characterizations from transmission electron microscopy (TEM), Raman spectroscopy, electrochemical impedance spectroscopy (EIS) and X-ray photoelectron spectroscopy (XPS) of the CMG materials have been reported in our previous work.²⁶ In brief, the *D/G* Raman signal ratio for graphene oxide (GO), thermally reduced graphene oxide (TRGO) and electrochemically reduced graphene oxide (ERGO) was reported to be 1.05, 1.35 and 1.08, respectively. XPS determined the C:O ratio to be 2.8, 23.3 and 5.1 for GO, TRGO and ERGO, respectively. In addition, EIS displayed charge transfer resistance (R_{ct}) values of 11.75, 3.05 and 4.71 $\text{k}\Omega$ for GO, TRGO and ERGO accordingly.

Apparatus

Differential pulse voltammetry (DPV) measurements were performed with a $\mu\text{Autolab}$ type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer and controlled by General Purpose Electrochemical System (GPES) software version 4.9. The parameters applied were: 60 s accumulation time at 0.2 V, 50 ms modulation time, 0.5 s interval time, 25 mV modulation amplitude and 5 mV step. The raw data produced from the DPV measurements were treated with a baseline correction of peak width 0.01 using the GPES software. All measurements were conducted at room temperature (25 $^\circ\text{C}$) by utilizing DEP electrodes with Ag/AgCl as a reference electrode and in 50 mM PBS buffer solution (pH 7.4) unless otherwise stated.

Protocol

PREPARATION OF A CMG MODIFIED DEP ELECTRODE. GO and TRGO were immobilized onto each DEP electrode surface *via* physical adsorption. 3 μL of CMG materials at a concentration of 1 mg ml^{-1} in ultrapure water was deposited on the electrode surface and left to dry at room temperature overnight. Excess of CMG materials that did not get adsorbed onto the electrode surface was then removed by gentle washing with ultrapure water. Prior to casting the CMG materials onto electrode surfaces, GO and TRGO suspensions were sonicated for 15 minutes in order to ensure maximum uniform dispersion of the materials. To obtain an ERGO modified electrode, a GO modified electrode was electrochemically reduced at -1.2 V for 900 s in PBS buffer solution (pH 7.4) at room temperature.

PHYSICAL IMMOBILIZATION OF INO-HPROBES. INO-Hprobes were next immobilized onto the CMG modified electrode surfaces by physical adsorption. 3 μL of INO-Hprobe in PBS buffer solution (pH 7.4) at a concentration of 10 $\text{pmol } \mu\text{L}^{-1}$ was deposited on the modified electrode surface and dried in an oven at 60 $^\circ\text{C}$ for 10 minutes. Subsequently, the electrode was washed with PBS buffer solution (pH 7.4) and ultrapure water to remove the excess INO-Hprobes which were not well adsorbed on the electrode surface.

INCUBATION WITH DNA SEQUENCES. DEP electrodes modified with INO-Hprobes were next incubated in Eppendorf tubes containing 5×SSC buffer solution (pH 7.0) with the desired concentrations of DNA target sequences (total volume: 100 μ l). The incubation was conducted at 42 °C for 30 minutes with gentle stirring. After which, two washings with 2×SSC buffer solution (pH 7.0) were performed to remove the excess of non-specific adsorbed DNA target sequences.

Results and discussion

In order to understand the mechanism of DNA hybridization based on physical immobilization of hairpin DNA probes, we investigated and compared the behaviour of various chemically modified graphene (CMG) platforms in this work. CMG materials such as graphene oxide (GO), thermally reduced graphene oxide (TRGO) and electrochemically reduced graphene oxide (ERGO) were utilized in this study.

Fig. 1 describes in summary the general scheme of the work. Inosine-substituted Hairpin probes (INO-Hprobes) were first physically immobilized on the surface of graphene. Upon incubation with (i) complementary ssDNA, it is commonly assumed from EIS data^{17,18} that part of the hybridized dsDNA is released from the graphenic surface. Secondly, upon incubation with (ii) mutant ssDNA, due to partial hybridization, a smaller portion of the non-completely hybridized dsDNA is released from the surface and non-hybridized mutant ssDNA target may also non-specifically adsorb onto the surface. Lastly, upon incubation with (iii) non-complementary ssDNA, there is no release of INO-Hprobes from the surface and non-complementary DNA can non-specifically adsorb onto the surface of graphene. We employed differential pulse voltammetry (DPV) in this study and determined the presence of guanine base in target DNA sequences (wild, mutant and non-complementary). Since DPV

draws electrons directly from guanine bases, only guanine bases adsorbed onto the surface of graphene give rise to a DPV signal. Fig. 1 exhibits the differential pulse voltammograms obtained in an experiment on graphene oxide surfaces with wild, mutant and non-complementary DNA sequences using INO-Hprobes. We focused on the guanine peak current value recorded at a peak potential of approximately 0.875 V, after each modification step in the protocol. As shown in Fig. 1, INO-Hprobe modified electrodes did not produce any observable guanine peak as they do not contain any guanine bases. After incubation with the various target sequences (wild, mutant or non-complementary), a significant guanine peak was observed. The peak current is greatest for the case of hybridization with the non-complementary sequence, followed by the mutant sequence and lastly the wild target sequence. These results confirm the previous ones obtained by EIS^{17,18} and can be thus unambiguously attributed to the partial release of INO-Hprobes from the electrode surface. When hybridization is effective, as in the case of the complementary wild target, most of the hybridized INO-Hprobes are released from the electrode surface and a relatively small guanine peak current is obtained. However, when hybridization is less effective, as in the case of the mutant target, a lower amount of hybridized INO-Hprobe is expected to be released from the electrode surface and thus a greater guanine peak current is recorded. For the case of a non-complementary target, poor hybridization is expected and the large guanine peak current observed can be reasonably attributed to the non-specific adsorption of the non-complementary target sequence onto the INO-Hprobe modified electrode surface.

In order to prove and confirm the mechanism of the DNA-graphene interactions, we proceed to evaluate which graphene platform is most suitable to be used as the transducing surface. Differential pulse voltammograms were recorded after hybridization with each target sequence for all three CMG (GO, ERGO

Fig. 1 Schematic of the protocol and differential pulse voltammograms of INO-Hprobes (blue), a complementary wild target (pink), a single base mismatch mutant target (green), and a negative control with a non-complementary (NC) target (black). The amount of INO-Hprobe and DNA targets used is 30 pmol. All measurements were performed in 50 mM PBS buffer solution (pH 7.4) with Ag/AgCl as a reference electrode.

and TRGO) surfaces. From the series of voltammograms obtained, the guanine peak currents were noted and are represented in a histogram, as shown in Fig. 2. From Fig. 2A, it can be deduced that for all three surfaces, with the exception of ERGO, the guanine peak current is the highest for the non-complementary target followed by a mutant target and lastly a complementary wild target. In addition, the overall relative standard deviation percentage values for GO, ERGO and TRGO surfaces for this set of experiment are 11.58, 13.60 and 23.76%, respectively. The disparity in the percentage values is most likely due to the difference in the degree of homogeneity of the suspensions. As GO has more oxygen-containing groups than TRGO,²⁶ it is subsequently more soluble in water. Hence, the GO suspension is comparatively more homogeneous than the TRGO suspension and the overall relative standard deviation percentage value for GO is much lower than TRGO. Consequently, GO was then selected to be the employed material for further experimental studies since it displays the best performance as compared to the other CMG materials.

In view that GO is the best platform for this type of hairpin probe based DNA detection, we further investigated the usage and necessary parameters of direct DPV oxidation of guanine for DNA sensing. INO-Hprobe optimization was next conducted on the GO platform to determine the optimum amount of INO-Hprobe to be physically immobilized onto the GO modified electrode. Differential pulse voltammograms were recorded after hybridization with complementary and non-complementary targets for a series of INO-Hprobe amounts. From the series of voltammograms obtained, the guanine peak currents were again noted and are represented in a histogram, as illustrated in Fig. 3.

Fig. 2 (A) Histogram representing a comparison of guanine peak currents on GO, ERGO and TRGO modified DEP electrodes when hybridized with wild complementary, single base mismatch mutant and non-complementary targets. Error bars correspond to triplicate experiments. (B–D) Differential pulse voltammograms of the guanine peak on GO, ERGO and TRGO modified DEP electrodes when hybridized with wild complementary, single base mismatch mutant and non-complementary targets. The amount of INO-Hprobes and DNA targets used is 30 pmol. All measurements were performed in 50 mM PBS buffer solution (pH 7.4) with Ag/AgCl as a reference electrode.

Fig. 3 Histogram representing a comparison of guanine peak currents when hybridized with wild complementary and non-complementary targets for a range of INO-Hprobe amounts. Error bars correspond to triplicate experiments. The amount of DNA targets used is 30 pmol. All measurements were performed in 50 mM PBS buffer solution (pH 7.4) with Ag/AgCl as a reference electrode.

As derived from Fig. 3, the percentage difference between wild complementary and non-complementary targets is 9.63, 10.2, 49.2, 26.6 and 5.40% for 3, 15, 30, 45 and 60 pmol of INO-Hprobe accordingly. Hence, at 30 pmol of INO-Hprobe, the best distinction between the two targets can be made. However, by taking the error bars into consideration, the best distinction is instead at 45 pmol of INO-Hprobe. Therefore, it is concluded that the optimum amount of INO-Hprobe to be immobilized onto the modified electrode surface to ensure the maximum surface coverage is 45 pmol.

Conclusions

In summary, differential pulse voltammetry was employed for the label-free detection of DNA hybridization and polymorphism. The results obtained confirm the previous assumption made to explain the change of the impedimetric signal in a similar protocol.^{17,18} The lower signal obtained for the guanine oxidation peak for complementary and mutant targets was due to the partial removal of hybridized dsDNA from the electrode surface during the hybridization event. Moreover, after comparing the performance of various chemically modified graphene surfaces, such as graphene oxide, electrochemically reduced graphene oxide and thermally reduced graphene oxide, we established that graphene oxide exhibits comparatively the best performance in terms of the ability to distinguish between complementary and non-complementary sequences and reproducibility of the results. We believe that our findings are very important in order to gain deeper insights into the mechanism of DNA biosensing.

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Thrombin aptasensing with inherently electroactive graphene oxide nanoplatelets as labels

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Graphene and its associated materials are commonly used as the transducing platform in biosensing. We propose a different approach for the application of graphene in biosensing. Here, we utilized graphene oxide nanoplatelets as the inherently electroactive labels for the aptasensing of thrombin. The basis of detection lies in the ability of graphene oxide to be electrochemically reduced, thereby providing a well-defined reduction wave; one graphene oxide nanoplatelet of dimension 50×50 nm can provide a reduction signal by accepting $\sim 22\,000$ electrons. We demonstrate that by using graphene oxide nanoplatelets as an inherently electroactive label, we can detect thrombin in the concentration range of 3 pM – $0.3\text{ }\mu\text{M}$, with good selectivity of the aptamer towards interferences by bovine serum albumin, immunoglobulin G and avidin. Therefore, the inherently electroactive graphene oxide nanoplatelets are a material which can serve as an electroactive label, in a manner similar to metallic nanoparticles.

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Introduction

Thrombin, an omnipresent protein in mammals, is a form of serine protease. Thrombin found in humans is comprised of two chains bound together by disulphide bridges, with an isoelectric point of 7.05 and a molecular weight of 36.7 kDa.¹ It has several crucial roles to perform in various physiological and pathological processes such as blood coagulation, inflammation, tissue restoration at the walls of blood vessels and platelet activation.^{2,3} In view of the above, the development of a biosensor towards thrombin detection with good selectivity, sensitivity, high throughput and simplicity is of immense importance in both clinical and research applications.^{4–6} Immunoassay systems based on the use of antibody as the recognition element are widely known to be flexible and robust tools for the analysis of various biomolecules in the areas of medical diagnostics and research. However, limitations in the fabrication, stability and variation of such assays have inhibited their extensive application.⁷ As a result, there is an emerging interest in the use of aptamers as the recognition element in recent times,^{8–10} especially for thrombin.^{11–13}

Aptamers are artificial single-stranded DNA or RNA oligonucleotides which are *in vitro* selected from nucleic acid libraries through SELEX (systematic evolution of ligands by exponential enrichment).^{14,15} In theory, it is probable to acquire all the different types of aptamers to recognize their respective target molecules with high affinity and specificity. As alternatives to antibodies, aptamers offer numerous unprecedented

advantages over the former. The advantages include easy isolation, modification, labeling and storage,^{16,17} good reproducibility, long-term stability and wide applicability.^{18–20}

Graphene and its associated materials, such as the oxidized form, graphene oxide, have experienced a surge in interest from researchers in the past decade.²¹ This is attributed to the excellent properties such as low production costs due to the simple synthetic routes, high electron and thermal conductivity, large surface area and strong mechanical strength which they possess.^{22–25} In particular, graphene is capable of adsorbing oligonucleotides due to the strong unique π – π interactions between graphene and the nitrogenous bases of nucleotides.²⁶ Thus, it is commonly used as a transducing platform for biosensing.^{27–31}

Herein, we employ a fresh approach for the application of graphene in biosensing, using graphene oxide nanoplatelets as the label for thrombin aptasensing. In contrast to graphene, graphene oxide materials are electroactive,^{32,33} *i.e.* they can undergo electrochemical reduction. Hence, the inherent reduction signal of graphene oxide nanoplatelets can be utilized as an analytical signal and the nanoplatelets can function as labels for the detection of thrombin, in a similar manner as metallic nanoparticles which were utilized in the past.³⁴

Experimental section

Materials

Stacked graphite nanofibers (GNFs) were purchased from Strem Chemicals (Newburyport, MA, USA).

Thrombin from human plasma (THR), thrombin aptamer (THR-APT-15) (5' GGT TGG TGT GGT TGG 3'), immunoglobulin G (IgG) from rabbit serum, albumin from bovine serum (BSA),

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avidin (Av), sulfuric acid (H_2SO_4) (95–98%), hydrochloric acid (HCl) (conc. 37%), potassium permanganate (KMnO_4), sodium nitrate (NaNO_3), hydrogen peroxide (H_2O_2), sodium phosphate dibasic salt (Na_2HPO_4), sodium chloride (NaCl), tris(hydroxymethyl)aminomethane ($\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$) (Tris), potassium hexacyanoferrate(II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$), and potassium hexacyanoferrate(III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$) were purchased from Sigma-Aldrich (Singapore).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of resistivity 18.2 M Ω cm.

Buffer solutions used in this study are as follows: PBS (0.01 M Na_2HPO_4 , 0.1 M NaCl, pH 7.0); Tris (0.025 M Tris, 0.3 M NaCl, pH 8.2).

Graphene oxide nanoplatelets (GONPs) were prepared with detailed characterization according to our previous report.³³ Briefly, GNFs were used as the starting material for the synthesis of GONPs. A mixture of NaNO_3 , H_2SO_4 and KMnO_4 was used for the oxidation of GNFs to yield graphite oxide nanofibers. Consequently, the obtained graphite oxide nanofibers were then dispersed in ultrapure water and ultrasonicated for 1 hour in order to acquire GONPs.

Disposable electrical printed (DEP) carbon electrodes were obtained from BioDevice Technology (Nomi, Japan). A three-electrode system was employed in this study which included a carbon-based working and counter electrode, and a Ag/AgCl reference electrode.

Equipment

All electrochemical measurements were performed with a μ Autolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer. Differential pulse voltammetry (DPV) measurements were controlled by General Purpose Electrochemical System (GPES) software version 4.9 and the parameters applied were 50 ms modulation time, 0.5 s interval time, 10 mV step potential, 50 mV modulation amplitude and 20 mV s^{-1} scan rate. DPV measurements were conducted in PBS buffer solution and the raw data produced were treated with a baseline correction of peak width 0.01 using the GPES software. Impedance measurements were controlled by NOVA software version 1.7 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The measurements were performed with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ (1 : 1 molar ratio) in a Tris buffer solution as the redox probe. A Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane. All electrochemical measurements were conducted at room temperature ($\sim 25^\circ\text{C}$) by utilizing DEP carbon electrodes unless otherwise stated.

Procedures

Thrombin aptamer (THR-APT-15) was immobilized onto the surface of a DEP electrode by dry physical adsorption. 3 μL of THR-APT-15 in PBS buffer solution at the optimum concentration of 10 μM was cast onto the clean bare electrode surface and dried in the oven at 60°C for 10 minutes. Subsequently, the

electrode was washed in PBS buffer solution for 5 minutes at 25°C with gentle stirring to remove the excess aptamer which was not adsorbed on the electrode surface.

DEP electrodes modified with THR-APT-15 were next incubated in Eppendorf tubes with Tris buffer solution containing desired concentrations of protein targets (total volume: 100 μL). The incubation was performed for 1 hour at 37°C with gentle stirring. Two washing steps were then conducted in Tris buffer solution at 37°C . Negative controls were carried out using immunoglobulin G, bovine serum albumin and avidin proteins.

Incubation with graphene oxide nanoplatelets (GONPs) was performed in Eppendorf tubes with PBS buffer solution containing desired concentrations of GONPs (total volume: 100 μL). The incubation was carried out for 20 minutes at 25°C with gentle stirring. This was then followed by two washing steps in PBS buffer solution at 25°C .

Results and discussion

Herein, we show the utility of graphene oxide as an inherently electroactive label for the electrochemical aptasensing of thrombin. Scheme 1 shows the illustration of the analytical protocol employed in this study. Thrombin aptamer (THR-APT-15) was first immobilized onto the surface of the electrode by dry physical adsorption.^{35–37} Subsequently, the aptamer-modified electrode was exposed to thrombin before it was incubated in a solution containing a fixed concentration of GONPs (A) or it was just incubated with the solution containing GONPs (B). In the case of (A), upon the exposure of the aptamer-modified electrode to thrombin, thrombin binds specifically to the immobilized THR-APT-15 and results in the partial removal of immobilized THR-APT-15 due to the conformational change of the aptamer when interacting with thrombin.³⁸ Hence, when the modified electrode underwent further incubation with GONPs, the GONPs conjugated to the remaining immobilized THR-APT-15 due to the strong π – π interactions between GONPs and THR-APT-15. In the case of (B), the aptamer-modified electrode was incubated with GONP solution directly and similar to (A), the GONPs conjugated to the immobilized THR-APT-15 due to the unique strong π – π interactions. For both cases, (A) and (B), differential pulse voltammograms were recorded and are demonstrated in Fig. 1.

As displayed in Fig. 1, it can be seen that when the THR-APT-15-modified electrode was exposed to thrombin prior to incubation with GONPs (red), a reduction peak of greater magnitude was attained as compared to a THR-APT-15-modified electrode which was incubated with GONPs directly (blue). Such observation can be related to the result of the partial removal of immobilized THR-APT-15 upon specific binding with thrombin. While the specific binding of thrombin to THR-APT-15 leads to the partial removal of the immobilized THR-APT-15, it also uncovers more of the electrode surface at the same time. Hence, when GONPs conjugate to the remaining immobilized THR-APT-15 subsequently, there is a larger electrode surface available for charge transfer and a greater reduction signal was resulted.

Scheme 1 Illustration of the utilization of graphene oxide nanoplatelets (GONPs) as inherently electroactive labels for the detection of thrombin (THR). (A) In the presence of THR, the aptamer (THR-APT-15) bound specifically to THR and resulted in partial removal of immobilized THR-APT-15 from the electrode surface due to conformational changes. GONPs then adsorbed onto the remaining immobilized THR-APT-15 due to the strong π - π interactions. (B) In the absence of THR, GONPs were adsorbed onto the immobilized THR-APT-15.

Fig. 1 Differential pulse voltammogram of the GONP reduction peak in the presence (red) and absence (blue) of THR. The concentration of THR-APT-15, THR and GONPs used was 10 μM , 40.5 nM and 0.2 mg mL^{-1} respectively. All measurements were conducted in PBS buffer solution (pH 7.0) at room temperature.

Fig. 2 Comparison of impedimetric responses towards the different concentrations of THR-APT-15 deposited on a bare DEP electrode surface. The signal is represented as $(R_{\text{ct aptamer}} - R_{\text{ct blank}})$. Error bars correspond to triplicate experiments. All measurements were conducted with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in Tris buffer solution (pH = 8.2) at room temperature.

In order to determine the optimum concentration of THR-APT-15 to be immobilized onto the electrode surface to ensure maximum surface coverage, THR-APT-15 optimization was next carried out using electrochemical impedance spectroscopy, which is a very sensitive technique for such task.¹³ Impedance spectra obtained were analyzed and illustrated as histograms as shown in Fig. 2.

From Fig. 2, it is examined that with increasing concentration of THR-APT-15, a larger impedimetric signal was obtained. A larger impedimetric signal signifies that there is a greater amount of THR-APT-15 immobilized on the electrode surface. This is because, THR-APT-15 being negatively charged due to the phosphate backbone, will repel the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe which is also negatively charged. Hence, with a higher amount of THR-APT-15 immobilized on the electrode surface, it will lead to a greater repulsion of the redox probe and this in turn causes a larger impedimetric signal. In addition, it is important to note that from 10 to 20 μM of THR-APT-15, the

slight increase in impedimetric signal is accompanied by a greater error bar. Therefore, 10 μM is determined to be the optimum concentration of THR-APT-15, which is to be immobilized on the electrode surface.

Since GONPs were used as the electroactive label in this study, it is hence crucial to determine the optimum concentration of GONPs to be employed. Fig. 3 depicts the voltammetric response of the proposed sensor in relation with the concentration of GONPs. It is noted that the increasing concentration of GONPs gave rise to an increasing voltammetric signal until a plateau was reached. The optimum concentration of GONPs is established to be 0.20 mg mL^{-1} as it produced the greatest peak height value with the smallest error bar.

The voltammetric response of the fabricated sensor towards different thrombin concentrations was next investigated to ascertain the range of detection. As depicted in Fig. 4, the voltammetric peak height increases with increasing concentration

Fig. 3 Histograms illustrating the voltammetric signal towards the GONP concentration. The concentration of THR-APT-15 and THR used was 10 μM and 40.5 nM respectively. Error bars correspond to triplicate experiments. All measurements were carried out in PBS buffer solution (pH 7.0) at room temperature.

Fig. 4 Voltammetric response towards THR concentrations. The concentration of THR-APT-15 and GONP used was 10 μM and 0.2 mg mL^{-1} respectively. Error bars correspond to triplicate experiments. All measurements were carried out in PBS buffer solution (pH 7.0) at room temperature.

of thrombin. For the range of 3 pM to 0.3 μM , the sensor displays good linearity as the R^2 value achieved is 0.937.

As it is of a high importance for future applications to real samples that a biosensing protocol shows selectivity for the target protein and not for other proteins, the selectivity of the sensor was assessed by conducting negative control experiments, with the acquired outcomes shown in Fig. 5. As presented in Fig. 5, incubation with thrombin yielded a voltammetric signal greater than that by a THR-APT-15-modified electrode while incubation with bovine serum albumin (BSA), immunoglobulin G (IgG) and avidin (Av), all as negative controls, produced voltammetric signals which are smaller than the THR-APT-15-modified electrode. This observation can be explained with the reason that as only thrombin binds to THR-APT-15, leading to the partial removal of the immobilized THR-APT-15 and consequently uncovering more electrode surface for charge transfer, a greater voltammetric signal was obtained as compared to that obtained by a THR-APT-15-modified electrode which did not undergo any incubation with a protein target. For the case of negative controls (BSA, IgG and Av), they do not interact with the immobilized THR-APT-15. As a result, there was no partial removal of the immobilized THR-APT-15. In addition, non-specific binding of the negative

Fig. 5 Histograms representing the peak height values obtained before and after incubation with various protein targets. BSA, IgG and Av were used as negative controls. The concentration of THR-APT-15 and THR used was 10 μM and 50 nM respectively. The concentration of BSA, IgG and Av used was 0.25 μM . The concentration of GONPs used was 0.2 mg mL^{-1} . Error bars correspond to triplicate experiments. All measurements were carried out in PBS buffer solution (pH 7.0) at room temperature.

controls to the electrode surface might have occurred, leading to further blocking of the electrode surface for charge transfer and thus, relatively smaller voltammetric signals were acquired. Therefore, the fabricated sensor can be deduced to be selective towards thrombin.

Conclusions

In conclusion, we show that graphene oxide nanoplatelets can serve as an excellent label for the aptasensing of thrombin, due to their extraordinary electrochemistry. Graphene oxide nanoplatelets, being much smaller than the traditional graphene sheets and with higher uniformity of the sizes, can lead to a high amplification of the voltammetric signal. Such nanoplatelets are capable of serving a similar function as metal nanoparticles, as an outstanding voltammetric label. However, graphene oxide nanoplatelets have a strong advantage over metal nanoparticles as labels. The advantage of graphene oxide nanoplatelets over metallic nanoparticles as labels is that for metallic nanoparticles labels, toxic chemicals are used for obtaining the analytical signal *via* a wet chemistry approach.^{39,40} The use of graphene oxide nanoplatelets, however, adopts solid state electrochemistry which does not involve the use of any corrosive or toxic chemicals.⁴¹ Furthermore, the biosensing protocol illustrated here shows that the graphene oxide nanoplatelets system is both sensitive and selective, with a range of detection determined to be 3 pM–0.3 μM , and a good discrimination towards interferences such as IgG, avidin and BSA. The use of graphene oxide nanoplatelets as labels, instead of using them as transducers, exhibits an immense potential for their widespread applications in biomedical fields.

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Inherently electroactive graphene oxide nanoplatelets as labels for specific protein-target recognition

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Graphene related materials have been widely employed as highly efficient transducers for biorecognition. Here we show a conceptually new approach of using graphene oxide nanoplatelets (50 × 50 nm) as voltammetric inherently active labels for specific protein-target molecule recognition. This proof-of-principle is demonstrated by biotin-avidin recognition, which displays that graphene oxide nanoplatelet labels show excellent selectivity. Therefore, it is expected that inherently electroactive graphene oxide nanoplatelet labels will play a similar role as electroactive gold nanoparticle labels which were developed more than a decade ago.

Introduction

Graphene, comprising of a monolayer of carbon atoms closely arranged in a two-dimensional honeycomb framework, has garnered immense attention from researchers since its discovery in 2004 owing to its exceptional thermal,¹ mechanical,² electronic,^{3,4} optical⁵ and electrochemical⁶ characteristics. In addition, graphene and its associated materials, such as graphene oxide,⁷ graphene nanoflakes⁸ and graphene platelets,^{9,10} are also becoming popular materials for sensing and biosensing purposes. Such a trend is attributed to their outstanding properties such as good thermal and electron conductivity, cheap production costs due to the straightforward synthetic processes, strong mechanical strength and high surface area which they exhibit.^{11–14}

Over the years, graphene has been generally employed as a transducing platform for sensing and biosensing.^{15–18} However, in recent times, there has been budding interest in using graphene as a label instead of as a transducing platform. Dong and co-workers¹⁹ presented a new electrochemical biosensing platform by exploiting the excellent electron transfer ability of graphene and its interactions with single-stranded DNA. Based on the proposed platform, they detected for adenosine triphosphate and mercury ions. The basis of detection is that in the absence of the target molecule, the immobilized aptamer could strongly adsorb the graphene label due to the robust π - π interactions, and could lead to a large decrease in the charge transfer resistance of the electrode. However, when the target molecule was present and bound to the aptamer, it inhibited the adsorption of the graphene label and charge transfer resistance could not be lowered. In a related report, Kim and co-workers²⁰ demonstrated an electrochemical mercury ion sensor employing, however,

graphene oxide as the indicator, and charge transfer resistance decreased with increasing concentration of mercury ions as graphene oxide functioned as an insulator in this context. Apart from acting as an insulator, graphene oxide also provides a strong inherent reductive signal which can be exploited for quantification of the material.^{21–23} Recently, a study performed by our group¹⁰ illustrated that graphene oxide nanoplatelets (GONPs) can also be employed as inherently electroactive labels for single nucleotide polymorphism detection. In that study, we utilized the reduction signal of GONPs for the detection of DNA hybridization and polymorphism and proved that single-stranded DNA interacted with GONPs differently from double-stranded DNA. This consequently resulted in a sensitive discrimination of single-base mutation. The cutting-edge novelty of our approach is in the use of inherently electroactive GONPs as labels. Being electroactive, GONPs are capable of providing electrochemical currents originating from the reduction of oxygen-containing groups on their surface. Furthermore, GONPs are not only a new label for bioassays, but also possess many advantages over metal nanoparticle labels. In particular, solid state electrochemistry enables single-step solid state readout of bioassay. This is in contrast to metal or quantum dot labels where toxic chemicals and multi-step protocols involving HBr/Br₂ or concentrated HCl/HNO₃ are employed.^{24–27}

Herein, we illustrate the proof-of-concept of using graphene oxide nanoplatelets as an inherently electroactive label for the sensing of protein-target binding whereby the characteristic reduction signal of nanoplatelets label is employed as an analytical signal.

Experimental section

Materials

Stacked graphite nanofibers (GNFs) were purchased from Strem Chemicals (Newburyport, MA, USA). Biotinylated DNA probe (biotin-probe) (5' biotin-TTT TTT TTT TTT GGT TGG TGT

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GGT TGG 3'), immunoglobulin G (IgG) from rabbit serum, albumin from bovine serum (BSA), avidin (Av), hydrochloric acid (HCl) (conc. 37%), sulfuric acid (H₂SO₄) (95–98%), potassium permanganate (KMnO₄), hydrogen peroxide (H₂O₂), sodium nitrate (NaNO₃), sodium phosphate dibasic salt (Na₂HPO₄), sodium chloride (NaCl), tris(hydroxymethyl)aminomethane (NH₂C(CH₂OH)₃) (Tris), potassium hexacyanoferrate(II) trihydrate (K₄[Fe(CN)₆]·3H₂O), and potassium hexacyanoferrate(III) (K₃[Fe(CN)₆]) were purchased from Sigma-Aldrich (Singapore). Ultrapure water used in this work was acquired from a Milli-Q ion exchange column (Millipore) of resistivity 18.2 MΩ cm. Buffer solutions used in this work are PBS (0.01 M Na₂HPO₄, 0.1 M NaCl, pH 7.0) and Tris (0.025 M Tris, 0.3 M NaCl, pH 8.2) solutions. Disposable electrical printed (DEP) carbon electrodes were acquired from BioDevice Technology (Nomi, Japan). A three-electrode system was adopted in this study and it includes a carbon-based working and counter electrode, and a Ag/AgCl electrode as the reference.

Equipment

All electrochemical measurements were performed with a μAutolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer. Differential pulse voltammetry (DPV) measurements were regulated by General Purpose Electrochemical System (GPES) software version 4.9 and the parameters applied were 50 ms modulation time, 0.5 s interval time, 10 mV step potential, 50 mV modulation amplitude and 20 mV s⁻¹ scan rate. DPV measurements were carried out with PBS buffer solution and the raw data obtained underwent a baseline correction of peak width 0.01 using the GPES software. Impedance measurements were regulated by NOVA software version 1.7 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The measurements were performed with 10 mM K₄[Fe(CN)₆]/K₃[Fe(CN)₆] (1 : 1 molar ratio) in Tris buffer solution as the redox probe. A Randles equivalent circuit was used to fit the acquired impedance spectra, represented as Nyquist plots in the complex plane. All electrochemical measurements were conducted at room temperature, utilizing DEP carbon electrodes unless otherwise stated.

Procedures

A biotinylated DNA probe (biotin-probe) was immobilized onto the surface of a DEP electrode by dry physical adsorption. 3 μl of biotin-probe in PBS buffer solution at the optimum concentration of 20 μM was drop-cast onto the clean bare electrode surface and left to dry in an oven at 60 °C for 10 minutes.²⁸ Subsequently, the electrode was washed in PBS buffer solution for 5 minutes at 25 °C with gentle stirring to remove the excess probe which was not successfully adsorbed onto the electrode surface.

DEP electrodes modified with the biotin-probe were next incubated in Eppendorf tubes with Tris buffer solution containing desired concentrations of protein targets (total volume: 100 μl). The incubation was conducted for 1 hour at 37 °C with gentle stirring. Two washing steps were then carried out in Tris

buffer solution at 37 °C. Negative controls were performed using immunoglobulin G and bovine serum albumin proteins.

Incubation with graphene oxide nanoplatelets (GONPs) was next carried out in Eppendorf tubes with PBS buffer solution containing desired concentrations of GONPs (total volume: 100 μl). The incubation was performed for 20 minutes at 25 °C with gentle stirring. This was then followed by two washing steps in PBS buffer solution at 25 °C.

Graphene oxide nanoplatelets (GONPs) were synthesized with detailed characterization according to our earlier studies.¹⁰ In summary, graphite nanofibers (GNFs) were used as the starting material for the synthesis of GONPs. A combination of NaNO₃, H₂SO₄ and KMnO₄ was used for the oxidation of GNFs to produce graphite oxide powder. After which, the attained graphite oxide powder was dispersed in ultrapure water and ultrasonicated for 1 hour in order to obtain GONPs.

Results and discussion

In this report, we demonstrate the proof-of-concept of employing graphene oxide nanoplatelets (GONPs) as an inherently electroactive label for the detection of protein-target interactions. As a model system, we have used a biotin-probe for the detection of avidin.²⁹

Our strategy is based on the use of a biotinylated DNA probe, instead of a simple biotin probe, to be immobilized on the electrode surface as the biorecognition element. This ensures both strong and stable adsorption of the probe to the electrode surface^{30,31} and a higher degree of freedom for the biotin label which will not be involved in any interactions with the electrode surface.

Scheme 1 depicts the analytical protocol followed in this work. Briefly, the biotinylated DNA was immobilized onto the surface of the electrode *via* a dry physical adsorption method.^{28,32,33} After which, the probe-modified electrode was either incubated in a solution containing a fixed concentration of avidin before it was incubated with GONP solution (A) or just directly incubated with GONP solution (B). In the instance of (A), during incubation with avidin, the biotin moiety on the immobilized biotin-probe bound specifically to avidin and led to the partial removal of the immobilized biotin-probe from the electrode surface.^{34–36} Therefore, as subsequent incubation with GONP solution followed, GONPs attached to the residual immobilized biotin-probe owing to the robust π–π interactions between GONPs and nitrogenous bases of the biotinylated DNA probe and a reduction peak stemming from the characteristic reduction of GONPs was achieved. On the other hand, in the instance of (B) where the probe-modified electrode was directly incubated in GONP solution, the GONPs attached to the immobilized biotin-probe as a result of the π–π interactions, analogous to (A), and gave rise to a reduction signal. For both instances, differential pulse voltammetry measurements were conducted and their respective voltammograms are exhibited in Fig. 1.

From Fig. 1, it is observed that when the probe-modified electrode was incubated with avidin prior to subsequent incubation with GONPs (blue), a reduction peak of higher intensity was achieved in contrast to a probe-modified electrode which

Scheme 1 Schematic diagram depicting the employment of graphene oxide nanoplatelets (GONPs) as a possible inherently electroactive label for the detection of avidin. (A) Upon incubation with avidin, the biotin-probe interacts specifically with avidin and partial removal of the immobilized biotin-probe from the electrode surface is resulted. GONPs subsequently adsorb onto the residual immobilized biotin-probe owing to robust π - π interactions. (B) Without incubation with avidin, GONPs are adsorbed directly onto the immobilized biotin-probe.

Fig. 1 Differential pulse voltammograms of GONPs reduction peak upon incubation (blue) and no incubation (red) with avidin. The concentration of the biotin-probe, avidin and GONPs used was 10 μM , 0.25 μM and 0.2 $mg\ ml^{-1}$ respectively. All measurements were performed with PBS buffer solution (pH 7.0) at room temperature.

underwent direct incubation with GONPs (red). These observations made can be attributed to the partial removal of the immobilized biotin-probe upon specific binding of the biotin moiety to avidin. Owing to the partial removal of the immobilized biotin-probe from the electrode surface, more of the electrode surface was uncovered and thus there was a larger electrode surface available for the charge transfer between GONPs and electrode when GONPs were subsequently attached onto the residual immobilized biotin-probe. As a result, a reduction signal of higher intensity was attained.

To affirm the optimum concentration of the biotin-probe to be immobilized onto the electrode surface in order to achieve maximum coverage of the surface, biotin-probe optimization

was performed employing electrochemical impedance spectroscopy, which is an exceptionally sensitive and appropriate technique for such tasks.^{32,37} Electrochemical impedance spectroscopy measurements were conducted with a range of different biotin-probe concentrations and the spectra obtained were examined and results were displayed as histograms in Fig. 2.

As presented in Fig. 2, a larger impedimetric signal was achieved with increasing concentration of the biotin-probe. The biotin-probe, consisting of a phosphate backbone, is negatively charged and will repel the redox probe which is also negatively charged. As such, with a greater amount of biotin-probe successfully immobilized on the electrode surface, it will give

Fig. 2 Histograms illustrating the different impedimetric responses towards the different concentrations of the biotin-probe immobilized on a bare DEP electrode surface. The signal is represented as $R_{ct}(\text{probe-blank})$. Error bars correspond to triplicate experiments. All measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in Tris buffer solution (pH = 8.2) at room temperature.

rise to a larger repulsion of the redox probe. This in turn brings about a greater impedimetric signal. Therefore, a greater impedimetric signal implies that a higher amount of biotin-probe was immobilized onto the electrode surface and more biotin-probe was immobilized on the electrode surface with increasing concentration of the biotin-probe. Furthermore, it is interesting to mention that the impedimetric signal increases rapidly for the initial concentrations before starting to plateau off from 10 μM . Therefore, 20 μM was determined as the optimum concentration of biotin-probe to be immobilized on the surface of the electrode to ensure maximal surface coverage.

Following which, the selectivity performance of the analytical protocol was assessed by performing negative control experiments and the results are depicted in Fig. 3.

As depicted in Fig. 3, a voltammetric signal greater than that of the probe-modified electrode was only acquired when incubation was performed with avidin. Incubation performed with bovine serum albumin (BSA) and immunoglobulin G (IgG), as negative controls, yielded voltammetric signals which are lower than that of the probe-modified electrode. Such observations can be related to the rationale that as only avidin is capable of specifically interacting with the biotin moiety in the biotin-probe, leading to partial removal of the immobilized biotin-probe and exposing more electrode surface for charge transfer, a larger voltammetric signal was achieved in contrast to a probe-modified electrode which did not undergo incubation with a protein target. For the instance of the two negative controls (BSA and IgG), they are incapable of resulting in partial removal of the immobilized biotin-probe as they do not interact with the immobilized biotin-probe. Moreover, non-specific binding of the negative controls could have taken place, giving rise to additional blocking of the electrode surface and comparatively lower voltammetric signals were obtained. Hence, the proposed analytical protocol was shown to be selective for avidin. To confirm our observation from a statistical point of view, a Student's *t*-test was performed in order to compare the results obtained for positive and negative controls. The difference between mean values of avidin and BSA was significant at the

Fig. 3 Histograms representing the peak height values obtained before and after incubation with various protein targets for 20 μM of biotin-probe. BSA and IgG were used as negative controls. The concentration of avidin, BSA, and IgG used was 0.25 μM . The concentration of GONPs used was 0.2 mg ml^{-1} . Error bars correspond to triplicate experiments. All measurements were performed with PBS buffer solution (pH 7.0) at room temperature.

Fig. 4 Comparison of the voltammetric signal towards GONP concentration. The concentration of the biotin-probe and avidin used was 20 μM and 0.25 μM respectively. Error bars correspond to triplicate experiments. All measurements were performed with PBS buffer solution (pH 7.0) at room temperature.

80% confidence level and 4 degrees of freedom ($t_{\text{calc}}(1.97) > t_{\text{tab}}(1.53)$). Moreover, the difference between mean values of avidin and IgG was significant at the 95% confidence level and 4 degrees of freedom ($t_{\text{calc}}(3.10) > t_{\text{tab}}(2.78)$).

Finally, as GONPs were employed as an electroactive label in this work, we then carried out an optimization experiment to determine the optimum concentration of GONPs to be used. In Fig. 4, the relationship between voltammetric response and concentration of GONPs is illustrated. From 0.2 to 0.3 mg ml^{-1} of GONPs, there is a steady increment in voltammetric signals and it is also noted that these concentrations produced relatively high peak height values with small error bars. Therefore, the optimum concentration of GONPs was concluded to be 0.25 mg ml^{-1} , which is the intermediate concentration for that range. In addition, it was also noted that when the GONP concentration was increased to 0.4 mg ml^{-1} , a decrease in peak height was obtained. This may be due to a reduced efficiency of the conjugation process of GONPs onto the modified electrode because of the increased concentration. Similar behaviour was previously observed for gold nanoparticles used for bio-conjugation purpose.^{38–40} We would like to point out that the strong avidin–biotin interactions are actually beneficial for the sensitivity of the proposed system. This is because, as a result of the strong interactions between avidin and biotin (which is among the strongest known non-covalent interaction ($K_d = 10^{-15} \text{ M}$)), partial removal of the immobilized biotin-probe bound to avidin can occur. This in turn uncovers more of the electrode surface and there is a larger electrode surface available for the charge transfer between graphene oxide nanoplatelets (GONPs) and electrode when GONPs were subsequently conjugated onto the residual immobilized biotin-probe. Hence, a reduction signal of higher intensity was attained compared to the case of no avidin, thereby conferring sensitivity on the system.

Conclusions

In conclusion, we have validated the proof-of-concept of using graphene oxide nanoplatelets as an inherently electroactive

label for the follow up of protein-target molecule recognition due to their exceptional electrochemical behaviour. The proposed analytical concept may also be extended to the detection of avidin associated biomolecules such as streptavidin. In this report, graphene oxide nanoplatelets serve effectively as a voltammetric label owing to their small dimensions and high consistency in sizes. In addition, the analytical protocol employed in this study has also proven to be selective, with a distinctive discrimination towards other proteins such as BSA and IgG at concentration levels similar to real samples.⁴¹ It is expected in the future that the inherently electroactive graphene oxide nanoplatelet labels will play the same role as gold nanoparticle labels that were developed more than a decade ago for the same purposes,⁴² albeit with safer and simpler protocols. The application of the presented concept is not only confined to the given system but can also be extended to be employed for the detection of microbes such as bacteria or fungi.⁴³

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Mycotoxin Aptasensing Amplification by using Inherently Electroactive Graphene-Oxide Nanoplatelet Labels

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We develop an electrochemical aptasensor for ochratoxin A (OTA) by utilizing graphene-oxide nanoplatelets (GONPs) as electroactive labels. OTA aptamer (OTA-apt), which serves as the recognition element, was first immobilized onto the electrode surface before the modified electrode underwent exposure to OTA. Subsequently, incubation with GONPs was performed and the nanoplatelets conjugated to the immobilized OTA-apt through π - π interactions. The principle of detection

lies in the ability of these nanoplatelets to be electrochemically reduced, thereby producing a well-defined reduction peak that was then employed as the analytical signal. The fabricated aptasensor is capable of detecting OTA in the concentration range of 310 fM to 310 pM, with good selectivity against common interferences. The obtained results from this study have the potential to contribute towards the aim of developing a biosensor for OTA, with high selectivity and sensitivity.

1. Introduction

Food safety is presently one of the most important global issues, owing to the rapid development of economics and improvement in the standards of living.^[1] As such, food safety analysis has attracted immense attention from researchers around the globe, where they generally place their point of focus on common food contaminants like mycotoxins.^[2,3] Mycotoxins are omnipresent toxins synthesized from the secondary metabolism of fungi. To date, more than 400 mycotoxins, with individual chemical structures and toxic effects, have been discovered.^[4] Among them, ochratoxin A (OTA) has been a key research interest, owing to its abundance and extreme toxicity,^[5] having nephrotoxic,^[6–8] teratogenic, hepatotoxic, and mutagenic effects on various mammalian species.^[9,10] In addition, clinical tests have also established that OTA could result in cancer in rats and mice and was involved in the pathogenesis of tumors of the upper urinary tract in humans.^[11]

To date, OTA has commonly been detected in a wide variety of food and beverages such as beans, spices,^[12] cereals,^[10,13,14] nuts, dried fruits,^[15] coffee,^[16,17] wine,^[18] and beer. Furthermore, it is not viable to reduce the levels of OTA in food products as it has a high resistance to food preparation processes including roasting, fermenting, and cooking.^[19] Hence, it is essential to acquire selective and sensitive detection of OTA to ensure food safety and minimize the potential health risk to humans as well as the environment.

Conventionally, chromatographic techniques have been adopted for the detection of OTA, owing to the fluorescent nature of the latter.^[20–22] However, despite their accuracy, these analytical methods are often expensive, time consuming, and involve complicated sample treatments that, in turn, require qualified personnel.^[23] Therefore, biochemical methods, which offer simplicity, low cost, rapid response, high sensitivity, and the possibility of miniaturization, prove to be an advantageous alternative. In general, these methods are based on specific interactions between a recognition element and its target, with the interactions translated into a quantifiable signal by employing a suitable transducer.^[24,25]

Aptamers, which are artificial, short, single-stranded oligonucleic acids or peptide molecules selected from a combinatorial library of sequences through the systematic evolution of ligands by exponential enrichment (SELEX) process based on their ability to recognize a target molecule with good specificity and affinity, serve as excellent recognition elements.^[5] In theory, aptamers can be generated according to a wide range of targets, including proteins, organic or inorganic molecules, and drugs. Moreover, aptamers also present unprecedented advantages such as ease of modification with a variety of functional tags, enabling the fabrication of flexible biosensing platforms, and good stability over a range of temperature and pH values.^[23,26,27] In view of the above factors, aptamers, coupled with electrochemical transducers, have been widely employed for the detection of OTA.^[28–31] Similarly, electrochemical transducers offer several plus-points such as the small sample size required and low matrix interferences.^[32]

Graphene and its associated materials, such as graphene oxide,^[33–35] have been a focus for researchers for the past decade, since their discovery, owing to the exceptional mechanical, optical, electronic, thermal, and electrochemical characteristics that they possess.^[36,37] In particular, graphene is capable of adsorbing oligonucleotides by forming strong π - π in-

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teractions with the nitrogenous bases of the nucleotides.^[38] Hence, graphene is being increasingly applied as a transducing platform for biosensing.^[39–43] In recent years, however, there has been a growing interest in employing graphene as a label instead of as a transducing platform.^[36,38,44]

In this study, we utilize graphene-oxide nanoplatelets (GONPs) as labels for the aptasensing of OTA by making use of the π - π interactions between the nanoplatelets and the nitrogenous bases of the nucleotides in an OTA aptamer. In addition, GONPs are inherently electroactive and can provide electrochemical currents, stemming from the reduction of oxygen-containing groups on their surface.^[45,46] Therefore, the inherent reduction signal of GONPs can be exploited as the analytical signal, and the nanoplatelets can serve as labels for OTA aptasensing. The use of GONPs as labels offers the advantage of large signal amplification, whereby a 50×50 nm-sized platelet correlates to about 22 000 withdrawn electrons, as GONPs contain a high density of oxygen functionalities.^[47]

2. Results and Discussion

Herein, we demonstrate the applicability of employing GONPs as inherently electroactive labels for the aptasensing of an important mycotoxin, OTA. Scheme 1 illustrates the analytical procedure utilized in this study. In summary, OTA aptamer (OTA-apt) was first immobilized onto the surface of the electrode, through dry physical adsorption, to serve as the selective biorecognition element. Subsequently, the OTA-apt-modified electrode underwent prior exposure to OTA before incubation with GONPs solution. In addition, a control experiment in the absence of OTA was carried out. For the latter instance, GONPs adsorbed onto the immobilized OTA-apt through π - π interactions. Whereas, for the former instance, upon exposure to OTA, the immobilized OTA-apt bound specifically to OTA, and conformational changes of the OTA-apt were induced.

Scheme 1. Representation of the application of GONPs as inherently electroactive labels for the aptasensing of OTA. In the absence of OTA, the GONPs were adsorbed onto immobilized OTA-apt through π - π interactions. In the presence of OTA, OTA-apt bound specifically to OTA and led to the partial removal of immobilized OTA-apt from the electrode surface, owing to conformational changes. GONPs then adsorbed onto the remaining immobilized OTA-apt. Inset: molecular structure of OTA.

Consequently, the induced conformational changes led to the partial removal of the immobilized OTA-apt from the electrode surface.^[48–50] Hence, when the modified electrode underwent subsequent incubation with GONPs, the GONPs conjugated to the remaining immobilized OTA-apt, owing to π - π interactions between OTA-apt and GONPs. An analytical signal was drawn from the inherent electrochemical reduction of GONPs and differential pulse voltammograms, as displayed in Figure 1, were recorded for both instances.

Figure 1. Differential-pulse voltammograms of the GONPs reduction peak in the presence (red) and absence (blue) of OTA. Concentration of OTA-apt, OTA, and GONPs used was 10 μ M, 0.309 μ M and 0.2 mg mL⁻¹ respectively. All electrochemical measurements were performed in PBS buffer solution (pH 7.0) at room temperature.

As indicated in Figure 1, a greater GONPs reduction signal was attained when the electrode modified with OTA-apt underwent exposure to OTA before incubation with GONPs (red line). This is in comparison with an OTA-apt-modified electrode, which was incubated with GONPs directly (blue line, control experiment). Such observations may be associated with the partial removal of immobilized OTA-apt upon specific binding with OTA. While the specific binding of OTA to OTA-apt leads to conformational changes and subsequent partial removal of the immobilized OTA-apt, it also uncovers more of the electrode surface simultaneously. Therefore, when GONPs subsequently conjugate to the remaining immobilized OTA-apt, there is a bigger electrode surface present for charge transfer and a greater reduction signal obtained.

To reinforce the proposed mechanism of partial removal of the immobilized OTA-apt upon specific binding with OTA, two sets of control experiments were conducted, with the find-

Figure 2. Histograms illustrating the charge-transfer resistance (R_{ct}) of a bare DEP electrode, OTA-apt-modified DEP electrode, and OTA-apt-modified DEP electrode after incubation with OTA. Concentration of OTA-apt and OTA used was 10 and 0.309 μM , respectively. Error bars correspond to replicate experiments. All electrochemical measurements were conducted with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in Tris buffer solution (pH 8.2) at room temperature.

ings illustrated in Figure 2 and Figure S1 (see the Supporting Information). For the first set of control experiments, electrochemical impedance spectroscopy (EIS) was utilized to measure the charge-transfer resistance (R_{ct}) between the electrode surface and negatively charged $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe for the successive modification steps. As depicted in Figure 2, R_{ct} increases drastically with the immobilization of OTA-apt, owing to electrostatic repulsion between similarly negatively charged OTA-apt and redox probe. Subsequently, upon incubating with OTA, R_{ct} experiences a decrease in magnitude, implying a partial removal of the immobilized OTA-apt. To substantiate the proposed mechanism, the second set of control experiments was performed with differential-pulse voltammetry (DPV). For this set of experiments, the guanine oxidation peak was exploited as the analytical signal, and the magnitude of the peak height is a measure of the amount of OTA-apt present on the electrode surface. From Figure S1, it is apparent that the peak height decreases after incubation with OTA, again signifying the removal of some immobilized OTA-apt. Therefore, the proposed mechanism was reasonably established.

Subsequently, OTA-apt, which functions as the biorecognition element, was optimized by employing EIS with the intention of determining the optimum concentration of OTA-apt to be deposited onto the electrode surface to ensure maximum coverage of the surface of the electrode. Impedance spectra were collected and analyzed for various concentrations of OTA-apt, with the results displayed as histograms in Figure 3.

From Figure 3, it can be discerned that the largest impedimetric signal was achieved for an OTA-apt concentration of 10 μM . The magnitude of the impedimetric signal is a measure of the amount of OTA-apt successfully immobilized onto the surface of the electrode. This rationale lies in OTA-apt being negatively charged, owing to the phosphate backbone. As such, there is electrostatic repulsion between OTA-apt and the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe, which is also similarly charged. Hence, with a higher amount of OTA-apt immobilized on the electrode surface, it will lead to a greater repulsion of the redox probe and a larger impedimetric signal is expected. Therefore, 10 μM was concluded to be the optimum concen-

Figure 3. Illustration of impedimetric responses towards various concentrations of OTA-apt deposited onto the surface of bare DEP electrode. OTA-apt in PBS buffer solution (3 μL , pH 7.0) at the desired concentrations was cast onto the clean, bare electrode surface. Signal was represented as ($R_{ct \text{ Apter}} / R_{ct \text{ Blank}}$). Error bars correspond to triplicate experiments. All electrochemical measurements were conducted with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in Tris buffer solution (pH 8.2) at room temperature.

tration of OTA-apt to be deposited onto the surface of the electrode so as to achieve maximum surface coverage. Furthermore, it is also worthy to highlight that the error bar was comparatively the smallest at 10 μM of OTA-apt.

The voltammetric response of the fabricated aptasensor towards various concentrations of OTA was next examined to define the range of detection. As demonstrated in Figure 4,

Figure 4. Comparison of voltammetric signals with different concentrations of OTA. Concentration of OTA-apt and GONPs utilized was 10 μM and 0.2 mg mL^{-1} , respectively. Error bars correspond to triplicate experiments. All electrochemical measurements were carried out in PBS buffer solution (pH 7.0) at room temperature.

the voltammetric response increased with increasing concentration of OTA until a plateau was reached around 310 pM. Beyond that concentration point, further increase of OTA concentration did not lead to any significant changes in the magnitude of the voltammetric signal. Therefore, the range of OTA detection was concurred to be from 310 fM to 310 pM. Furthermore, it should be noted that our acquired detection range is comparatively more sensitive than other proposed electrochemical OTA aptasensors^[51–53] and our detection assay is relatively more straightforward.^[54]

To establish the selectivity performance of the fabricated aptasensor towards OTA, ascorbic acid, which is a common com-

ponent in several foodstuffs, was employed as the negative control. As presented in Figure S2 (see the Supporting Information), the voltammetric signal derived for ascorbic acid was considerably lower than OTA. Furthermore, a Student's t -test was performed at 95% confidence interval and four degrees of freedom, with $t_{\text{cal}} = 3.719 > t_{\text{tab}} = 2.776$ (where t_{cal} is the calculated t value and t_{tab} is the tabulated critical t value). Hence, the voltammetric signals for ascorbic acid and OTA can be considered to be significantly different. Such observations could be justified on the grounds of OTA-apt only binding to OTA and, thus, stimulating conformational changes of its structure, leading to an enhancement of GONPs adsorption, and a relatively high voltammetric response was attained for OTA. Therefore, the aptasensor was deduced to be selective for OTA. Lastly, it is also worthy to highlight that the average relative standard deviation derived for this study was 11.8%, suggesting good reproducibility.

3. Conclusions

This study has demonstrated the potential of employing GONPs as inherently electroactive labels for the aptasensing of OTA. GONPs prove to be an effective electroactive label, owing to their exceptional electrochemistry and small, uniform size. The proposed analytical procedure exhibits that the GONP approach is sensitive and selective, with an established detection range of 310 fM to 310 pM, and a good discrimination against ascorbic acid. These findings are expected to have a profound impact on the fabrication of future biosensors related to food safety analysis.

Experimental Section

Materials

Stacked graphite nanofibers (GNFs) were purchased from Strem Chemicals (Newburyport, MA, USA), OTA was purchased from *Petromyces albertensis*, and OTA-apt (5' GAT CGG GTG TGG GCG TAA AGG GAG CAT CGG ACA 3'), L-ascorbic acid, sulfuric acid (H_2SO_4) (95–98%), hydrochloric acid (HCl) (conc. 37%), potassium permanganate (KMnO_4), sodium nitrate (NaNO_3), hydrogen peroxide (H_2O_2), sodium phosphate dibasic salt (Na_2HPO_4), sodium chloride (NaCl), tris(hydroxymethyl)aminomethane [$\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$] (Tris), potassium hexacyanoferrate (II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$), potassium hexacyanoferrate (III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$) were purchased from Sigma-Aldrich (Singapore). Ultrapure water used in this study was obtained from a Milli-Q ion-exchange column (Millipore) of resistivity of 18.2 M Ω cm.

Buffer solutions used in this study were PBS (0.01 M Na_2HPO_4 , 0.1 M NaCl, pH 7.0) and Tris (0.025 M Tris, 0.3 M NaCl, pH 8.2).

GONPs were prepared with detailed characterization according to our previous report.^[36] In summary, GNFs were used as the starting material for the synthesis of GONPs. A mixture of NaNO_3 , H_2SO_4 , and KMnO_4 was used for the oxidation of GNFs to yield graphite-oxide powder. Subsequently, the obtained graphite-oxide powder was dispersed in ultrapure water and subjected to ultrasonication for 1 h to acquire GONPs.

Disposable electrical printed (DEP) carbon electrodes were obtained from BioDevice Technology (Nomi, Japan). A three-electrode system was employed in this study, which included a carbon-based working and counter electrode and a Ag/AgCl reference electrode.

Equipment

All electrochemical measurements were performed with a μ Autolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer. DPV measurements were controlled by General-Purpose Electrochemical System (GPES) software, version 4.9, and the parameters applied were 50 ms modulation time, 0.5 s interval time, 10 mV step potential, 50 mV modulation amplitude, and 20 mVs⁻¹ scan rate. DPV measurements were conducted in PBS buffer solution and the raw data produced were treated with a baseline correction of peak width 0.01 by using GPES software. Impedance measurements were controlled by NOVA software version 1.7 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The measurements were performed with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$ / $\text{K}_3[\text{Fe}(\text{CN})_6]$ (1:1 molar ratio) in Tris buffer solution as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane. All electrochemical measurements were conducted at room temperature (ca. 25 °C) by utilizing DEP carbon electrodes, unless otherwise stated.

Procedures

OTA-apt was immobilized on the surface of a DEP electrode through dry physical adsorption. OTA-apt in PBS buffer solution at the optimum concentration of 10 μM (3 μL) was cast onto the clean, bare electrode surface and dried in an oven at 60 °C for 10 min. Subsequently, the electrode was washed in PBS buffer solution for 5 min at 25 °C with gentle stirring to remove the excess aptamer that was not adsorbed on the electrode surface.

DEP electrodes modified with OTA-apt were incubated in Eppendorf tubes with Tris buffer solution containing the desired concentrations of OTA (total volume: 100 μL). The incubation was performed for 1 h at 37 °C with gentle stirring. Two washing steps were then conducted in Tris buffer solution at 37 °C.

Incubation with GONPs was performed in Eppendorf tubes with PBS buffer solution containing desired concentrations of GONPs (total volume: 100 μL). The incubation was carried out for 20 min at 25 °C with gentle stirring. This was then followed by two washing steps in PBS buffer solution at 25 °C.

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CVD graphene based immunosensor†

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Graphene synthesis by chemical vapour deposition (CVD) method has been receiving much attention from researchers. This is due to the fact that high quality graphene can be obtained at relatively low production costs. While there has been much advancement in CVD synthesis of graphene, little study has been done on the biosensing applications of CVD graphene. Herein, we aim to draw attention to employing CVD grown graphene as a potential platform for immunosensing of IgG. Using electrochemical impedance spectroscopy (EIS), we obtained a sensitive detection of rabbit IgG in the range of 0.1–100 $\mu\text{g ml}^{-1}$ with untreated CVD graphene as the electrode interface for direct immobilization of the recognition antibodies. From our report, it can be concluded that CVD grown graphene exhibits great potential to be utilized as a platform for immunosensing applications.

Introduction

Comprising of a single layer of carbon atoms that are densely packed in a honeycomb two-dimensional matrix, graphene has been the key interest of many researchers since its discovery in 2004.¹ The enormous attention which graphene is receiving is due to the extraordinary properties it displays. Examples of these properties include superior electron and thermal conductivity, robust mechanical strength and large surface area.^{2–5} As a result of the above mentioned remarkable properties, graphene has also been dubbed as a “miracle material”.⁶ However, some of these mentioned outstanding properties can only be achieved by samples exhibiting the highest quality, such as mechanically exfoliated graphene.¹ Presently, no graphene prepared *via* other techniques has demonstrated equivalent characteristics. Nevertheless, these synthetic methods are rapidly improving.

The methods for the preparation of graphene can be broadly categorized into two main approaches; bottom-up or top-down. For the instance of top-down approach, the working principle is based on stripping individual sheets of graphene from a graphite source material and this approach encompasses techniques such as oxidation of graphite to graphite oxide with subsequent thermal, chemical or electrochemical reduction, and mechanical or liquid-phase exfoliation of graphite.^{7–9} On the other hand, for the case of bottom-up approach, the working principle is based on employing small carbon sources to fabricate graphene. The bottom-up approach comprises of epitaxial growth on silicon carbide¹⁰ and chemical vapour deposition.¹¹

In recent times, much effort has been channelled into research on graphene synthesis by chemical vapour deposition (CVD) method, which can generate high quality graphene with controlled number of layers and possibly at low mass production costs.¹² Although metal catalysts such as iridium, ruthenium, cobalt, platinum and iron have been successfully employed to grow graphene, nickel and copper represent the current most frequently adopted metal catalysts due to their lower cost and ease of controlling the number of graphene layers.^{13–17} CVD grown graphene has been advantageously adopted for the fabrication of transparent electrodes, touch screens, and electronic devices.¹⁸ This is in contrary to thermally/chemically reduced graphenes, which are in the form of powders. However, the use of CVD graphene for biosensing applications has not been fully explored yet. In particular, there have only been a few attempts on the utilization of CVD graphene as an electrochemical biosensing platform and these works include the detection of DNA hybridization and glucose sensing.^{19–21} Till date, there has been no study conducted with the immunology systems.

Hence, in this work, electrochemical impedance spectroscopy (EIS) was employed to monitor the specific interactions between anti-rabbit IgG probes, which were immobilized on a CVD graphene platform, and rabbit IgG protein targets. A stable and uniform immobilization of the recognition element, anti-rabbit IgG, was achieved without any pre-treatment performed on the CVD graphene. In addition, a sensitive and specific detection of rabbit IgG protein, adopted as the model analyte, was obtained. Hence, this work demonstrates great promises for the future use of CVD grown graphene as a biosensing platform.

Experimental section

Materials

Immunoglobulin G from rabbit serum (rabbit IgG), anti-rabbit immunoglobulin G produced in goat (anti-rabbit IgG), albumin

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from bovine serum (BSA), avidin, human hemoglobin, hydrochloric acid (conc. 37%), sodium phosphate dibasic, sodium chloride, Tween® 20, potassium hexacyanoferrate(II) trihydrate and potassium hexacyanoferrate(III) were purchased from Sigma-Aldrich (Singapore).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of resistivity 18.2 MΩ cm.

Buffer solutions used in this study are as follows: PBS (0.01 M phosphate, 0.135 M sodium chloride, pH 7.4), PBS-B (0.01 M phosphate, 0.135 M sodium chloride, 1% BSA, pH 7.4) and PBS-T (0.01 M phosphate, 0.135 M sodium chloride, 0.05% Tween® 20, pH 7.4).

Multilayer graphene (105 nm thick on average) on nickel foil (CVD graphene) was purchased from Graphene Laboratories Inc (Calverton, New York).

Equipment

All electrochemical measurements were conducted with a μAutolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer. Impedance measurements were controlled by NOVA software version 1.8 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The obtained impedance spectra, presented as Nyquist plots in the complex plane, underwent electrochemical circle fitting. All electrochemical measurements were performed at room temperature with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1 : 1 molar ratio) in PBS buffer solution as the redox probe, and Ag/AgCl as the reference electrode.

Raman spectra were acquired by using a confocal micro-Raman LabRam HR instrument (Horiba Scientific) in back-scattering geometry with a CCD detector. A 514.5 nm Ar laser and a 100× objective lens mounted on a Olympus optical microscope were employed for the focusing of the samples. The initial calibration was conducted at 0 and 520 cm^{-1} with a silicon wafer as the reference to give a peak position resolution of less than 1 cm^{-1} .

Scanning electron microscope images were attained by utilizing a JSM-7600F Schottky Field Emission Scanning Electron Microscope (JEOL, Japan) at 5 kV accelerating voltage.

Atomic force microscopy was performed with a MultiMode 8 atomic force microscope (Bruker, Singapore) controlled by Nanoscope 8.15 software and using the ScanAsyst mode.

X-ray photoelectron spectroscopy data were obtained with a Phoibos 100 spectrometer and an Mg X-ray radiation source (SPECS, Germany) for the measurement of wide-scan survey spectra and high-resolution N 1s spectra.

Procedures

Commercial CVD graphene was washed gently with ultrapure water before use.

Anti-rabbit IgG was immobilized onto the surface of CVD graphene by dry physical adsorption. 50 μl of anti-rabbit IgG in PBS buffer solution at a concentration of 100 μg ml⁻¹ was deposited onto CVD graphene and left to dry under the lamp for 30 minutes. Subsequently, the modified surface underwent

gentle washings with PBS-T buffer solution, PBS buffer solution and ultrapure water to remove the excess anti-rabbit IgG that was not well adsorbed on the surface.

50 μl of PBS-B buffer solution was next drop-cast on the anti-rabbit IgG modified CVD graphene surface and placed under the lamp for 10 minutes for drying. After which, gentle washings with PBS-T buffer solution, PBS buffer solution and ultrapure water were performed to remove the excess BSA which was not well adsorbed on the surface.

CVD graphene modified with anti-rabbit IgG and BSA then underwent incubation with rabbit IgG in PBS-T buffer solution. The incubation was performed at 37 °C for 1 hour in the oven. Finally, gentle washings with PBS-T buffer solution, PBS buffer solution and ultrapure water were performed to remove the excess of non-specifically adsorbed species. For selectivity study, negative controls were conducted using BSA, hemoglobin and avidin.

Results & discussions

We investigate here the analytical proof-of-concept of performing immunosensing of IgG on CVD graphene. First of all, in order to gain a better understanding of the material employed as the sensing platform, characterizations by scanning electron microscopy (SEM), atomic force microscopy (AFM) and Raman spectroscopy were conducted with CVD graphene.

Characterization of CVD graphene was first performed with SEM to acquire information regarding its surface morphology. The SEM images obtained are shown in Fig. S1 (ESI).† Imaging of CVD graphene was carried out at two different magnifications of 370× (Fig. S1A†) and 10 000× (Fig. S1B†). From the SEM images, it can be observed that the CVD graphene, which was grown on nickel foil, demonstrates several grain boundaries between continuous islands of graphene. In addition, different colours ranging from white to dark grey, indicating regions with different number of graphene layers, can also be seen. Such observation is a common occurrence in samples grown on thin nickel films.²² The homogeneous colour distribution, however, suggests a homogeneous graphene film thickness. Fig. S1B† clearly shows the edges of the graphene islands grown during the catalytic process. The edges are indicated by white lines which also account for folded graphene. On the other hand, the darker lines seen in Fig. S1B† may indicate structural discontinuity or cracks.

In order to gain more insights about the surface of the CVD graphene employed, AFM analysis was next conducted. Fig. S2 (see ESI†) presents the AFM characterization of CVD graphene. The two-dimensional and three-dimensional profiles of CVD graphene are depicted in Fig. S2A and B† respectively. From the two figures, it can be confirmed that several of the boundaries visible in SEM as white lines, resemble folded graphene sections which emerge from the film surface at a height of approximately 60 nm (Fig. S2C†).

Lastly, Raman spectroscopy was carried out to attain further structural information such as the presence of defects and the number of layers in CVD graphene. Fig. S3† displays the Raman

spectrum obtained in this study and it was noted that there is an absence of the D band ($\sim 1350\text{ cm}^{-1}$) in the spectrum. This suggests that there is no significant presence of structural defects in the sp^2 lattice of CVD graphene. The average ratio of the intensity of G band ($\sim 1560\text{ cm}^{-1}$) to 2D band ($\sim 2700\text{ cm}^{-1}$) was calculated to be approximately 2.21. This implies that the CVD graphene is of multilayer structure.²³ Moreover, another piece of evidence which indicates the multilayer property is the slight shoulder observed at 2D band as single layer graphene will exhibit a symmetrical 2D band.²⁴

Following the various characterizations, CVD graphene was employed as the transducing platform for the immunosensing of rabbit IgG by using electrochemical impedance spectroscopy (EIS) as the detection technique.²⁵ Scheme 1 illustrates the analytical protocol adopted. In summary, anti-rabbit IgG probes were first immobilized onto the surface of CVD graphene by physical adsorption. The successful attachment of anti-rabbit IgG probes onto CVD graphene was characterized by the appearance of a N 1s peak in X-ray photoelectron spectroscopy (XPS) study after the anti-rabbit IgG immobilization process (see Fig. S4, ESI†). After which, BSA blocking was performed by drop-cast method in order to block off the remaining CVD graphene surface and deter non-specific binding. Lastly, incubation with rabbit IgG target was carried out. For each stage of the analytical procedure, impedance measurement was conducted and the respective Nyquist plots are shown in Scheme 1.

Briefly, bare CVD graphene demonstrated relatively the lowest charge transfer resistance as it was entirely accessible to the redox probe. Subsequently, anti-rabbit IgG probes were immobilized onto the surface of CVD graphene and the magnitude of charge transfer resistance increased owing to the decrease in accessibility of CVD graphene by the redox probe. After which, blocking of the remaining CVD graphene surface with BSA was performed and this further lowered the accessibility by redox probe and charge transfer resistance was further

enhanced. Last of all, incubation with rabbit IgG, which binds specifically to anti-rabbit IgG, was carried out and it led to another increase in charge transfer resistance. This can be either attributed to the additional steric hindrance caused by the bulky rabbit IgG protein molecule, or the electrostatic interactions between rabbit IgG and redox probe.^{26,27} It should be noted that in our previous article, we have compared physical adsorption, chemical linker and biotin/avidin linker for apta-sensing on graphenes and we found that the highest sensitivity was exhibited by physical adsorption.²⁸

Selectivity is a fundamental requirement of a functional sensor. Hence, the selectivity performance of the proposed immunosensing platform was investigated by conducting negative control experiments with BSA, hemoglobin and avidin. The conclusions from the experiments are exemplified in Fig. 1.

From Fig. 1, it can be noticed that the impedimetric signal ($R_{\text{ct Target-Probe}}$) of rabbit IgG is much greater than that of the negative control proteins, BSA, hemoglobin and avidin. This indicates that the negative control proteins, BSA, hemoglobin and avidin, have negligible interactions with the immobilized anti-rabbit IgG probes. As such, the increase in charge transfer resistance after incubation with the negative control proteins is small, resulting in the low impedimetric signals. Therefore, the proposed immunosensing platform was deduced to be selective for rabbit IgG.

Subsequently, to assess the sensitivity of the proposed sensing system and to evaluate the range of detection, the variation of impedimetric signal with rabbit IgG concentration was examined. As depicted in Fig. 2, the impedimetric signal increases with increasing concentration of rabbit IgG and the linear range of detection was determined to be from 0.1 to $100\text{ }\mu\text{g mL}^{-1}$ with the limit of detection assessed to be $0.136\text{ }\mu\text{g mL}^{-1}$. With our acquired range of detection, the immunosensor has the potential to detect IgG in human samples as IgG is present in human serum and plasma samples in the range of $4\text{--}16\text{ mg mL}^{-1}$.

With the selectivity and sensitivity aspects of the proposed sensing platform studied, we next move on to the optimization of the analytical protocol employed.

Scheme 1 Schematic illustration of the protocol and Nyquist plots ($-Z''$ vs Z') of CVD graphene (black box), CVD graphene with immobilized anti-rabbit IgG (red circle), anti-rabbit IgG modified CVD graphene after BSA blocking (blue triangle) and anti-rabbit IgG modified CVD graphene after BSA blocking and incubation with rabbit IgG (pink pentagon). All measurements were performed with $10\text{ mM K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in PBS buffer solution (pH 7.4) at room temperature with Ag/AgCl as reference electrode.

Fig. 1 Illustration of the impedimetric response towards different protein. BSA, hemoglobin and avidin were employed as negative controls. Signal is represented as $R_{\text{ct Target-Probe}}$. The error bars correspond to replicate experiments. All measurements were performed with $10\text{ mM K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in PBS buffer solution (pH 7.4) at room temperature with Ag/AgCl as reference electrode.

Fig. 2 Impedimetric response towards different concentration of rabbit IgG. Signal is represented as $R_{ct \text{ Target-Probe}}$. The error bars relate to replicate experiments. All measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution (pH 7.4) at room temperature with Ag/AgCl as reference electrode. Units of concentration: $\mu\text{g ml}^{-1}$.

Anti-rabbit IgG optimization was carried out to establish the optimum concentration of the anti-rabbit IgG probe to be deposited onto the surface of CVD graphene in order to achieve maximum surface coverage. Impedance measurements were performed for a series of anti-rabbit IgG concentration and the spectra acquired were analysed and displayed as histograms shown in Fig. 3.

As exhibited in Fig. 3, the impedimetric signal ($R_{ct \text{ Probe/Blank}}$) increases progressively as the concentration of anti-rabbit IgG increases from 0.01 to 10 $\mu\text{g ml}^{-1}$. However, when the concentration of anti-rabbit IgG further increases to 100 $\mu\text{g ml}^{-1}$, a decrease in the signal can be observed. A higher impedimetric signal correlates to a greater amount of anti-rabbit IgG being successfully immobilized onto the surface of CVD graphene. For that reason, 10 $\mu\text{g ml}^{-1}$ is determined to be the optimum concentration of anti-rabbit IgG to be deposited onto the surface of CVD graphene as it ensures the greatest coverage of the surface.

CVD graphene offers several advantages. For example, the previous research on chemically modified graphenes exhibited

Fig. 3 Comparison of impedimetric response towards different concentration of anti-rabbit IgG. Signal is represented as $R_{ct \text{ Probe/Blank}}$. The error bars correspond to replicate experiments. All measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution (pH 7.4) at room temperature with Ag/AgCl as reference electrode.

poorer performance, with a detection range of 0.3–7 $\mu\text{g ml}^{-1}$.²⁹ In addition, here we compare the selectivity with BSA, hemoglobin and avidin while previously the comparison of selectivity was done only with BSA.²⁹ Moreover, CVD graphene is transparent and it can be easily integrated into transparent electronic devices.

In addition, in order to ensure that the physiological pH (7.4) employed for the EIS measurements was suitable for the proposed immunosensing concept, a pH dependent EIS study was conducted (see Fig. S5, ESI†). From the obtained results, it can be confirmed that pH 7.4 is the most appropriate pH to be utilized for this study.

Last of all, the stability of the fabricated immunosensor was assessed by performing a stability study over a time period of seven days (see Fig. S6, ESI†). The results indicate that after seven days, the recorded signal decrease was 48% with an increase in the % RSD value.

Conclusions

In summary, we have demonstrated in this study the proof-of-principle of performing immunosensing of IgG on CVD graphene. The proposed sensing concept was shown to be selective for IgG, with good discrimination from hemoglobin, avidin and BSA. Furthermore, the linear range of detection was determined to be from 0.1 to 100 $\mu\text{g ml}^{-1}$, thereby conferring reasonable sensitivity to the proposed system. Probe optimization study concluded that 10 $\mu\text{g ml}^{-1}$ is the optimum concentration of anti-rabbit IgG to be deposited on the surface of CVD graphene. CVD graphene based immunosensors should find their way into biomedical applications in the near future.

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Short communication

Exfoliated transition metal dichalcogenides (MoS₂, MoSe₂, WS₂, WSe₂): An electrochemical impedance spectroscopic investigation

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ABSTRACT

Owing to the enthralling properties which transition metal dichalcogenides present, they are facing immense scientific interest from researchers. Till date, these two-dimensional materials have been assessed for a wide array of different applications and there are various synthetic methods of attaining them in their respective bulk and exfoliated forms. Herein, we explore the effects of lithium ion intercalation exfoliation process on the charge transfer resistance of transition metal dichalcogenide materials (MoS₂, MoSe₂, WS₂ and WSe₂). We also show that electrochemical activation of the transition metal dichalcogenides results in decreased resistance towards charge transfer, as demonstrated by electrochemical impedance spectroscopy.

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1. Introduction

Two-dimensional (2D) materials, existing in the bulk form as stacks of strongly bonded layers with weak interlayer interactions; allowing exfoliation into single, atomically thin layers [1], have garnered immense interest from researchers for decades. An example of a 2D material which is currently attracting the most attention is graphene, the monolayer associate of graphite [2]. Nonetheless, with the success of graphene, other forms of 2D materials such as transition metal dichalcogenides (TMDs) [3,4] are experiencing a surge of scientific and engineering interest.

TMDs are a class of materials with the generalized formula of MX₂, whereby M represents a transition metal element from group 4, 5 or 6 and X represents a chalcogen. These materials are layered compounds of similar structure to graphite and parallel to graphene, individual layers of TMDs can be isolated from the bulk form via mechanical cleavage [5–7], liquid exfoliation [8,9] or ion intercalation [10–13] methods. In particular, ion intercalation methods have been demonstrated to yield a higher percentage of monolayers [11] as compared to the other separation techniques. The monolayer of TMD materials is constituted of a plane of metal atoms sandwiched by two hexagonal planes of chalcogen atoms which are covalently bonded to the metal atoms [2]. On the other hand, adjacent layers are weakly held together by van der Waals interactions to form the bulk structure.

While TMD materials in general, are capable of displaying a wide range of optical, electronic, chemical, mechanical and thermal properties

[4,14], it is crucial to highlight that the 2D exfoliated counterparts of TMD materials often present properties which differ vastly from those of the bulk materials [11], such as sizable bandgaps and chemical versatility [15].

In view of the intriguing properties which TMD materials offer, they have found their applications in advanced energy storage [16], such as lithium ion battery [17] and supercapacitors [18], electrochemical catalysis, such as hydrogen evolution [19–21] and hydrosulfurization, toxicology studies [22], and molecular sensing, such as gas and biomolecule sensors [23–27].

However, despite the elaborate study on synthesis methods and development of applications of TMD materials, there is presently a lack in fundamental knowledge of the inherent electrochemical characteristics of TMDs. This is especially the case after exfoliation of their bulk structure into few or single layers [28].

In view of the current interest to employ TMDs for a wide array of applications, a fundamental study on the effects of exfoliation on the inherent electrochemical properties of TMD materials is crucial.

For this aim, we perform electrochemical impedance spectroscopy to characterize TMD materials which have undergone exfoliation employing lithium ion intercalation method with lithium intercalants of differing strengths, in order to gain some insights on the implications of the exfoliation process on the charge transfer resistance of TMD materials.

2. Experimental section

2.1. Materials

Hydrochloric acid (HCl) (conc. 37%), sodium phosphate dibasic salt (Na₂HPO₄), sodium chloride (NaCl), potassium hexacyanoferrate (II)

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trihydrate ($K_4[Fe(CN)_6] \cdot 3H_2O$) and potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$) were purchased from Sigma-Aldrich (Singapore). Molybdenum sulfide (MoS_2), methyl lithium (1.6 M in diethyl ether), *n*-butyl lithium (1.6 M in hexane) and *t*-butyl lithium (1.7 M in pentane) were obtained from Sigma-Aldrich (Czech Republic). Hexane was obtained from Lach-ner (Czech Republic). Molybdenum selenide ($MoSe_2$), tungsten sulfide (WS_2) and tungsten selenide (WSe_2) were obtained from Alfa Aesar (Germany). Argon (99.9999% purity) was obtained from SIAD (Czech Republic).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of resistivity 18.2 MΩ cm.

Disposable electrical printed (DEP) carbon chips were obtained from BioDevice Technology (Nomi, Japan). A three-electrode system was employed in this study which included a carbon-based working and counter electrode, and a Ag/AgCl reference electrode.

The following transition metal dichalcogenide (TMD) materials were employed in this study:

Molybdenum sulfide (MoS_2) (bulk, methyl lithium exfoliated, *n*-butyl lithium exfoliated and *t*-butyl lithium exfoliated forms)

Molybdenum selenide ($MoSe_2$) (bulk, methyl lithium exfoliated, *n*-butyl lithium exfoliated and *t*-butyl lithium exfoliated forms)

Tungsten sulfide (WS_2) (bulk, methyl lithium exfoliated, *n*-butyl lithium exfoliated and *t*-butyl lithium exfoliated forms)

Tungsten selenide (WSe_2) (bulk, methyl lithium exfoliated, *n*-butyl lithium exfoliated and *t*-butyl lithium exfoliated forms)

2.2. Procedures

Exfoliation of TMD materials (MoS_2 , $MoSe_2$, WS_2 , WSe_2) was carried out by suspending 3 g of TMD bulk powder in 20 mL of 1.6 M methyl lithium in diethyl ether, 20 mL of 1.6 M *n*-butyl lithium in hexane, or 20 mL of 1.7 M *t*-butyl lithium in pentane. The solution was then stirred for 72 h at 25 °C under argon atmosphere. The Li-intercalated material was next separated by suction filtration under argon atmosphere and the intercalating compound was washed several times with hexane (dried over Na). Subsequently, the separated TMD with intercalated Li

was placed in water (100 mL) and repeatedly centrifuged (18 000 g). Finally, the material was dried in a vacuum oven at 50 °C for 48 h prior to further use.

DEP carbon chip surface was modified with TMD materials by physical adsorption. 3 μL of TMD materials at a concentration of 1 mg/mL in ultrapure water was deposited onto each carbon chip surface and left to dry under ambient conditions overnight. The excess of TMD materials which was not well adsorbed on the chip surface was then removed by gentle rinsing with ultrapure water. Prior to depositing the materials onto the surface of the chips, TMD suspensions were sonicated for 15 min to achieve maximum dispersion of the materials.

2.3. Equipment

All electrochemical measurements were performed with a μAutolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer.

Impedance measurements were controlled by NOVA software version 1.9 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The measurements were performed with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1 molar ratio) in 0.1 M PBS buffer solution (0.1 M NaCl + 10 mM Na_2HPO_4 , pH 7.0) as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane.

All electrochemical measurements were conducted under ambient conditions by utilizing DEP carbon chips unless otherwise stated.

3. Results & discussions

Electrochemical impedance spectroscopy (EIS) represents a powerful, sensitive and non-destructive tool which is widely employed for investigating interfacial properties of a broad range of materials [29,30]. Specifically, EIS is capable of assessing the ability of the material to transfer and exchange charges with molecules in proximity.

Fig. 1. Nyquist plots ($-Z''$ vs. Z') of the different transition metal dichalcogenide (TMD) materials: (A) molybdenum sulfide; (B) molybdenum selenide; (C) tungsten sulfide; and (D) tungsten selenide, in their bulk (black) and various exfoliated forms (red, blue and pink). All measurements were conducted under ambient conditions with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS buffer solution as the redox probe.

Fig. 2. Histograms illustrating the impedimetric signal of the different transition metal dichalcogenide (TMD) materials in their bulk and various exfoliated forms. Impedimetric signal is represented as $R_{ct}(\text{TMD} - \text{Bare})$. Error bars correspond to triplicate experiments. All measurements were conducted under ambient conditions with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in PBS buffer solution as the redox probe.

Therefore, EIS was performed to evaluate the resistance of the various transition metal dichalcogenide (TMD) materials towards heterogeneous charge transfer, with the Nyquist plots attained from the EIS measurements depicted in Fig. 1. The diameter of the semi-circle in a Nyquist plot corresponds to the charge transfer resistance value, which is inversely proportional to the rate of heterogeneous charge transfer.

From the acquired Nyquist plots, the respective charge transfer resistance values were inferred and analyzed, with the results of the analysis demonstrated as histograms, as represented in Fig. 2.

As illustrated in Fig. 2, the different TMD materials in their bulk and various exfoliated forms, produced impedimetric signals of varying intensities. Furthermore, from the histograms plotted, a few conclusions can be drawn. First of all, selenide TMDs (MoSe_2 and WSe_2) present faster heterogeneous electron transfer, while sulfide TMDs (MoS_2 and WS_2) show a slower rate of heterogeneous electron transfer towards the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe. Such conclusion is inferred from the overall lower impedimetric signals demonstrated by MoSe_2 and WSe_2 , as compared to MoS_2 and WS_2 . Another conclusion which can be derived from Fig. 2 is that while the impedimetric signals of the various exfoliated forms for MoSe_2 and WSe_2 do not seem to follow any particular trend with lithium intercalants of increasing strength ($\text{MeLi} < \text{BuLi} < \text{t-BuLi}$), a general decreasing trend and a slight increasing trend can be observed for the case of MoS_2 and WSe_2 respectively. In addition, it is also worthy to highlight that the average relative standard deviation percentage value obtained for the series of experiments illustrated in Fig. 2 is 7.17%.

Fig. 3. Histograms depicting the impedimetric signal of the different *t*-BuLi exfoliated transition metal dichalcogenide (TMD) materials with (yellow) and without (pink) prior activation step. Impedimetric signal is represented as $R_{ct}(\text{TMD} / \text{Bare})$. Error bars correspond to replicate experiments. All measurements were conducted under ambient conditions with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ in PBS buffer solution as the redox probe.

Subsequently, in order to assess the potential impact which electrochemical activation, via the cycling of potential, has on the charge transfer resistance of the TMD materials, comparison experiments were conducted with *t*-BuLi exfoliated TMD materials. Electrochemical activation was achieved by using cyclic voltammetry over a potential range of 1 to -1 V, at a scan rate of 0.1 V/s and for two scans. Fig. 3 depicts the obtained results from the comparison study.

From Fig. 3, it is apparent that electrochemical activation of the TMD materials led to a lower impedimetric signal and hence weaker charge transfer resistance towards the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe. Such observations can be elucidated by changes in the surface chemistry of the TMD materials upon electrochemical activation, hence resulting in lesser repulsion of the redox probe and facilitating heterogeneous electron transfer. On another note, the average relative standard deviation percentage value determined for this set of experiments is 18.3 %.

4. Conclusions

In summary, we have evaluated the resistance of transition metal dichalcogenide (TMD) materials, which have undergone exfoliation employing lithium ion intercalation method with lithium intercalants of differing strengths, towards heterogeneous charge transfer by utilizing electrochemical impedance spectroscopy. From the study, it is demonstrated that selenide TMD materials generally display a faster rate of heterogeneous electron transfer as compared to the sulfide counterparts. In addition, it is also discovered that electrochemical activation, via the cycling of potential, of TMD materials lead to an enhancement in the rate of heterogeneous electron transfer. These conclusions are expected to provide constructive insights on the implications of the exfoliation process on the inherent electrochemistry of TMD materials.

Conflict of interest

The authors declare that there is no conflict of interest.

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Transitional Metal/Chalcogen Dependant Interactions of Hairpin DNA with Transition Metal Dichalcogenides, MX_2 **

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Owing to the attractive properties that transition metal dichalcogenides (TMDs) display, they have found recent application in the fabrication of biosensing devices. These devices involve the immobilization of a recognition element such as DNA onto the surface of TMDs. Therefore, it is imperative to examine the interactions between TMDs and DNA. Herein, we explore the effect of different transition metals (Mo and W) and chalcogens (S and Se) on the interactions between hairpin DNA and TMDs of both bulk and *t*-BuLi exfoliated forms. We discovered that the interactions are strongly dependent on the metal/chalcogen composition in TMDs.

Layered and two-dimensional (2D) materials have experienced an upsurge of research interest in the past decade, as demonstrated by the rapid rise of graphene.^[1–3] Nonetheless, it should be highlighted that graphene itself is just a piece of the pie in the research field of layered 2D materials and with the success of graphene, other forms of layered 2D materials such as transition metal dichalcogenides (TMDs) are also attracting immense attention from researchers, owing to the broad range of interesting properties they display.^[4,5]

TMDs exhibit a structure analogous to graphite.^[6,7] Hence, individual layers of TMDs can be isolated from the bulk form through a wide array of methods such as ion intercalation,^[8–10] liquid exfoliation,^[11,12] and mechanical cleavage.^[13–15] A single layer of TMDs is comprised of a layer of transition metal atoms sandwiched between two layers of chalcogen atoms, which are covalently bonded to the transition metal atoms. In contrast, between the individual layers of TMDs, the interactions are of a van der Waals nature.^[16]

Owing to the extensive range of electronic, chemical, and optical properties that TMD materials exhibit,^[17,18] the employment of TMD materials for the development of biosensors has recently been proposed. TMD materials can either serve as a transducing platform or as electroactive labels for the fabrication of biosensors.^[19]

The fabrication of biosensors with the utilization of TMD materials as the transducing platform generally involves the immobilization of a DNA sequence onto the surface of TMD materials to serve as a recognition element for the targeted biomolecules. Immobilization of a DNA sequence onto the surface of TMD materials is achieved through the van der Waals forces between the nucleobases and basal plane of the TMD materials.^[20–22] In view of the current budding interest in employing TMDs for the construction of biosensors, it is therefore crucial to investigate the interactions between DNA and TMD materials.

For this purpose, we investigate the interactions of hairpin DNA (Hp DNA) molecules with the surface of different TMD materials (MoS_2 , MoSe_2 , WS_2 , and WSe_2) (Figure 1).^[23–25] In par-

Figure 1. Schematics illustrating the physical immobilization of Hp DNA molecules onto TMD materials (MoS_2 , MoSe_2 , WS_2 , and WSe_2).

ticular, we assess Hp DNA interactions with both the bulk (non-exfoliated) form as well as the exfoliated (*t*-BuLi exfoliated) form of layered TMDs.^[23] Electrochemical impedance spectroscopy (EIS), which is capable of evaluating the resistance of a surface towards heterogeneous charge transfer,^[26–29] is utilized to study the interactions between Hp DNA and TMD materials in this work. Specifically, the effect of different transition metals (Mo and W) and chalcogens (S and Se) on the interactions between Hp DNA and TMD materials is examined.

In this work, EIS measurements were performed before and after the physical immobilization of Hp DNA molecules onto the surface of TMD materials to assess the change in charge transfer resistance (R_{ct}) upon the Hp DNA immobilization process.

Upon the immobilization of Hp DNA molecules onto the surface of TMD materials, an enhancement in the R_{ct} is observed. This occurs because Hp DNA molecules contain a negatively charged phosphate backbone and this will in turn lead to re-

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[**] M = Mo, W; X = S, Se

Figure 2. Histograms demonstrating the impedimetric signals of the different TMD materials in their bulk (purple) and *t*-BuLi exfoliated (cyan) forms after the physical immobilization of Hp DNA molecules. Impedimetric signal is represented as $R_{ct(Hp\ DNA-TMD)}$. Error bars relate to replicate experiments. All measurements were performed under ambient conditions with 10 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in PBS solution as the redox probe.

pulsion with the similarly negatively charged redox probe, and further hinder charge transfer. Hence, an increase in R_{ct} results. The increase in R_{ct} upon the physical immobilization of Hp DNA molecules ($R_{ct(Hp\ DNA-TMD)}$) is then utilized as the analytical impedimetric signal, as demonstrated in Figure 2.

The interactions of Hp DNA with two types of TMD materials (bulk and *t*-BuLi exfoliated) were studied (Figure 2). A few important conclusions can be inferred from the plotted histograms, which reflect the increase in R_{ct} , and thus, the amount of Hp DNA at the solid/liquid interface, as represented by the TMD and aqueous redox probe phase. First of all, for both Mo and W, the difference in impedimetric signals between their sulfides and selenides is more pronounced in the bulk form (Figure 2; purple), as compared to the *t*-BuLi exfoliated form (Figure 2; cyan). This implies that in the bulk form, the selenide TMDs (MoSe₂ and WSe₂) have much stronger interactions with Hp DNA molecules than their sulfide counterparts (MoS₂ and WS₂). However, upon undergoing exfoliation with *t*-BuLi, the difference in the strength of interactions becomes much smaller.

Secondly, for both bulk and *t*-BuLi exfoliated forms, selenide TMDs consistently display relatively greater impedimetric signals, as compared to sulfide TMDs. Such observation suggests that the increase in R_{ct} after the physical immobilization of Hp DNA molecules is higher for selenide TMDs than for sulfide TMDs. Hence, the physical immobilization of Hp DNA molecules is more favorable on the surface of selenide TMDs than sulfide TMDs, and selenide TMDs have stronger interactions with Hp DNA than sulfide TMDs.

On another note, by assessing molybdenum and tungsten, it can be noticed that molybdenum TMDs (MoS₂ and MoSe₂) also constantly exhibit larger impedimetric signals than that of their tungsten associates (WS₂ and WSe₂) for both bulk and *t*-BuLi exfoliated forms. Therefore, this signifies that molybdenum TMDs have greater interactions with Hp DNA in comparison with tungsten TMDs.

To summarize, we assessed the influence of different transition metals (Mo and W) and chalcogens (S and Se) on the in-

teractions between Hp DNA and TMD materials. From the study, it was revealed that selenide and molybdenum TMD materials present stronger interactions with Hp DNA in contrast to sulfide and tungsten TMD materials, respectively. In addition, it is also noted that selenide TMDs have much greater interactions with Hp DNA molecules than their sulfide counterparts in the bulk form. However, upon undergoing exfoliation, the difference in the degree of interactions becomes less significant. These findings are anticipated to contribute useful insights on the fabrication of any DNA-based architectures at TMD platforms, including those for biosensing devices.

Experimental Section

Materials

Hydrochloric acid (HCl; conc. 37%), sodium phosphate dibasic salt (Na₂HPO₄), sodium chloride (NaCl), potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6] \cdot 3H_2O$), potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$), and hairpin DNA (Hp DNA) were purchased from Sigma-Aldrich (Singapore). Molybdenum sulfide (MoS₂) and *tert*-butyl lithium (1.7 M in pentane) were obtained from Sigma-Aldrich (Czech Republic). Molybdenum selenide (MoSe₂), tungsten sulfide (WS₂), and tungsten selenide (WSe₂) were obtained from Alfa Aesar (Germany). Argon (99.9999% purity) was obtained from SIAD (Czech Republic). Hexane was obtained from Lachner (Czech Republic).

The sequence of Hp DNA is as follows:

5' ATG GAG ACC AGG CGG CCG CAC ACG TCC TCC AT 3'

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) of resistivity 18.2 MΩ cm.

Disposable electrical printed (DEP) carbon chips were obtained from BioDevice Technology (Nomi, Japan). A three-electrode system was employed in this study that included a carbon-based working and counter electrodes, and a Ag/AgCl reference electrode.

The following transition metal dichalcogenide (TMD) materials were employed in this study: molybdenum sulfide (MoS₂; bulk and *tert*-butyl lithium exfoliated forms), molybdenum selenide (MoSe₂; bulk and *tert*-butyl lithium exfoliated forms), tungsten sulfide (WS₂; bulk and *tert*-butyl lithium exfoliated forms), and tungsten selenide (WSe₂; bulk and *tert*-butyl lithium exfoliated forms).

Procedures

The exfoliation of TMD materials (MoS₂, MoSe₂, WS₂, and WSe₂) was carried out by suspending TMD bulk powder (3 g) in 1.7 M *tert*-butyl lithium in pentane (20 mL). The solution was then stirred for 72 h at 25 °C under an argon atmosphere. The Li-intercalated material was next separated by suction filtration under an argon atmosphere and the intercalating compound was washed several times with hexane (dried over Na). Subsequently, the separated TMD with intercalated Li was placed in water (100 mL) and repeatedly centrifuged (18 000 g). Finally, the material was dried in a vacuum oven at 50 °C for 48 h prior to further use.

The DEP carbon chip surface was modified with TMD materials by physical adsorption. TMD (3 μL) at a concentration of 1 mg mL⁻¹ in ultrapure water was deposited onto each carbon chip surface and

left to dry under ambient conditions overnight. The excess of TMD, which was not well adsorbed on the chip surface, was then removed by gentle rinsing with ultrapure water. Prior to depositing the materials onto the surface of the chips, TMD suspensions were sonicated for 15 min to achieve maximum dispersion.

To physically immobilize Hp DNA molecules, an aliquot of Hp DNA molecules (3 μL) at a concentration of 10 $\text{pmol } \mu\text{L}^{-1}$ was deposited onto the TMD-modified electrodes and dried in the oven at 60 $^{\circ}\text{C}$ for 10 min. Subsequently, two washing steps were conducted in phosphate-buffered saline (PBS) solution to remove Hp DNA molecules that were not well adsorbed.^[30]

Equipment

All electrochemical measurements were performed with a $\mu\text{Autolab}$ type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer.

Impedance measurements were controlled by NOVA software (version 1.9) and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The measurements were performed with 10 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ (1:1 molar ratio) in 0.1 M PBS solution (0.1 M $\text{NaCl} + 10 \text{ mM } \text{Na}_2\text{HPO}_4$, pH 7.0) as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane.

All electrochemical measurements were conducted under ambient conditions by utilizing DEP carbon chips unless otherwise stated.

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Keywords: electrochemical impedance spectroscopy • hairpin DNA • immobilization • nanomaterials • transition metal dichalcogenides

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Strong dependence of fluorescence quenching on the transition metal in layered transition metal dichalcogenide nanoflakes for nucleic acid detection

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In recent years, the application of transition metal dichalcogenides for the development of biosensors has been receiving widespread attention from researchers, as demonstrated by the surge in studies present in the field. While different transition metal dichalcogenide materials have been employed for the fabrication of fluorescent biosensors with superior performance, no research has been conducted to draw comparisons across materials containing different transition metals. Herein, the performance of MoS₂ and WS₂ nanoflakes for the fluorescence detection of nucleic acids is assessed. It is discovered that, at the optimal amount, MoS₂ and WS₂ nanoflakes exhibit a similar degree of fluorescence quenching, at 75% and 71% respectively. However, MoS₂ nanoflakes have better performance in the areas of detection range and selectivity than WS₂ nanoflakes. The detection range achieved with MoS₂ nanoflakes is 9.60–366 nM while 13.3–143 nM with WS₂ nanoflakes. In the context of selectivity, MoS₂ nanoflakes display a signal difference of 97.8% between complementary and non-complementary DNA targets, whereas WS₂ nanoflakes only exhibit 44.3%. Such research is highly beneficial as it delivers vital insights on how the performance of a fluorescent biosensor can be affected by the transition metal present. Furthermore, these insights can assist in the selection of suitable transition metal dichalcogenide materials for utilization in biosensor development.

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Introduction

For decades, research on layered two-dimensional materials has attracted immense interest.^{1,2} Specifically, transition metal dichalcogenides (TMDs), representing a type of inorganic two-dimensional materials with the general formula of MX₂ (M: transition metal, X: chalcogen), have experienced a surge in research growth in recent years.^{3,4} From a structural perspective, a single layer of TMDs consists of a layer of transition metal atoms sandwiched between two layers of chalcogen atoms, with the chalcogen atoms covalently bonded to the transition metal atoms. Van der Waals interactions then hold the individual layers of TMDs together to yield the layered bulk form.⁵ The recent tremendous growth in the research on TMDs can be attributed to the broad range of interesting electronic, mechanical, chemical and optical behaviors.^{6,7} Consequently, TMDs have been put to use for applications in advanced energy storage⁸ such as lithium ion batteries⁹ and

supercapacitors,¹⁰ in electrochemical catalysis, such as hydrogen evolution^{11–13} and hydrosulfurization, and in toxicology studies.¹⁴ Nonetheless, the emerging trend in the utilization of TMDs lies in the development of sensing and biosensing devices, exploiting the large specific surface area, electrical conductivity, fast heterogeneous electron transfer and fluorescence quenching.¹⁵

To date, there have been a number of studies performed on the employment of TMDs for fabrication of biosensors based on the fluorescence quenching ability of TMDs. In these studies, different TMD materials such as single-layer WS₂, MoS₂, TaS₂ and TiS₂ nanosheets were utilized as the fluorescence quenching platform and a fluorophore-labeled single-stranded DNA acts as the biorecognition element for the detection of vital target biomolecules.^{16–19} These target biomolecules comprise of DNA, miRNA, thrombin and adenosine triphosphate (ATP). The principle of the sensing strategy lies in the different affinities of TMD materials towards the biorecognition element and the biorecognition element–target biomolecule complex. Hence, the difference in fluorescence signal, before and after the biorecognition process, can be exploited for detection.

However, to our knowledge, there is currently no study performed to investigate how the transition metal present in TMD

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materials will affect their potential to be utilized as a fluorescence quenching platform for the biosensing of nucleic acids. To this aim, we examine the performance of two types of TMD nanoflakes, namely MoS_2 and WS_2 , for the fluorescence detection of nucleic acids, based on a signal-on sensing approach. In addition, it is also worth highlighting that MoS_2 and WS_2 nanoflakes are chosen for comparison as the transition metals (Mo and W) belong to the same periodic group and would supposedly have similar chemical properties.

Experimental section

Materials

Molybdenum disulfide (MoS_2) and tungsten disulfide (WS_2) nanoflakes solution was purchased from Graphene Laboratories Inc. (Calverton, NY, USA). Magnesium chloride hexahydrate was purchased from Quality Reagent Chemical (Auckland, New Zealand). Tris(hydroxymethyl)aminomethane, potassium chloride, sodium chloride, hydrochloric acid (conc. 37%) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA oligonucleotide sequences, relating to Alzheimer's disease, are as follows: FAM-Lprobe – 5' [6-FAM (6-carboxyfluorescein)] ACC AGG CGG CCG CAC ACG TCC TCC AT 3'; DNA target (complementary) – 5' ATG GAG GAC GTG TGC GGC CGC CTG GT 3'; negative control DNA (non-complementary) – 5' AAA AAA AAA AAA AAA AAA AAA AA 3'. Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) with a resistivity of 18.2 M Ω cm. A 20 mM Tris-HCl buffer solution (pH 7.4, 100 mM NaCl, 5 mM KCl, 15 mM MgCl_2) was employed in this study.

Procedures

For fluorescence quenching, the working solution of the fluorescent DNA oligonucleotide (FAM-Lprobe) was prepared by diluting the stock solution to about 40 nM with Tris-HCl buffer solution. An aliquot at the optimum volume of TMD nanoflakes was then added to the Tris-HCl buffer solution containing the FAM-Lprobe and allowed to incubate for 20 minutes at room temperature.

For the detection of DNA hybridization, the DNA target was first added to 40 nM of the FAM-Lprobe with incubation at 50 °C for 30 minutes. After the incubation, TMD nanoflakes at the optimum volume were introduced and left for 20 minutes at room temperature.²⁰ After which, fluorescence measurement of the mixture was conducted. The final concentration of the DNA target in the mixture ranged from 0.004 nM to 1000 nM.

Equipment

Fluorescence measurements were performed at room temperature on a Varian Cary Eclipse fluorescence spectrophotometer. The excitation wavelength was fixed at 490 nm, with the emission spectra recorded over the range of 500 to 800 nm.

Results and discussion

Herein, we investigate the effect of different transition metals in layered transition metal dichalcogenide (TMD) nanoflakes on their potential as the fluorescence sensing platform for nucleic acid detection. To this aim, the performance of two types of TMD nanoflakes, specifically molybdenum disulfide (MoS_2) and tungsten disulfide (WS_2), was evaluated. The general principle behind the utilization of TMD nanoflakes as a fluorescence nucleic acid sensing platform, as depicted in Scheme 1, is based on the adsorption of the fluorescently labeled single-stranded DNA (ssDNA) probe, FAM-Lprobe, onto the surface of the TMD nanoflakes, resulting in fluorescence quenching. On the other hand, when the FAM-Lprobe undergoes prior hybridization with its complementary DNA target to form double-stranded DNA (dsDNA), the extent of interactions between dsDNA and TMD nanoflakes is so low such that the dsDNA is distant from the nanoflakes, thereby leading to retention of the fluorescence from the FAM-Lprobe.

The different affinities of TMD nanoflakes towards the FAM-Lprobe and dsDNA can be elucidated by the following. The FAM-Lprobe can be readily adsorbed onto TMD nanoflakes due to van der Waals forces of attraction between the basal plane of nanoflakes and the exposed nitrogenous bases of the FAM-Lprobe, giving rise to significant fluorescence quenching. In contrast, upon hybridization with its complementary DNA target to form dsDNA, the nitrogenous bases become effectively shielded by the negatively charged phosphodiester backbone of dsDNA, leading to weak interactions between dsDNA and TMD nanoflakes. Hence, dsDNA is far away from the nanoflakes, resulting in low fluorescence quenching.

With the aim of substantiating the adsorption mechanism illustrated in Scheme 1 and to examine the effect of different transition metals in layered TMD nanoflakes on their potential of being employed as a nucleic acid fluorescence sensing platform, the FAM-Lprobe was subjected to different experimental conditions with MoS_2 and WS_2 nanoflakes accordingly.

Scheme 1 Schematic representation of transition metal dichalcogenide (TMD) nanoflakes as a fluorescence sensing platform for the detection of DNA.

Fig. 1 Fluorescence emission spectra of FAM-Lprobe (40 nM) under various experimental conditions for (A) MoS₂ nanoflakes and (B) WS₂ nanoflakes. Black: Tris-HCl buffer solution; red: TMD nanoflakes; blue: FAM-Lprobe; pink: FAM-Lprobe + TMD nanoflakes and green: FAM-Lprobe + DNA target + TMD nanoflakes.

From Fig. 1, it is observed that in the absence of TMD nanoflakes, the FAM-Lprobe exhibits intense fluorescence emission (Fig. 1, blue line). However, upon the introduction of MoS₂ and WS₂ nanoflakes, about 75% and 71% quenching of the FAM-Lprobe fluorescence emission resulted, respectively (Fig. 1, pink line). Hence, this suggests that both TMD nanoflakes have similar quenching efficiencies and can adsorb the FAM-Lprobe to a comparable extent. In contrast, when the FAM-Lprobe undergoes prior hybridization with its complementary DNA target to form dsDNA, the fluorescence emission is significantly retained in the presence of TMD nanoflakes (Fig. 1, green line). It is interesting to note that, for the same amount of complementary DNA target, there is lesser quenching of the fluorescence signal by WS₂ nanoflakes (22%) than MoS₂ nanoflakes (59%). This indicates that the extent of interactions between DNA and WS₂ nanoflakes is much weaker than that of the MoS₂ nanoflakes. Therefore, this indicates that the type of transition metal in layered TMD nanoflakes has a strong influence on the strength of interactions between DNA and TMD nanoflakes, and this will in turn affect their potential for being utilized for nucleic acid detection. In addition, it is also crucial to point out that both TMD nanoflakes do not display any fluorescence emission in the region of interest, and therefore do not lead to any interference.

Moving on, since TMD nanoflakes serve as the nanoquenchers in this study, it is imperative to ascertain the optimum amount of MoS₂ and WS₂ nanoflakes to be adopted for the detection assay. As such, the fluorescence emission spectra of the FAM-Lprobe upon exposure to various volumes of MoS₂ and WS₂ nanoflakes were recorded and demonstrated in Fig. 2.

Fig. 2 Fluorescence emission spectra of FAM-Lprobe (40 nM) upon the introduction of increasing volumes of (A) MoS₂ nanoflakes and (B) WS₂ nanoflakes.

As depicted in Fig. 2, it is evident that with increasing volume of TMD nanoflakes introduced, the fluorescence intensity of the FAM-Lprobe decreases, signifying an increase in the degree of quenching. Saturation in quenching is achieved at 60 μ L for MoS₂ nanoflakes (Fig. 2A) and 200 μ L for WS₂ nanoflakes (Fig. 2B). Hence, the optimum volume of MoS₂ and WS₂ nanoflakes to be employed is established to be 60 μ L and 200 μ L respectively.

Next, the calibration experiment with different concentrations of the complementary DNA target was conducted to assess the dynamic range of detection for MoS₂ and WS₂ nanoflakes. As displayed in Fig. 3, at the region of low complementary DNA target concentrations, there are no significant variations in the fluorescence signal. Subsequently, as the complementary DNA target approaches higher concentrations, substantial enhancement in the fluorescence intensity is detected before saturation is attained. Drawing conclusions from Fig. 3, it can be inferred that MoS₂ nanoflakes impart a wider linear range of detection as compared to WS₂ nanoflakes. It is estimated that MoS₂ nanoflakes exhibit a detection range of 9.60–366 nM while WS₂ nanoflakes display a detection range of 13.3–143 nM. Hence, the transition metal present in TMD nanoflakes has an impact on the detection range and can affect the sensitivity of the fluorescence sensing platform.

Lastly, in order to assess the selectivity performance of the two types of TMD nanoflakes, a hybridization experiment was performed with complementary and non-complementary DNA targets, and the findings are demonstrated in Fig. 4. For both MoS₂ and WS₂ nanoflakes, it is noted that the non-complementary DNA target gives rise to a lower fluorescence intensity than the complementary DNA target. This is because the

Fig. 3 Calibration curves for the detection of DNA hybridization with (A) MoS₂ nanoflakes and (B) WS₂ nanoflakes.

Fig. 4 Fluorescence emission peak intensity of FAM-Lprobe (40 nM) upon incubation with complementary and non-complementary DNA targets, in the presence of (A) MoS₂ nanoflakes and (B) WS₂ nanoflakes.

non-complementary DNA target undergoes inefficient hybridization with the FAM-Lprobe, leading to a large amount of the remaining FAM-Lprobe which is subsequently adsorbed onto TMD nanoflakes and has its fluorescence quenched. In contrast, the complementary DNA target hybridizes with the FAM-Lprobe effectively to yield dsDNA which has weak affinity with TMD nanoflakes and thus retains its fluorescence. Furthermore, it is crucial to highlight that the disparity in signal between complementary and non-complementary DNA targets is more pronounced for MoS₂ nanoflakes (97.8% difference)

than for WS₂ nanoflakes (44.3% difference). In addition, for the case of MoS₂ nanoflakes, the fluorescence of the non-complementary DNA target is more comparable to the blank control than WS₂ nanoflakes. Hence, MoS₂ nanoflakes display enhanced selectivity over WS₂ nanoflakes.

Conclusions

In summary, the influence of the transition metal in layered transition metal dichalcogenides on their application as a fluorescence sensing platform for nucleic acid detection is examined by comparing the performance of MoS₂ and WS₂ nanoflakes. From the findings in this study, it is deduced that WS₂ nanoflakes have weaker interactions with nucleic acids than MoS₂ nanoflakes. This can then be related to the narrower range of detection and lower selectivity demonstrated by WS₂ nanoflakes. Specifically, it is reported that MoS₂ and WS₂ nanoflakes exhibit a detection range of 9.60–366 nM and 13.3–143 nM, respectively. In addition, MoS₂ nanoflakes show 97.8% disparity with negative control while WS₂ nanoflakes only exhibit 44.3%. Hence, the transition metal in layered transition metal dichalcogenide nanoflakes has a strong effect on their fluorescence quenching efficiency. The conclusions derived from this investigation are anticipated to provide invaluable insights on the selection of TMD materials for the future fabrication of biosensors.

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Molybdenum disulfide (MoS₂) nanoflakes as inherently electroactive labels for DNA hybridization detection†

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The detection of specific DNA sequences plays a critical role in the areas of medical diagnostics, environmental monitoring, drug discovery and food safety. This has therefore become a strong driving force behind the ever-increasing demand for simple, cost-effective, highly sensitive and selective DNA biosensors. In this study, we report for the first time, a novel approach for the utilization of molybdenum disulfide nanoflakes, a member of the transition metal dichalcogenides family, in the detection of DNA hybridization. Herein, molybdenum disulfide nanoflakes serve as inherently electroactive labels, with the inherent oxidation peak exploited as the analytical signal. The principle of detection is based on the differential affinity of molybdenum disulfide nanoflakes towards single-stranded DNA and double-stranded DNA. The employment of transition metal dichalcogenide nanomaterials for sensing and biosensing purposes represents an upcoming research area which holds great promise. Hence, our findings are anticipated to have significant contributions towards the fabrication of future DNA biosensors.

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Introduction

Single-layered transition metal dichalcogenides (TMDs), with a similar two-dimensional (2D) structure as graphene, demonstrate chemical versatility. Hence, single-layered TMDs hold great potential for fundamental and technological research in a wide range of areas consisting of energy storage, catalysis, sensing and electronic devices.¹ Single-layered TMDs is comprised of transition metal atoms surrounded in a sandwich structure by chalcogen atoms which are covalently bonded to the transition metal atoms, whereas the interactions between the individual layers are of van der Waals type. Parallel to graphene, individual layers of TMDs are isolated from their bulk forms through intercalation methods or mechanical cleavage^{2,3} and exfoliation of these materials into single or few-layers generally leads to additional characteristics as a result of confinement effects.^{4–6} Hence, it is with high expectations that the chemistry of TMD materials presents prospects for uncovering new research directions for inorganic 2D materials.¹

As one of the more studied TMDs, molybdenum disulfide (MoS₂) nanosheets have garnered considerable attention from researchers due to their large specific surface area and excellent optical, mechanical, and electronic properties.^{7–12} From a

structural point of view, the MoS₂ nanosheet is composed of three atomic layers: one Mo metal layer sandwiched between two S layers by covalent bonds. Such three-layer stacks are then held together by weak van der Waals interactions.¹³ As a result of this layered structure, MoS₂ presents interesting electronic properties which are different along the plane from those in the perpendicular direction. In addition, significant electrochemical and catalytic differences can be found between the basal plane portion of the material and its edges.¹⁴ This is because the 2D electron–electron correlations among Mo atoms would assist in the enhancement of planar electric transportation properties.¹⁵ In view of these exceptional features, MoS₂ nanosheets have been employed in various areas such as sensors,^{16,17} fuel cells¹⁴ and transistors¹⁸ thus far. In particular, the application of MoS₂ nanosheets in sensing and biosensing devices represents an upcoming research direction.

To date, there have been a number of studies performed on the utilization of MoS₂ nanosheets in the research field of sensing and biosensing. In these studies, the MoS₂ nanosheet is mainly involved as a fluorescence quencher^{19,20} or as the transducing platform, either by itself^{7,21} or in the form of a composite with other components such as nanoparticles^{22,23} and polymers.^{24,25} Herein, we report a novel approach of employing MoS₂ nanoflakes as electroactive labels for the voltammetric detection of DNA hybridization, correlating to the diagnosis of Alzheimer's disease. The rationale behind our approach lies in the inherent electroactivity of MoS₂ nanoflakes, originating from the oxidation of the nanoflakes, as well as the differential affinity of the nanoflakes towards single and double-stranded DNA.^{19,20}

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Results and discussion

Herein, we investigate the interactions between molybdenum disulfide (MoS_2) nanoflakes and DNA strands for the detection of DNA hybridization, correlating to the diagnosis of Alzheimer's disease. It is demonstrated that MoS_2 nanoflakes have different degrees of interactions with single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA).¹⁹ Hence, by utilizing the different degrees of interactions which MoS_2 nanoflakes exhibit with DNA strands, as well as the electrochemical behavior of MoS_2 nanoflakes, we propose a sensing strategy for DNA hybridization with MoS_2 nanoflakes functioning as electroactive labels.

The electrochemical behaviour of MoS_2 nanoflakes was investigated by performing cyclic voltammetry between 2.0 and -2.0 V. As demonstrated in Fig. S1 (ESI[†]), a series of oxidation waves were observed in the first scan only. This suggests that the oxidation of MoS_2 nanoflakes is chemically irreversible. Subsequently, the oxidation wave at ~ 0.62 V was employed as the analytical signal for the aim of DNA sensing, as oxidation waves at other potentials faced interference. In addition, differential pulse voltammetry was then adopted over cyclic voltammetry due to improved sensitivity.

Scheme 1 illustrates the sensing strategy employed in this study. First of all, the ssDNA probe was immobilized onto the surface of a bare disposable electrical printed (DEP) carbon chip *via* physical adsorption. Upon the addition of complementary DNA target (Path B), the ssDNA probe hybridized with the complementary DNA target to form a dsDNA duplex. Subsequently, the modified DEP chips (Path A and B) were exposed to MoS_2 nanoflakes for conjugation. As demonstrated in Scheme 1, MoS_2 nanoflakes conjugated to the modified DEP chips' surfaces to different extents depending on the presence of complementary DNA target. As such, different amounts of MoS_2 nanoflakes were affixed onto the modified DEP chips' surfaces and voltammetric signals of varying intensities were attained.

Specifically, for the case of Path A, MoS_2 nanoflakes could effectively adsorb onto the immobilized ssDNA probe *via* van der Waals forces of attractions between the nucleobases and the basal plane of MoS_2 nanoflakes. In contrast, when the ssDNA probe underwent hybridization with its complementary DNA

target (Path B), the nucleobases involved in the formation of the double helix became buried deep within the densely negatively charged helical phosphate backbone, and the degree of interactions between the resulting dsDNA duplex and MoS_2 nanoflakes was severely reduced. Hence, ssDNA has a higher affinity with MoS_2 nanoflakes as compared to dsDNA and a significantly higher amount of MoS_2 nanoflakes was conjugated to the ssDNA probe modified DEP chip surface relative to the dsDNA duplex modified DEP chip surface and thus, a greater voltammetric signal, stemming from the oxidation of MoS_2 nanoflakes, was achieved for the former case.

In order to validate that MoS_2 nanoflakes indeed have different degrees of interactions with ssDNA and dsDNA, DNA hybridization experiments were conducted with three different DNA sequences (complementary, single-base mismatch and non-complementary targets) with the attained results displayed in Fig. 1.

In detail, when the hybridization experiment was conducted using the complementary sequence, a comparatively lowest oxidation voltammetric signal was produced (black line). For the instance of a single-base mismatch sequence, a higher voltammetric signal was achieved (red line) and finally for the instance of a non-complementary sequence, the highest voltammetric signal was obtained (blue line). This signifies that the smallest amount of MoS_2 nanoflakes was conjugated to the modified DEP chip surface when hybridization was performed with a complementary sequence, followed by a single-base mismatch sequence and lastly a non-complementary sequence.

The observed trend could be attributed to different degrees of hybridization efficiency demonstrated by the three sequences. Explicitly, the complementary sequence demonstrated the highest hybridization efficiency and the modified DEP chip surface consisted mainly of dsDNA strands upon the

Scheme 1 Schematic illustration of the experimental approach adopted.

Fig. 1 Differential pulse voltammograms of the MoS_2 nanoflake oxidation peak when the hybridization step was performed with a complementary sequence (black), single-base mismatch sequence (red) and non-complementary sequence (blue). The concentration of DNA probes, DNA targets and MoS_2 nanoflakes employed was $10 \mu\text{M}$, 300 nM and 0.018 mg mL^{-1} , respectively. All electrochemical measurements were conducted in 0.1 M PBS buffer solution ($\text{pH } 7.0$) under ambient conditions.

hybridization step. In contrast, for the case of a non-complementary sequence, the modified DEP chip surface consisted primarily of non-hybridized ssDNA probe strands due to its poor hybridization efficiency. As described earlier, ssDNA possesses higher affinity with MoS₂ nanoflakes as compared to dsDNA, and hence, the highest amount of MoS₂ nanoflakes would be affixed onto the modified DEP chip which had undergone incubation with the non-complementary sequence. Conversely, for the instance of a complementary sequence, the amount of conjugated MoS₂ nanoflakes would be the lowest. Therefore, the largest voltammetric signal was displayed after incubation with the non-complementary sequence. In the case of a single-base mismatch sequence, its voltammetric signal was greater than the complementary sequence due to the occurrence of partial hybridization. Hence, through these control experiments, it was validated that MoS₂ nanoflakes have different extents of interactions with ssDNA and dsDNA, with ssDNA having a higher affinity for the nanoflakes than dsDNA, and the intensity of the voltammetric signal reflects the amount of MoS₂ nanoflakes which were conjugated to the modified DEP chips' surfaces.

To determine the optimum concentration of the DNA probe to be deposited onto the DEP chip surface in order to achieve maximum surface coverage and to reduce the effect of non-specific binding subsequently, probe optimization experiments were performed using electrochemical impedance spectroscopy (EIS) which is widely known to be highly sensitive for such tasks.^{26–28}

A series of DNA probe concentrations were employed for the optimization study and their impedimetric responses were recorded and represented as histograms in Fig. 2. As depicted in Fig. 2, it can be observed that the intensity of impedimetric signal increases from 0.001 μM to 10 μM before experiencing a slight decrease at 20 μM . The impedimetric signal arises from the charge transfer resistance between the Fe(CN)₆^{3–/4–} redox

probe and the DEP chip surface. With the immobilization of the DNA probe onto the surface of the DEP chip, enhancement of the charge transfer resistance is expected. This is because the DNA probe contains a negatively charged phosphate backbone which will in turn lead to repulsion with the similarly negatively charged redox probe and further hinder charge transfer, resulting in the charge transfer resistance to increase and thus a larger impedimetric signal. Therefore, with the increasing concentration of the DNA probe being immobilized onto the DEP chip surface, there will be greater repulsion of the redox probe and the magnitude of impedimetric signal will increase. As such, at 10 μM of the DNA probe which produced the greatest impedimetric signal, it has the highest amount of DNA probe immobilized on the DEP chip surface and thereby capable of providing the maximum coverage of the DEP chip surface. Hence, the optimum concentration of the DNA probe to be deposited onto the DEP chip surface was established to be 10 μM .

Since the voltammetric signal stemming from the oxidation of MoS₂ nanoflakes was utilized as the analytical signal in this report, it is hence crucial to determine the appropriate concentration of MoS₂ nanoflakes to be employed for the final labelling step.

To do so, a calibration with various concentrations of MoS₂ nanoflakes, obtained by serial dilution from the stock solution, was carried out. The histograms illustrated in Fig. 3 represent the voltammetric peak height of MoS₂ nanoflake oxidation in relation to the different concentrations of MoS₂ nanoflakes. For each individual concentration, DNA hybridization was performed with the complementary (yellow), single-base mismatch (light blue) and non-complementary (pink) sequences. From Fig. 3, it is noticed that the voltammetric peak height is the greatest at 0.018 mg mL^{–1}. In addition, at 0.018 mg mL^{–1}, there is also a maximum discrimination among the three different sequences. Henceforth, this concentration of MoS₂ nanoflakes was used for subsequent experimental studies.

Fig. 2 Impedimetric response towards different concentrations of the DNA probe deposited on the surface of the bare DEP chip. Signal is displayed as $R_{\text{ct}}(\text{Probe-blank})$. Error bars correspond to the standard deviations obtained from triplicate experiments. All electrochemical measurements were performed with 10 mM K₄Fe(CN)₆/K₃Fe(CN)₆ in 0.1 M PBS buffer solution (pH 7.0) under ambient conditions.

Fig. 3 Histograms representing the voltammetric response towards different concentrations of MoS₂ nanoflakes employed. Error bars correspond to the standard deviations obtained from triplicate experiments. The concentration of DNA probes and DNA targets used was 10 μM and 300 nM, respectively. All electrochemical measurements were conducted in 0.1 M PBS buffer solution (pH 7.0) under ambient conditions.

Fig. 4 Differential pulse voltammograms of MoS₂ nanoflake oxidation peak when DNA hybridization was conducted with 0.03 (black), 0.3 (red), 3 (blue), 30 (pink) and 300 (green) nM of the complementary DNA sequence. The inset demonstrates the calibration curve of the voltammetric response to the concentration of the complementary DNA sequence. Error bars correspond to the standard deviations obtained from triplicate experiments. The concentration of the DNA probe and MoS₂ nanoflakes utilized was 10 μM and 0.018 mg mL⁻¹, respectively. All electrochemical measurements were conducted in 0.1 M PBS buffer solution (pH 7.0) under ambient conditions.

The voltammetric response of the proposed biosensor towards various concentrations of the complementary DNA sequence was next assessed with the aim of defining the range of detection. As displayed in Fig. 4, the voltammetric peak height decreases with increasing concentration of the complementary DNA sequence for the range of 0.03 nM to 300 nM with good linearity. Furthermore, it should also be highlighted that the percentage relative standard deviation (% RSD) values exhibited for the calibration plot are lower than 10%, suggesting good reproducibility. Therefore, the range of detection was determined to be from 0.03 nM to 300 nM, which is of four orders of magnitude.

Conclusion

In summary, we have shown that the differential affinity of molybdenum disulfide nanoflakes towards single-stranded DNA and double-stranded DNA, coupled with its electrochemical behavior, can be exploited for the detection of DNA hybridization, correlating to the diagnosis of Alzheimer's disease, with the nanoflakes playing the role of electroactive labels. The electrochemical behavior of molybdenum disulfide nanoflakes, specifically the oxidation peak, was utilized as the analytical signal in the proposed biosensing assay. Based on the proposed biosensing assay, the optimum concentration of the DNA probe to be immobilized onto the disposable electrical printed chip surface was determined to be 10 μM while the optimum concentration of molybdenum disulfide nanoflakes to be employed as electroactive labels was verified to be 0.018 mg mL⁻¹. In addition, the range of detection was established to be from 0.03 nM to 300 nM, which is of four orders of magnitude.

A novel approach for the utilization of molybdenum disulfide in the field of biosensing was demonstrated in this study, whereby molybdenum disulfide nanoflakes were employed as electroactive labels. The use of transition metal dichalcogenides such as molybdenum disulfide for sensing and biosensing purposes is a recent research field which holds great potential. Hence, the findings presented in this report are expected to have an immense influence on future work.

Experimental section

Materials

Molybdenum disulfide (MoS₂) nanoflakes solution was purchased from Graphene Laboratories Inc. (Calverton, New York), with the lateral size ranging from 100 to 400 nm and thickness of 1 to 8 layers.

Hydrochloric acid (HCl) (conc. 37%), sodium phosphate dibasic salt (Na₂HPO₄), sodium chloride (NaCl), potassium hexacyanoferrate(II) trihydrate (K₄Fe(CN)₆·3H₂O), potassium hexacyanoferrate(III) (K₃Fe(CN)₆) and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA sequences are as follows:

DNA probe: 5'-ACC AGG CGG CCG CAC ACG TCC TCC AT-3'.

Complementary target: 5'-ATG GAG GAC GTG TGC GGC CGC CTG GT-3'.

Single-base mismatch target: 5'-ATG GAG GAC GTG CGC GGC CGC CTG GT-3'.

Non-complementary target: 5'-AAA AAA AAA AAA AAA AAA AAA AAA AA-3'.

Trisodium citrate dihydrate (Na₃C₆H₅O₇·2H₂O) was purchased from Alfa Aesar (Singapore).

Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) with a resistivity of 18.2 MΩ cm.

The following buffers were used in this study:

0.1 M PBS (0.1 M NaCl + 10 mM Na₂HPO₄, pH 7.0).

TSC 1 (0.75 M NaCl + 75 mM trisodium citrate, pH 7.0).

TSC 2 (0.30 M NaCl + 30 mM trisodium citrate, pH 7.0).

Disposable electrical printed (DEP) carbon chips were obtained from BioDevice Technology (Nomi, Japan). A three-electrode system was employed in this study which included a carbon-based working and counter electrode, and a Ag/AgCl reference electrode.

Equipment

All electrochemical measurements were performed with a μAutolab type III electrochemical analyzer (Eco Chemie, Utrecht, The Netherlands) connected to a personal computer.

Differential pulse voltammetry (DPV) measurements were controlled by using General Purpose Electrochemical System (GPES) software version 4.9 and the parameters applied were: 50 ms modulation time, 0.5 s interval time, 10 mV step potential, 50 mV modulation amplitude and 20 mV s⁻¹ scan rate. DPV measurements were conducted in 0.1 M PBS buffer solution (pH 7.0) and the raw data produced were treated with a baseline correction of peak width 0.01 using the GPES software.

Impedance measurements were controlled by NOVA software version 1.9 and recorded between 0.1 MHz and 0.1 Hz at a sinusoidal voltage perturbation of 10 mV amplitude. The measurements were performed with 10 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ (1 : 1 molar ratio) in 0.1 M PBS buffer solution (pH 7.0) as the redox probe. Randles equivalent circuit was used to fit the obtained impedance spectra, represented as Nyquist plots in the complex plane.

All electrochemical measurements were conducted under ambient conditions by utilizing DEP carbon chips unless otherwise stated.

Procedures

The DNA probe was immobilized on the surface of the DEP carbon chip by dry physical adsorption. A 3 μ L aliquot of DNA probe solution in PBS buffer at the optimum concentration of 10 μ M was deposited onto the DEP chip surface and left in the oven for 10 minutes at 60 °C for drying. After which, the DNA probe modified DEP chip was washed twice in PBS buffer solution under gentle stirring at 25 °C in order to remove the excess DNA probe which was not well adsorbed.

The DNA probe modified DEP chip then underwent incubation with hybridization solution (TSC 1 buffer) containing desired concentrations of DNA targets, with the total volume fixed at 100 μ L. The incubation step was performed at 42 °C for 30 minutes under gentle stirring. Subsequently, two washing steps were conducted in TSC 2 buffer.

Conjugation with MoS_2 nanoflakes was next carried out by incubating the modified DEP chip with 100 μ L of MoS_2 nanoflakes solution at the desired concentration in PBS buffer. The incubation was performed for 20 minutes at 25 °C under gentle stirring. After which, two washing steps in PBS buffer were conducted.

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Carboxylic Carbon Quantum Dots as a Fluorescent Sensing Platform for DNA Detection

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Supporting Information

ABSTRACT: The demand for simple, sensitive, affordable, and selective DNA biosensors is ubiquitous, due to the important role that DNA detection performs in the areas of disease diagnostics, environment monitoring, and food safety. A novel application of carboxylic carbon quantum dots (cCQD) is highlighted in this study. Herein, cCQD function as a nanoquencher in the detection of nucleic acid based on a homogeneous fluorescent assay. To that purpose, the performance of two types of cCQD, namely, citric acid QD and malic acid QD, is evaluated. The principle behind the sensing of nucleic acid lies in the different propensity of single-stranded DNA and double-stranded DNA to adsorb onto the surface of cCQD. For both types of cCQD, a superior range of detection of at least 3 orders of magnitude is achieved, and the potential to distinguish single-base mismatch is also exhibited. These findings are anticipated to provide valuable insights on the employment of cCQD for the fabrication of future DNA biosensors.

KEYWORDS: carbon quantum dot, carboxylic groups, biosensor, nucleic acid, fluorescence, quenching

INTRODUCTION

The intensive development of sensitive, selective, cost-effective, and rapid systems for the detection of nucleic acid is imperative due to their widespread applications in various fields such as clinical disease diagnostics and treatments, genetic profiling, food safety, and forensic studies.^{1–3} In recent years, considerable effort has been channelled into fabricating fluorescent sensors for nucleic acid detection owing to the inherent advantages which they exhibit. These advantages include ease of operation, high sensitivity, and in situ imaging properties.^{4,5}

Interesting to note, sensors belonging to this category are often based on fluorescence resonance energy transfer (FRET) or quenching mechanisms for detection,^{6–8} and they usually comprise of a fluorophore and a quencher molecule attached to the opposite ends of a DNA probe molecule. In the absence of the target molecule, the fluorophore and quencher molecules are in close proximity and fluorescence is suppressed. Upon the occurrence of hybridization with the target molecule, the quencher molecule becomes physically separated from the fluorophore molecule and fluorescence is released. However, despite the extensive utilization of fluorescent sensors in a wide

array of applications,^{9–11} sensors of such design still suffer from one major shortcoming, which is they require careful selection of fluorophore–quencher pair in order to ensure optimum efficiency.⁷

To circumvent such a drawback, nanomaterials have been employed as quenchers in recent years as they are capable of quenching various fluorophores of different emission wavelengths and improving the signal-to-noise ratio.^{2,12} To date, gold nanoparticles,^{13,14} transition metal dichalcogenides,¹⁵ carbon nanotubes,^{16,17} graphene oxide,^{18,19} and metal–organic frameworks²⁰ have been adopted as efficient nanoquenchers to develop fluorescent sensors. Although these nanomaterials have been successfully implemented as nanoquenchers for the detection of nucleic acids, the preparation of some of these nanomaterials is often sophisticated and strict, with difficulty in ensuring uniform size dispersion. Furthermore, some of these nanomaterials display cytotoxicity behaviors.^{21–23} Therefore,

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the search for new nanomaterials as replacements is ever important.

Carbon quantum dots (CQD), belonging to a new class of carbon nanomaterials with sizes below 10 nm, are gradually becoming a rising star in the research of nanomaterials.^{24,25} The rationale behind the much acquired interest can be attributed to the superior and unique properties which CQD present. These properties include ease of functionalization and preparation, yielding particles with small and well-defined sizes, biocompatibility, low cytotoxicity, and good stability against photobleaching. Another vital characteristic of CQD is their high water solubility, thereby allowing homogeneous sensing assays which are greatly preferred for the aim of nucleic acid detection.²⁴ While many studies on their synthesis, properties, and potential applications have been reported, the fluorescence quenching ability of CQD has not been investigated. Some of the reported potential applications include bioimaging^{26,27} and biomedicine delivery systems^{28,29} (due to the biocompatibility and low cytotoxicity), solar cells³⁰ and supercapacitors,³¹ and catalysts.³² In addition, it is important to point out that although there have been some works on the application of carbon nanoparticles^{1,8,33} for the detection of nucleic acids by fluorescence spectroscopy, these nanoparticles are larger than CQD and challenging to synthesize.⁸

Herein, we demonstrate the proof-of-concept on employing carboxylic CQD (cCQD), functioning as a nanoquencher, as a fluorescent sensing platform for nucleic acid detection. The performance of two variations of cCQD, derived from citric acid and malic acid precursors specifically, is also assessed. Both cCQD exhibit a good detection range and potential selectivity down to single-base mismatch. In addition, the cCQD also offer well-defined sizes and a simple synthetic method, thereby allowing large scale production. Moreover, the presence of carboxylic groups on the surface of CQD presents versatile anchoring points which can be exploited via numerous approaches.^{34,35} Hence, this work is expected to serve as a basis for future applications of carboxylic acid-enriched CQD in biosensing.

■ RESULTS AND DISCUSSION

The general principle behind the proposed fluorescent nucleic acid sensing platform, as illustrated in Scheme 1, is based on the

Scheme 1. Schematic Illustration of the Carboxylic Carbon Quantum Dots (cCQD) Based Fluorescent Detection of DNA

adsorption of a fluorescently labeled single-stranded DNA (ssDNA) probe, FAM-Lprobe, onto the cCQD, leading to fluorescence quenching. In contrast, when FAM-Lprobe undergoes prior hybridization with its complementary DNA target to form double-stranded DNA (dsDNA), the interactions between dsDNA and cCQD are so weak that the dsDNA is away from the surface of cCQD, leading to retention of the fluorescence of FAM-Lprobe.

Interesting to note, the interactions between DNA and cCQD represent a constant competition of electrostatic repulsion and hydrophobic π - π interactions. The hydrophobic π - π interactions originate from the π - π stackings between DNA nucleobases and the hydrophobic regions of the cCQD surfaces, while electrostatic repulsion arises from the repulsion between the carboxylic groups of cCQD and the negative phosphodiester backbone of DNA. Therefore, the difference in electrostatic/hydrophobic properties of the FAM-Lprobe and dsDNA will result in a different propensity to adsorb onto the surface of cCQD. Theoretically, such an effect will in turn impart sensitivity and selectivity to the detection assay.³³ Under the conditions that the hydrophobic interactions are greater than the electrostatic repulsion, the DNA will be adsorbed onto the cCQD.

To understand the properties of the synthesized cCQD, material characterizations were performed with X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FT-IR), atomic force microscopy (AFM), and transmission electron microscopy (TEM). In addition, the optical properties were also investigated by photoluminescence measurement and UV-vis spectroscopy.

XPS, being a very sensitive and semiquantitative analysis method for probing the surface of a material, is capable of providing surface elemental composition and bonding arrangements of the synthesized cCQD. Hence, survey scans, from a binding energy of 1200 to 0 eV, were recorded for both citric acid QD and malic acid QD in order to find out their overall elemental compositions. As illustrated in Figure 1, carbon-to-oxygen (C/O) ratios of 2.12 and 1.39 for citric acid QD and malic acid QD, respectively, were obtained. This suggests that citric acid QD had undergone a greater degree of carbonization as compared to malic acid QD. Subsequently, high resolution scans were performed, and it was uncovered that citric acid QD produce C=C, C-C, C-O, C=O, and O-C=O peaks of intensities 12.97, 42.51, 16.85, 12.81, and 14.85, respectively, while malic acid QD yield 3.49, 33.98, 27.16, 8.19, and 27.15 comparatively. These values highlight the presence of carboxylic moieties and hydrophobic regions on the surface of the synthesized cCQD. Specifically, malic acid QD are deduced to contain more carboxylic moieties than citric acid QD while citric acid QD have more hydrophobic regions as compared to malic acid QD.

From the FT-IR spectra in Figure 2, it can be observed that both cCQD demonstrate peaks corresponding to the C=O (1720 cm^{-1}) and -OH (3380 cm^{-1}) stretching modes of the -COOH groups, and the C=C (1598 cm^{-1}) graphitic stretching mode. Hence, the presence of hydrophobic regions and carboxylic groups on the surface of both cCQD is further established.

To gain insights on the morphology of cCQD, TEM and AFM were utilized with the results demonstrated in Supporting Information (SI) Figures S1 and S2. In both cases, intensive aggregation of cCQD was observed after drying for measurement. From the TEM images in SI Figure S1, the round shape

Figure 1. X-ray photoelectron spectroscopy (XPS) spectra for survey and high resolution C 1s core-level scans of (A) citric acid QD and (B) malic acid QD.

Figure 2. Fourier transform infrared spectroscopy (FT-IR) spectra on malic acid QD and citric acid QD.

of cCQD with typical dimensions in the range of 10–20 nm can be seen. Moreover, the round shaped cCQD have a platelet profile which can be spotted on the images of aggregated cCQD by AFM (SI Figure S2). The thickness of the platelets was determined to be in the range of 5–10 nm.

Moving on, the optical properties of the synthesized cCQD were subsequently assessed with photoluminescence measurement and UV–vis spectroscopy. In SI Figure S3, the excitation wavelength dependent photoluminescence spectra defined the maximum photoluminescence to be 430 nm for malic acid QD and 465 nm for citric acid QD. Furthermore, for both cCQD, it can be noticed that maximum photoluminescence was obtained with an excitation wavelength of approximately 370 nm. Interesting to note, the photoluminescence is significantly narrower for malic acid QD as compared to citric acid QD. In addition, the intensive luminescence of cCQD is also displayed

Figure 3. Fluorescence emission spectra of FAM-Lprobe (40 nM) under different experimental conditions for (A) citric acid QD and (B) malic acid QD: black, Tris-HCl buffer solution; red, cCQD; blue, FAM-Lprobe; pink, FAM-Lprobe + cCQD; purple, FAM-Lprobe + 4 nM DNA target + cCQD.

Figure 4. Fluorescence intensity of FAM-Lprobe (40 nM) upon the introduction of different volumes of (A) citric acid QD and (B) malic acid QD.

Figure 5. (A) Calibration curve for the detection of DNA hybridization based on citric acid QD. (B) Corresponding fluorescence emission spectra in the presence of different concentrations of DNA target (0.4, 2, 4, 20, 40, 200, and 400 nM).

Figure 6. (A) Calibration curve for the detection of DNA hybridization based on malic acid QD. (B) Corresponding fluorescence emission spectra in the presence of different concentrations of DNA target (0.04, 0.4, 2, 20, 40, 200, and 400 nM).

in SI Figure S4. On another note, UV-vis spectra of cCQD in SI Figure S5 demonstrate that malic acid QD showed an absorption edge at 421 nm while citric acid QD at 469 nm.

With the aim of validating the adsorption mechanism as described in Scheme 1, the fluorescence behavior of FAM-Lprobe under different experimental conditions was next investigated. The performance of the two types of cCQD, citric acid QD and malic acid QD, was also evaluated, with the findings displayed in Figure 3.

From Figure 3, it is demonstrated that, in the absence of cCQD, FAM-Lprobe exhibits strong fluorescence emission (Figure 3, blue line). However, the presence of citric acid QD and malic acid QD leads to about 23% and 39% quenching of the FAM-Lprobe fluorescence emission, respectively (Figure 3, pink line). This indicates that malic acid QD can adsorb FAM-

Lprobe better and quench the fluorescent dye more effectively. However, when FAM-Lprobe was hybridized with its complementary DNA target to form dsDNA, its fluorescence was significantly retained in the presence of cCQD (Figure 3, purple line). In addition, it is also worth noting that cCQD (Figure 3, red line) do not display any fluorescence emission in the region of interest and thus do not cause any interference.

Since the cCQD function as the nanoquenchers in this work, it is therefore vital to determine the optimum amount of citric acid QD and malic acid QD to be adopted for the sensing platform. To this aim, the fluorescence intensities of FAM-Lprobe toward various volumes of citric acid QD and malic acid QD introduced were measured and plotted in Figure 4.

As illustrated in Figure 4, with a greater volume of cCQD introduced, there is an increase in the extent of quenching of

Figure 7. Fluorescence emission spectra of FAM-Lprobe upon DNA target hybridization in the presence and absence of (A) citric acid QD and (B) malic acid QD.

FAM-Lprobe fluorescence emission until saturation is attained. Drawing conclusions from Figure 4A,B, the optimum volumes of citric acid QD and malic acid QD to be utilized are determined to be 30 and 40 μ L accordingly.

Subsequently, a calibration experiment with different concentrations of complementary DNA target was performed to evaluate the range of detection for citric acid QD and malic acid QD. As seen in both Figures 5A and 6A, with increasing concentration of DNA target, enhancement in fluorescence intensity is observed. This can be attributed to the reason that, with more complementary DNA target added, a higher degree of DNA hybridization occurs and more dsDNA is formed. Consequently, this gives rise to a lesser extent of adsorption onto cCQD surface and thus lesser quenching, resulting in a higher fluorescent signal being acquired. The range of detection is established to be 0.4–400 nM for citric acid QD while 0.04–400 nM for malic acid QD. In addition, the limit of detection was calculated to be 45.6 nM for citric acid QD and 17.4 nM for malic acid QD. The wider range of detection and lower detection limit for malic acid QD, as compared to citric acid QD, may be due to the higher amount of carboxylic groups the malic acid QD exhibit, hence imparting enhanced sensitivity. Interesting to note, in two separate studies by Li and co-workers whereby carbon nanoparticles and carbon nanospheres were employed instead, the range of detection was determined to be 33–300 nM and 0.1–300 nM, respectively.^{1,8}

In order to confirm that the complementary DNA target does not have any inherent quenching or fluorescent effect on the fluorescence emission by FAM-Lprobe and further substantiate the proposed sensing mechanism, a control study was conducted with the outcomes depicted in Figure 7. As shown in Figure 7, the addition of solely a DNA target has negligible effect on the fluorescence emission by FAM-Lprobe. On the other hand, when cCQD are present together with the DNA target, quenching in FAM-Lprobe fluorescence emission is reflected. Hence, it can be concluded that the DNA target itself does not have any effect on the fluorescent signal derived and cCQD indeed function as the nanoquencher in this work.

Last of all, to investigate the selectivity performance of the proposed detection assay, single nucleotide polymorphism analysis was performed for both citric acid QD and malic acid QD. For the case of citric acid QD, the enhancement in fluorescent signal, relative to when no DNA target was introduced, is 17.0 au when complementary DNA target was added while just 12.6 au when single-base mismatch DNA was added. For the case of malic acid QD, the enhancement in fluorescent signal is 27.3 au for complementary DNA target

while 23.2 au for single-base mismatch DNA. This suggests that the proposed detection assay has the potential to distinguish single-base mismatch DNA from complementary DNA target. However, further studies have to be conducted in order to optimize the selectivity performance.

CONCLUSION

In summary, we have demonstrated the proof-of-concept of a novel application of carboxylic carbon quantum dots (cCQD). In this work, cCQD, functioning as a nanoquencher, are employed as a fluorescent sensing platform for the detection of nucleic acid. In addition, the performance of two variations of cCQD, citric acid QD and malic acid QD, is assessed. For both cCQD, the proposed sensing platform demonstrates superior range of detection and potential selectivity down to single-base mismatch. The linear dynamic range of detection is determined to be 0.4–400 nM for citric acid QD and 0.04–400 nM for malic acid QD, with detection limits of 45.6 and 17.4 nM for citric acid QD and malic acid QD, respectively. The conclusions from this study act as the foundation for future works on utilizing cCQD for fluorescent detection of essential biomolecules and disease diagnostics.

EXPERIMENTAL SECTION

Materials. Citric acid, malic acid, ethylenediaminetetraacetic acid, and sodium hydroxide were obtained from Lach-Ner (Neratovice, Czech Republic). Dialysis membranes (1 kDa cutoff) were obtained from P-LAB (Prague, Czech Republic). Magnesium chloride hexahydrate was purchased from Quality Reagent Chemical (Auckland, New Zealand). Tris(hydroxymethyl)aminomethane, potassium chloride, hydrochloric acid (concn 37%), and DNA oligonucleotides were purchased from Sigma-Aldrich (Singapore). The DNA oligonucleotide sequences are as follows: FAM-Lprobe, 5' [6FAM] ACC AGG CGG CCG CAC ACG TCC TCC AT 3'; DNA target, 5' ATG GAG GAC GTG TGC GGC CGC CTG GT 3'; single-base mismatch DNA, 5' ATG GAG GAC GTG CGC GGC CGC CTG GT 3'. Ultrapure water used in this study was obtained from a Milli-Q ion exchange column (Millipore) with a resistivity of 18.2 M Ω cm. A 0.1 M amount of Tris-HCl buffer solution (pH 7.2, 50 mM KCl, 10.0 mM MgCl₂) was employed in this study.

Procedures. *Preparation of Carboxylic Carbon Quantum Dots.* A 6 g amount of citric acid/malic acid, in a glass beaker covered with a lid, was heated at 205 $^{\circ}$ C until the solid melted completely, and the temperature was held for another 15 min. After cooling to room temperature, 50 mL of 0.25 M sodium hydroxide (NaOH) was added to the reaction mixture. The resulting solution was then adjusted to pH 6.0 with 0.05 M NaOH, followed by dialysis using a 1 kDa cutoff membrane for 3 days before subsequent use. The colloidal solution of cCQD was next evaporated to 100 mL by vacuum evaporator at 40 $^{\circ}$ C.

The size of the synthesized cCQD was subsequently measured with DLS and determined to be 11.3 nm (full width at half-maximum (fwhm) of size distribution, 3.7 nm) for citric acid QD and 24.7 nm (fwhm of size distribution, 7.1 nm) for malic acid QD.

To determine the concentration of cCQD, TOC analyzer was used to measure the carbon concentration before utilizing XPS composition analysis to recalculate to cCQD concentration. The concentration of cCQD obtained from citric acid QD is 81.9 mg/L and the concentration of cCQD obtained from malic acid QD is 352 mg/L.

Fluorescence Quenching and Detection of DNA Hybridization. For fluorescence quenching, the working solution of the fluorescent DNA oligonucleotide (FAM-Lprobe) was prepared by diluting the stock solution to about 40 nM with Tris-HCl buffer solution. An aliquot at the optimum volume of cCQD was then added to the Tris-HCl buffer solution containing FAM-Lprobe and allowed to incubate for 20 min at room temperature.

For the detection of DNA hybridization, DNA target was first added to 40 nM FAM-Lprobe with incubation at 50 °C for 30 min. After the incubation, cCQD at the optimum volume were introduced and left for 20 min at room temperature, after which, fluorescence of the mixture was measured. The final concentration of DNA target in the mixture ranged from 0.04 to 400 nM.

Equipment. High resolution X-ray photoelectron spectroscopy (XPS) was performed on an ESCAProbeP (Omicron Nanotechnology Ltd., Taunusstein, Germany) spectrometer equipped with a monochromatic aluminum X-ray radiation source (1486.7 eV). Survey and high resolution C 1s core-level scans were performed. The relative sensitivity factors were used in the evaluation of the carbon-to-oxygen (C/O) ratios from the survey spectra. Samples were prepared by drop casting of cCQD solution on gold coated silicon wafer.

Fourier transform infrared spectroscopy (FT-IR) spectra were recorded using a Nicolet iS50R spectrometer (Thermo Scientific, Waltham, MA, USA) with Smart SAGA specular reflectance accessory. Samples were prepared by drop casting of cCQD solution on gold coated silicon wafer.

Dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, England). The samples were measured at 20 °C using disposable PMMA cuvettes.

Fluorescence measurements were performed at room temperature on a Varian Cary Eclipse fluorescence spectrophotometer. The excitation wavelength was fixed at 490 nm, with the emission spectra recorded over the range of 500–800 nm. 2D photoluminescence spectra were measured using Fluorolog 3 Extreme (HORIBA Scientific, France). The measurement was performed using quartz glass cuvette. Excitation wavelength was from 200–800 nm with 10 nm step and emission spectra were measured in the range of 200–800 nm.

Transmission electron microscopy (TEM) was performed with a JEOL JEM-1010 instrument at accelerating voltage of 80 kV. Images were taken by SIS MegaView III digital camera (Soft Imaging Systems) and analyzed with AnalySIS v2.0 software.

Concentration of cCQD was determined with liquiTOC II (Elementar Analysensysteme, Hanau, Germany) by measurement of the total carbon concentration.

For atomic force microscopy (AFM) images, the dialyzed cCQD sample was ultrasonicated for 5 min before the suspension was drop casted onto a freshly cleaved mica substrate. The measurements were carried out on an Ntegra Spectra instrument from NT-MDT, and surface scans were performed in tapping (semicontact) mode. A cantilever with a strain constant of 1.5 kN/m equipped with a standard silicon tip with curvature radius lower than 10 nm was used for all measurements. The measurements were conducted under ambient condition with a scan rate of 1 Hz and a scan line of 512.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b10160.

Figures showing TEM images of cCQD, AFM images and photoluminescence spectra of citric acid and malic acid QDs, luminescence of the QDs excited by a blue laser pointer, and UV–vis spectra of citric acid and malic acid QDs (PDF)

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Notes

The authors declare no competing financial interest.

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