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Naziah Mohamad Latiff

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**LAYERED MATERIALS FOR ENERGY APPLICATIONS:
ELECTROCHEMISTRY AND TOXICITY STUDIES**

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APPLICATIONS: ELECTROCHEMISTRY AND
TOXICITY STUDIES**

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School of Physical & Mathematical Sciences

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement
for the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from 7 papers published in the following peer-reviewed journal(s) / from papers accepted at conferences in which I am listed as an author.

Part of Chapter 2 is published as 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. DOI: 10.1039/c6cs00136j.

The contributions of the co-authors are as follows:

- Assoc. Prof Martin Pumera initiated the review and edited the drafts.
- I prepared the section on hydrogen evolution reaction. Dr Chua Chun Kiang assisted in its editing.
- Dr Adriano Ambrosi, Dr. Chua Chun Kiang, Dr. Adeline Hui Ling Loo, Dr Colin Hong An Wong, Dr. Alex Yong Sheng Eng and Dr. Alessandra Bonnani prepared and edited the other sections of the review.
- Dr Adriano Ambrosi compiled the whole review.

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The contributions of the co-authors are as follows:

- Assoc Prof Martin Pumera provided the idea, suggested the materials and edited the manuscript drafts.
- Prof Adrian C. Fisher supported the work financially.
- I wrote the manuscript drafts, conducted the electrochemical measurements and characterized the materials using scanning electron microscopy (SEM), Raman spectroscopy, X-ray photoelectron spectroscopy (XPS).
- Dr. Lu Wang provided guidance on carrying out capacitance studies and material characterization.
- Dr. Carmen Clotilde Mayorga-Martinez mentored the electrochemical experiments after Dr Lu Wang left the group and revised the manuscript drafts.
- Prof Zdenek Sofer synthesized the materials, performed combustible elemental analysis and inductively coupled plasma optical emission spectrometry (ICP-OES), as well as edited the manuscript.

Chapter 4 is published as Latiff, N. M.; Wang, L.; Mayorga-Martinez, C. C.; Sofer, Z.; Fisher, A. C.; Pumera, M. Valence and oxide impurities in MoS₂ and WS₂ dramatically change their electrocatalytic activity towards proton reduction. *Nanoscale* **2016**, 8, 16752. DOI: 10.1039/c6nr03086f.

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- Assoc Prof Martin Pumera provided the idea, suggested the materials and edited the manuscript drafts.
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- I wrote the manuscript drafts, conducted the electrochemical measurements and characterized the materials using scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS) and X-ray photoelectron spectroscopy (XPS).
- Dr. Lu Wang mentored me on how to conduct hydrogen evolution reaction measurements, analyze the data and carry out material characterizations.
- Dr. Carmen Clotilde Mayorga-Martinez guided me on electrochemical impedance measurements and helped to revise the manuscript drafts.
- Prof Zdenek Sofer synthesized MoS₂, WS₂ and WS₃ materials, as well as edited the manuscript.

Chapter 5 is published as Latiff, N.; Teo, W. Z.; Sofer, Z.; Huber, S.; Fisher, A. C.; Pumera, M. Toxicity of layered semiconductor chalcogenides: beware of interferences. *RSC Adv.* **2015**, 5, 67485. DOI: 10.1039/c5ra09404f.

The contributions of the co-authors are as follows:

- Assoc Prof Martin Pumera provided the idea, suggested the materials and edited the manuscript drafts.
- Prof Adrian C. Fisher supported the work financially.
- I wrote the manuscript drafts, conducted the toxicity experiments and performed material characterization via scanning electron microscopy (SEM) and scanning transmission electron microscopy (STEM).
- Dr. Wei Zhe Teo mentored the toxicity experiments and revised the manuscript drafts.
- Prof Zdenek Sofer synthesized the materials, performed combustible elemental analysis and inductively coupled plasma optical emission spectrometry (ICP-OES), as well as edited the manuscript.
- Dr Stepan Huber assisted with the X-ray diffraction (XRD) data.

Chapter 6 is published as Latiff, N. M.; Teo, W. Z.; Sofer, Z.; Fisher, A. C.; Pumera, M. The cytotoxicity of layered black phosphorus. *Chem. Eur. J.* **2015**, *21*, 19991. DOI: 10.1002/chem.201502006.

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- Prof Adrian C. Fisher supported the work financially.
- I wrote the manuscript drafts, conducted the toxicity experiments and performed material characterization.
- Dr. Wei Zhe Teo mentored the toxicity experiments and revised the manuscript drafts.
- Prof Zdenek Sofer synthesized the materials and edited the manuscript drafts.

Chapter 7 is published as Latiff, N. M.; Sofer, Z.; Fisher, A. C.; Pumera, M. Cytotoxicity of exfoliated layered vanadium dichalcogenides. *Chem. Eur. J.* **2017**, 22, 18810. DOI: 10.1002/chem.201604430.

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- Prof Zdenek Sofer synthesized the materials and edited the manuscript drafts.

Chapter 8 is published as Latiff, N. M.; Mayorga-Martinez, C. M.; Khezri, B.; Szokolova, K.; Sofer, Z.; Fisher, A. C.; Pumera, M. Cytotoxicity of layered metal phosphorus chalcogenides (MPX_Y) nanoflakes; FePS₃, CoPS₃, NiPS₃. FlatChem 2018, 12, 1. DOI: 10.1016/j.flatc.2018.11.003.

The contributions of the co-authors are as follows:

- Assoc Prof Martin Pumera provided the idea, suggested the materials and edited the manuscript drafts.
- Prof Adrian C. Fisher supported the work financially.
- I wrote the manuscript drafts, conducted the toxicity experiments and performed material characterization via scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS) and scanning transmission electron microscopy (STEM).
- Dr. Carmen Clotilde Mayorga-Martinez
- Prof Zdenek Sofer synthesized the materials, helped to provide data for high resolution transmission electron microscopy (HR TEM-EDS), X-ray diffraction (XRD), Raman spectroscopy and edited the manuscript.
- Dr Katerina Szokolova helped with the X-ray photoelectron spectroscopy (XPS) measurement, analysis and writing.
- Dr Bahareh Khezri assisted with inductively coupled plasma mass spectroscopy (ICP-MS) measurement.

Chapter 9 is published as Latiff, N. M.; Mayorga-Martinez, C. M.; Sofer, Z.; Fisher, A. C.; Pumera, M. Cytotoxicity of phosphorus allotropes (black, violet, red). *Appl. Mater. Today* **2018**, *13*, 310. DOI: 10.1016/j.apmt.2018.09.010.

The contributions of the co-authors are as follows:

- Assoc Prof Martin Pumera provided the idea, suggested the materials and edited the manuscript drafts.
- Prof Adrian C. Fisher supported the work financially.
- I wrote the manuscript drafts, conducted the toxicity experiments and performed material characterization via scanning electron microscopy (SEM), scanning transmission electron microscopy (STEM), dynamic light scattering (DLS) as well as X-ray photoelectron spectroscopy (XPS).
- Dr Carmen Clotilde Mayorga-Martinez performed inherent electrochemistry and Raman spectroscopy measurements, mentored the project and revised the manuscript drafts.
- Prof Zdenek Sofer synthesized the materials and edited the manuscript.

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Table of Contents

	Page
Summary.....	i
List of Publications	ii
Chapter 1: Objective of the Thesis	1
Chapter 2: Introduction and Literature Review	7
2.1. Introduction on Layered Materials	11
2.1.1. A Brief History	11
2.1.2. Preparation of Layered Materials.....	12
2.2. Layered Materials and Energy-related Applications	14
2.2.1. Materials under Study.....	17
2.2.2. Electrochemistry of Layered Materials and Energy Applications	21
2.2.3. Supercapacitors.....	22
2.2.4. Hydrogen Evolution Reaction (HER)	28
2.3. Layered Materials and their Safety Aspects.....	33
2.3.1. A Brief History	33
2.3.2. Current Progress	35

PART I – Electrochemical Studies of Layered Materials for Energy Applications

Chapter 3: Effect of Doping Graphene on their Capacitance.....	49
Chapter 4: Effect of Valence and Oxide Impurities in MoS ₂ and WS ₂ on HER.....	77

PART II – Layered Materials and their Safety Aspects

Chapter 5: Cytotoxicity of Semiconductor Chalcogenides.....	109
Chapter 6: Cytotoxicity of Black Phosphorus	135
Chapter 7: Cytotoxicity of Vanadium Dichalcogenides.....	153
Chapter 8: Cytotoxicity of Metal Phosphorus Chalcogenides	181
Chapter 9: Cytotoxicity of Black Phosphorus and its Allotropes	213
Chapter 10: Conclusion and Future Outlook	249

Summary

With climate change, global warming, depleting amount of fossil fuels and rising energy demands, it becomes critical for us to search for practical energy solutions. In this endeavor, layered materials such as graphene, transition metal dichalcogenides (TMDs), semiconductor chalcogenides, metal phosphorus chalcogenides and black phosphorus have demonstrated promising properties as new materials for applications in enhanced energy storage and generation systems such as batteries, supercapacitors and fuel cells. In the heart of these devices, electrochemistry plays a central role in their operation. Therefore, we are interested to investigate the electrochemical properties of layered materials to develop on their performance for such applications. To further advance our current understanding in these areas, we studied the effect of dopants in microwave exfoliated graphene on their capacitive performance, as well as the effect of valence and oxide impurities in two common TMDs (i.e. MoS_2 and WS_2) on their electrocatalytic hydrogen production for use in fuel cells. Besides technological advancement, we also recognize that there is growing concern over the potential health and environmental hazards posed by these layered materials. As such, we have investigated the cytotoxicity of several new emerging layered materials to conduct preliminary toxicological studies. We have chosen vanadium dichalcogenides, semiconductor chalcogenides (i.e. GaSe, GeS), metal phosphorus chalcogenides and black phosphorus to address this research gap. This was performed by incubating the test materials with human lung cancer epithelial cells (A549) for 24 hours and subsequently measuring the remaining cell viabilities using two well-established assays; water-soluble tetrazolium salt (WST-8) and methyl-thiazolyldiphenyl-tetrazolium bromide (MTT). The A549 cell line was chosen since the lung is most likely to be the first organ of contact when they enter the body through inhalation. These electrochemical and cytotoxicity studies would be useful for the progression of our endeavour towards sustainable clean energy.

List of publications

First author articles

1. Toxicity of layered semiconductor chalcogenides: beware of interferences

Latiff, N.; Teo, W. Z.; Sofer, Z.; Huber, S.; Fisher, A. C.; Pumera, M. *RSC Adv.* **2015**, *5*, 67485.

2. The cytotoxicity of layered black phosphorus

Latiff, N. M.; Teo, W. Z.; Sofer, Z.; Fisher, A. C.; Pumera, M. *Chem. –Eur. J.* **2015**, *21*, 19991.

3. Valence and oxide impurities in MoS₂ and WS₂ dramatically change their electrocatalytic activity towards proton reduction

Latiff, N. M.; Wang, L.; Mayorga-Martinez, C. C.; Sofer, Z.; Fisher, A. C.; Pumera, M. *Nanoscale* **2016**, *8*, 16752.

4. Cytotoxicity of exfoliated layered vanadium dichalcogenides

Latiff, N. M.; Sofer, Z.; Fisher, A. C.; Pumera, M. *Chem. –Eur. J.* **2017**, *22*, 18810.

5. Microwave irradiated N- and B,Cl-doped graphene: Oxidation method has strong influence on capacitive behavior

Latiff, N. M.; Mayorga-Martinez, C. C.; Wang, L.; Sofer, Z.; Fisher, A. C.; Pumera, M. *Appl. Mater. Today* **2017**, *9*, 204.

6. Cytotoxicity of layered metal phosphorus chalcogenides (MPX_Y) nanoflakes; FePS₃, CoPS₃, NiPS₃

Latiff, N. M.; Mayorga-Martinez, C. M.; Khezri, B.; Szokolova, K.; Sofer, Z.; Fisher, A. C.; Pumera, M. *FlatChem* **2018**, *12*, 1.

7. Cytotoxicity of phosphorus allotropes (black, violet, red)

Latiff, N. M.; Mayorga-Martinez, C. M.; Sofer, Z.; Fisher, A. C.; Pumera, M. *Appl. Mater. Today* **2018**, *13*, 310.

Co-authored articles

1. Black phosphorus nanoparticle labels for immunoassays via hydrogen evolution reaction mediation

Mayorga-Martinez, C. C.; **Latiff, N. M.**; Eng, A. Y. S.; Sofer, Z.; Pumera, M. *Anal. Chem.* **2016**, *88*, 10074.

2. Boron and nitrogen doped graphene via microwave exfoliation for simultaneous electrochemical detection of ascorbic acid, dopamine and uric Acid

Thearle, R. A.; **Latiff, N. M.**; Sofer, Z.; Mazanek, V.; Pumera, M. *Electroanalysis* **2016**, *29*, 45.

3. Cytotoxicity of Group 5 transition metal ditellurides (MTe₂; M=V, Nb, Ta)

Chia, H. L.; **Latiff, N. M.**; Sofer, Z.; Pumera, M. *Chem. –Eur. J.* **2017**, *23*, 206.

4. In vitro cytotoxicity of covalently protected layered molybdenum disulfide

Rosli, N. F.; **Latiff, N. M.**; Sofer, Z.; Fisher, A. C.; Pumera, M. *Appl. Mater. Today* **2018**, *11*, 200.

5. Layered PtTe₂ matches electrocatalytic performance of Pt/C for oxygen reduction reaction with significantly lower toxicity

Rosli, N. F.; Mayorga-Martinez, C. C.; **Latiff, N. M.**; Rohaizad, N.; Sofer, Z.; Fisher, A. C.; Pumera, M. *ACS Sustainable Chem. Eng.* **2018**, *6*, 7432–7441.

6. Triazine- and heptazine-based carbon nitrides: Toxicity

Dong, Q.; **Latiff, N. M.**; Mazanek, V.; Rosli, N. F.; Chia, H. L.; Sofer, Z.; Pumera, M. *ACS Appl. Nano Mater.* **2018**, *1*, 4442.

7. Cytotoxicity of shear exfoliated pnictogen (As, Sb, Bi) nanosheets

Chia, H. L.; **Latiff, N. M.**; Gusmao, R.; Sofer, Z.; Pumera, M. *Chem. –Eur. J.* **2019**, *25*, 2242.

Review articles

1. Graphene and its electrochemistry - an update

Ambrosi, A.; Chua, C. K.; **Latiff, N. M.**; Loo, A. H.; Wong, C. H. A.; Eng, A. Y. S.; Bonanni, A.; Pumera, M. *Chem. Soc. Rev.* **2016**, 45, 2458.

Chapter 1 – Objective of the Thesis

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The overall objective of this thesis titled “*Layered Materials for Energy Application: Electrochemistry and Toxicity Studies*” is to develop on our current understanding of layered materials for energy applications. Energy has been specified here for us to address a more pressing concern of meeting future higher energy demands despite depleting supply of fossil fuel. Two aspects would be looked at in this thesis; first is exploring the electrochemical properties of layered materials for energy applications, and second is studying their toxicity to understand the possible health hazards that they can pose to humans. These two aspects are chosen as electrochemistry is a simple yet powerful tool that can provide solutions to our energy concerns, and toxicity studies are important to prepare for the widescale production of these materials in the future.

Before embarking on addressing the challenges in these fields, it is important for us to understand their background and current research progress first. Therefore, this thesis begins with an introduction and literature review (**Chapter 2**) that lays down the necessary fundamentals required. This chapter can be broken down to three sections:

1. The first one introduces various layered materials such as graphene, transition metal dichalcogenides (TMDs), semiconductor chalcogenide and black phosphorus in terms of their structure, synthesis methods, properties and applications.
2. The second section explains how studying the electrochemistry of materials can bring insight to their development in energy-related applications (specifically in supercapacitors and as electrocatalysts for hydrogen evolution reaction).
3. The third section covers the importance of investigating the toxicity of nanomaterials as well as the current progress in the field.

Following the introduction and literature review, projects performed herein are divided into two parts.

Part I covers electrochemical studies of layered materials for energy applications. There are two projects presented in this section. **Chapter 3** explores the effect of doping graphene on its capacitance while **Chapter 4** investigates the effect of valence and oxide impurities on two transition metal dichalcogenides (TMDs; molybdenum disulphide and tungsten disulphide).

Thereafter, **Part II** discusses projects pertaining to safety aspects of various layered materials. It begins with **Chapter 5** which attempts to investigate the cytotoxicity of semiconductor chalcogenides (gallium selenide and germanium sulfide). **Chapter 6** follows with an investigation on a more recent layered material (black phosphorus) while **Chapter 7** explores the cytotoxicity of vanadium dichalcogenides which are part of the TMDs family. Later, **Chapter 8** covers metal phosphorus chalcogenides and the section concludes with **Chapter 9** on cytotoxicity studies of black phosphorus again but here with comparison to its other allotropes (red and violet phosphorus). The chapters here are arranged in chronological order with the most recent project presented last. This organization was chosen as there are slight modifications in the methodology used in the latter projects, and presenting them in chronological order can allow readers to have a better understanding for the modifications implemented. Chapters 6 and 9 are presented separately despite them sharing the same material under study (i.e. black phosphorus) due to this reason.

Part II focuses more on the recent layered materials (semiconductor chalcogenides, black phosphorus, vanadium dichalcogenides and metal phosphorus chalcogenides) as their health hazards are much less understood compared to the earlier discovered layered materials (i.e. graphene, molybdenum disulphide, tungsten disulphide). On the other hand, Part I touches on the earlier discovered layered materials such as graphene, molybdenum disulphide and tungsten disulphide to further deepen our understanding on the electrochemistry of these materials. This approach was used to address different research gaps of the respective fields for a more varied contribution to the scientific community.

The thesis then ends with concluding remarks and comments on the future directions of the field in **Chapter 10**.

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Chapter 2 –

Introduction and Literature Review

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Short selected excerpts of this introduction and literature review have also been published in the following review article. All discussions herein are written by the author of the thesis and reproduced here with permission.

Graphene and Its Electrochemistry – An Update

Ambrosi, A.; Chua, C. K.; **Latiff, N. M.**; Loo, A. H.; Wong, C. H. A.; Eng, A. Y. S.; Bonanni, A.; Pumera, M. *Chem. Soc. Rev.* **2016**, *45*, 2458.

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2.1. Introduction on Layered Materials

2.1.1. A Brief History

The explosion of interest in research on layered materials can be traced back to the first isolation of single layer graphene from layered graphite by Professor Geim and Professor Novoselov of the University of Manchester in 2004 using a simple “scotch-tape” mechanical cleavage method.¹ After its isolated, remarkable properties of graphene started to be unravelled.

Graphenes are two-dimensional sheets of strong sp^2 covalently bonded carbon atoms linked together in a honeycomb lattice structure.² In its bulk form, these flat layers are held together by weak van der Waals interaction, which can be separated into single, few- or multi-graphene layers by a variety of exfoliation techniques including mechanical, thermal, chemical and electrochemical methods.³ Graphenes have large theoretical specific surface area, flexibility, high intrinsic mobility, fast heterogeneous electron transfer, and potentially low cost of large-scale manufacture.⁴⁻⁷ It was reported that electrons can travel through graphene with minimal resistances, uncommon to other materials, possibly due to its pristine structure that contains negligible defects to scatter electrons.⁸ This gives rise to many interesting physical phenomena such as measurements of extremely high electron mobility and detection of single molecule adsorption.^{9,10}

Moreover, changing the number of layers and using different synthesis methods can alter the properties of graphene thereby providing tunability of its properties to suit a specific application. Through creative synthesis methods, a large myriad of graphene derivatives was developed such as zero dimensional buckyball, one dimensional carbon nanotubes, graphene oxide, reduced graphene oxide, graphene nanoribbons, doped graphene, halogenated graphene, functionalized graphene, graphane, graphitic carbon nitride and many more. This further makes the field more exciting as there are greater avenues for its possibilities and applications. These discoveries have potential to revolutionize our future.^{9,11,12} It is not surprising that Professor Geim and Professor

Novoselov even received the Nobel Prize in Physics in 2010 for pioneering the breakthroughs made from the first isolation of graphene.

Despite its fascination, graphene has its drawbacks. Graphene has a zero-band gap that restricts its performance in certain applications.^{13,14} This had spurred exploration on other layered materials, like transition metal dichalcogenides (TMDs), semiconductor chalcogenides, metal phosphorus chalcogenides and black phosphorus, that makes the field more colourful. With many possibilities in technological advances arising from these layered materials, the field continues to grow rapidly.

2.1.2. Preparation of Layered Materials

Several synthetic routes have been established for the preparation of layered materials. They can be categorized into two general approaches; top-down and bottom-up synthesis methods.¹⁵ An emphasis on graphene is given as it has pioneered the research on layered materials. The other layered materials generally follow these approaches as well.

Top-down methods

Top-down synthesis methods of layered materials would involve an exfoliation process. As the layers in graphite and other layered materials are held together by weak forces of interaction, they can be separated through mechanical cleavage or chemical means.¹⁵ The scotch-tape method used by Professors Geim and Novoselov is an example of exfoliation by a mechanical process. Although this method can yield pristine graphene which is important for fundamental studies, it lacks scalability and has low reproducibility. As such, exfoliation via chemical method was explored. This involves intercalation of ions and molecules (i.e. sulphuric acid, nitric acid, ammonia, Li ions, tetrabutyl ammonium hydroxide, bovine serum albumin) in between the layers to increase their interlayer

distances, followed by separation of the layers (i.e. sonication, heating, microwave). In the case of graphene, oxygen is often introduced through various oxidation methods such as the use of sulphuric acid and nitric acid under presence of strong oxidizing agents. The Staudenmaier, Hoffman and Hummers' oxidation methods are well-established methods for the oxidation of graphite.¹⁶ The exfoliation method via chemical process is often preferred due to its ease of scaling up for mass production.

Bottom-up methods

In contrast to top-down approaches where the bulk layered structures become exfoliated into single or few-layer sheets through exfoliation, bottom-up methods involve building of individual sheets from simple elements or compounds in the presence of catalysts. Special conditions and the use of supports can be used to facilitate their formation.¹⁶

Chemical vapour deposition (CVD) is one example where high temperatures are used to transport one or more volatile precursors via the vapour phase to react and decompose on a substrate in a reaction chamber.¹⁷ This method is known to produce layered materials of single or few sheets with large surface area and of high quality.^{18,19} However, this method can be costly for large scale production.

Alternatively, wet-chemistry can be used to prepare layered materials through reacting compounds chemically in solution. This is also often referred to as hydrothermal or solvothermal processes. For graphene, it can be synthesized by combining polycyclic aromatic hydrocarbon molecules together. However, the size of growth is restricted to a few nanometers as further growth becomes impeded by its insolubility in most organic solvents.²⁰

Graphene derived from most of these synthetic routes generally deviates from the International Union for Pure and Applied Chemistry (IUPAC) definition of graphene (“a single carbon layer of graphite structure, describing its nature by analogy to a polycyclic aromatic hydrocarbon of quasi-infinite size”).²¹ The common deviations to this definition is that the number of layers referred to the term ‘graphene’ is often more than one, and that oxygen and other heteroatoms are frequently present.²² As the trend of referring all ‘graphene’ derivatives as graphene is unlikely to be reversible, it is crucial to accompany all graphene- related studies (and all layered materials in general) with full characterizations of the material for readers to be well-informed of the true nature of the material reported.

Characterizations of layered materials

From the different synthesis routes available, a large variability of individual types of layered materials (i.e. graphene, TMDs, metal phosphorus chalcogenides, black phosphorus) with varying properties have been reported. A combination of several characterization techniques are often used to gather a clear picture of the materials produced. The commonly used methods are scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy dispersive spectroscopy (EDS), Raman spectroscopy, X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM).²² These characterizations are important to probe into the material’s size and morphology as well as the existence and bonding states of atoms on the surface of the material.

2.2. Layered materials and energy-related applications

Research to address energy-related issues has been of high interest due to critical concerns over climate change. Through many advances brought about from industrial revolution, it has produced an exponential increase in emission of greenhouse gases in the earth’s atmosphere (i.e. CO₂, CH₄ and NO_x). In a report by the Intergovernmental

Panel on Climate Change (IPCC) in 2014, there was a scientific consensus regarding the reality of anthropogenic climate change.²³ They presented that our industrial activities have significantly contributed to rapid increments in the world's average temperature and apparent rise in sea levels in the past decades (Figure 2.1).^{24,25} It is projected that if this trend continues to follow, heat waves and extreme weather events (i.e. heat waves, wildfires, droughts, floods, and cyclones) will become more frequent and intense in many regions. These events have already been evident, and they pose threats to our food security globally.

Figure 2.1. Global trends in (a) average combined land and sea surface temperature anomaly, (b) average sea level change and (c) average greenhouse gas concentrations. Adapted from reference 24.

Realizing the heavy consequences of climate change, a global collective effort must be made to improve our future. As such, many government agencies have implemented policies to reduce the carbon footprint within their countries. This includes allocating funding for research and development that addresses this challenge. In Singapore's context, Cambridge Centre for Advanced Research and Education in Singapore (CARES) in Campus for Research Excellence and Technological Enterprise (CREATE) is one example of a research entity established to "tackle the environmentally relevant and complex problem of assessing and reducing the carbon footprint of the integrated petro-chemical plants and electrical network on Jurong Island in Singapore".²⁶ A broad spectrum of research efforts can be done to achieve this aim, from improving our efficiency of energy usage, capturing carbon dioxide from industrial processes, progressing utilization of renewable clean energy and developing better performing energy storage systems. Besides climate change, many other factors like increasing demand for energy and decreasing supply of fossil fuel also contribute to propel such research in the energy sector.

Layered materials have been well demonstrated by many reports to show superior electronic, magnetic, electrochemical properties suitable for enhanced energy-related devices such as supercapacitors, lithium ion batteries, solar cells and electrocatalysts for clean fuel production.²⁷⁻⁴⁰ In the next section, we will demonstrate this by presenting specific layered materials as well as their properties and potential usages in energy applications.

2.2.1. Materials under Study

Graphene

As one of the first prototypes of layered structures, graphene and its derivatives have dominated research in layered materials. Graphene is a two-dimensional (2D) material which comprises of carbon atoms covalently bonded together in a hexagonal configuration by sp^2 bonds (Figure 2.2).² Its extraordinary properties include large surface area ($2630 \text{ m}^2/\text{g}$), flexibility, high elasticity, high mechanical strength as well as good thermal conductivity.⁴¹ These are favourable for many applications in the fields of electronics, thermal materials, photonics, sensing, electrocatalysis, energy storage and generation, drug delivery, bioimaging, diagnostics, environmental remediation, and the list is still expanding.⁴²⁻⁵⁰ Different forms of graphene are possible such as carbon nanotubes, fullerenes, nanoplatelets/nanosheets/nanoflakes, nanoribbons and quantum dots.

Figure 2.2. Structure of graphene

Transition metal dichalcogenides (TMDs)

Transition metal dichalcogenides (TMDs) is a promising alternative to graphene for its band gap. Unlike graphene, TMDs consist of more than one element (Figure 2.3A). TMDs represent a group of materials with formula AB_2 , where A is a d-block transition metal (e.g. molybdenum, tungsten, vanadium, niobium, tantalum) and B is a group 16 chalcogen (e.g. sulphur, selenium, tellurium).⁵¹ Among the various TMDs, MoS_2 and WS_2 are most widely studied. TMDs also exhibit favourable electronic and mechanical properties which can be applied to similar fields like graphene, such as

electronics, sensing, biomedical, electrocatalysis and batteries, supercapacitors and environmental control.⁵²⁻⁵⁹ Interestingly, there can be different kinds of polymorphs possible for TMD structures; 1T phase, 2H phase as well as 3R phase (Figure 2.3B).⁶⁰ The numbers refer to the number of layers per unit cell while letters indicate trigonal, hexagonal and rhombohedral arrangements respectively. The different polymorphs can have varying properties such as metallic character.^{61,62}

Figure 2.3. (A) Side view of a layer of TMD (top) and various transition metals and chalcogens that are part of the TMD family (bottom). Adapted from reference 39. (B) Structural representation of 1T, 2H and 3R TMD polymorphs.

Semiconductor chalcogenides

Some layered inorganic compounds containing metalloid elements (i.e. Ga, Ge) and chalcogens (i.e. S, Se) in the ratio of 1 : 1 have been identified to possess suitable properties for semiconductor applications.⁶³⁻⁶⁶ We have termed this group of materials as semiconductor chalcogenides. GaSe and GeS are two examples of semiconductor chalcogenides that show remarkable properties as new materials in solar cells and enhanced lithium ion batteries with improved performances.⁶⁷⁻⁷³ In comparison with graphite, GaSe and GeS have more than one element present and each layer shows a more complex structure (Figure 2.4).

Figure 2.4. Structures of GaSe (A) and GeS (B).

Metal phosphorus chalcogenides

Beyond graphene, TMDs and semiconductor chalcogenides, metal phosphorus chalcogenide present a new group of trielement layered material with a general formula of MPX_n where M is a metal (i.e. Mn, Fe, Co, Ni), X is a chalcogen and n is typically 3 or 4.⁷⁴ They have strong anisotropic properties arising from their layered structures and can host compounds for intercalation.⁷⁵ Besides that, interesting magnetic and antiferromagnetic properties of several MPX_n have been reported.⁷⁶⁻⁷⁸ In addition, this

class of materials also display a broad band gap which would be useful for optoelectronic applications that requires a broad range of wavelength for operation.⁷⁴ In terms of structure, a variety of crystal structures have been identified for different combinations of MPX_n (Figure 2.5).^{79,80} Besides optoelectronics, metal phosphorus chalcogenides have shown potential applications for sensing and electrocatalysis in energy-related fields.^{79,80}

Figure 2.5. Crystal structures of several metal phosphorus chalcogenide materials with a general formula MPX_n . Adapted from reference 79.

Black phosphorus (BP)

BP has recently been reintroduced as a valuable layered material.⁸¹ It is mono-elemental with various forms of allotropes, similar to graphite.^{82,83} Its structure, however differs from graphite, with each sheet having a unique puckered arrangement as displayed in Figure 2.6.⁸² This new addition to the layered materials family have shown attractive properties and much potential in various energy-related applications such as photocatalysis, and rechargeable batteries.⁸²⁻⁸⁷

Figure 2.6. Structures of black phosphorus. Adapted from reference ⁸⁸.

2.2.2. Electrochemistry of Layered Materials and Energy Applications

Electrochemistry is a branch in chemistry which studies relations between electrical and chemical processes. Chemical composition can be altered by applying electron flow and the converse is also true; electricity can be generated through changes in chemical composition.⁸⁹ Electrochemical reactions occur in many energy generation and storage systems such as fuel cells, batteries and capacitors.

The integration of renewable sources of energy such as solar and wind power into the electrical grid system is a challenge due to their fluctuating and intermittent nature. Electrochemical energy storage devices such as batteries and capacitors can potentially be a reliable system for bulk energy storage and distribution due to their high power efficiency, large energy density, good recyclability and ease of maintenance.⁹⁰ When electricity is needed, this energy can be used to power electrolyzers which split water into hydrogen and oxygen gas without any production of greenhouse gases. The hydrogen gas produced can then be utilized in fuel cells to produce electricity cleanly with only water as its by-product. This hydrogen economy system is envisioned as a promising solution to reduce our carbon footprint in the future.⁹¹

Therefore, investigating the electrochemistry of layered materials is important in developing their potential and application in energy systems. We will be focussing on two energy-related applications in this thesis; namely supercapacitors and electrocatalyst for hydrogen evolution reaction.

2.2.3. Supercapacitors

The increasing demand for energy spurs researchers to develop enhanced energy storage devices. Owing to their fast charge-discharge character, long cycling life and high-power density, supercapacitors have attracted significant research attention. Presently, they commonly are used alongside batteries for additional power supply. They are not preferred for stand-alone units due to their lower energy densities relative to batteries. To illustrate, the energy density of lithium ion batteries is reported to be greater than 180 W h kg^{-1} whereas that of supercapacitors is lower than 10 W h kg^{-1} .^{92,93} Currently, much research efforts are devoted to enhancing the performance of supercapacitors by developing on their energy density to meet the performance of present rechargeable batteries while maintaining its advantages of long cycling life and high power density.

Types of supercapacitors

There are two types of supercapacitors depending on their charge storage mechanism; (1) electrochemical double-layer capacitors (EDLCs), and (2) pseudocapacitors (or oxide supercapacitors). Typically, EDLC electrodes consist of carbon-based materials such as graphene or activated carbon whereas pseudocapacitors are generally made of inorganic compounds or conducting polymers.⁹⁴ EDLCs store charges by reversible adsorption of electrolyte ions onto the surface of electrode materials (non-Faradaic charge transfer) without redox and diffusion limitations.^{92,93,95-98} On the other hand, pseudocapacitors can store charges in two ways: (1) by Faradaic electron transfer i.e. redox reaction with two or more states of metal centres, and (2) by non-Faradaic charge storage i.e. adsorption of ions on the electrical double layer at the surface of electrode material.⁹⁹

Both these Faradaic and non-Faradaic processes are characterized by the equation $i = Cv$ where i represents current (in A), C is for capacitance (in F) and v is the

charge/discharge rate (in V/s). The total charge (Q in Coulombs) stored can be calculated by the equation: $Q = C \times \Delta E$, where ΔE is the change in potential window in which the two processes are occurring. These equations only hold for materials that display rectangular-shaped cyclic voltammograms, and linear voltage responses for charging/discharging tests under constant current.⁹⁹

In contrast, the charge storage of batteries primarily depends on Faradaic processes through intercalation of cations (i.e. Li^+ or H^+) that enable redox reactions within the crystalline framework of electrode materials, but it is diffusion-controlled and hinders its rate of charge and discharge.¹⁰⁰⁻¹⁰² As the charge storage mechanism of pseudocapacitors lie between that of EDLCs and batteries, they hold great promise of achieving a good cycling life and power density of EDLCs together with high battery-level energy density.

As charges are stored on the surface of electrode materials, having an electrode of larger surface area would significantly improve its capacitance. Consequently, there have been many reports of developing nano-sized and porous electrode materials for supercapacitors.⁹⁶ Owing to their large surface area, layered materials make excellent candidates as electrode materials for supercapacitors. This has been demonstrated by many recent reports of graphene and TMDs revealing high capacitive performance.¹⁰³⁻¹⁰⁵

How to evaluate capacitive performance

The capacitive performance of a material can be evaluated by various electrochemical measurements to help us to understand its charge storage mechanism. This includes cyclic voltammetry (current versus voltage curves) and galvanostatic charge/discharge test (voltage versus time curves) performed in basic electrolytes. It is important to first classify the material properly as incorrect evaluation of its capacitive performance can mislead the research community.

Under cyclic voltammetry (CV), EDLCs typically show a potential-independent relationship with current or rectangular-shaped voltammograms (Figure 2.7a,b) whereas batteries display distinctly separated oxidation and reduction peaks (Figure 2.7g,h). In the case of galvanostatic constant current discharging measurements, EDLCs present a linear response between voltage and time (Figure 2.7c). On the other hand, battery-type electrodes show clear plateaus of constant potential with time (Figure 2.7i). Meanwhile, pseudocapacitors demonstrate intermediate features between EDLCs and batteries for these electrochemical evaluations (Figure 2.7d-f).⁹⁹ When measuring at different scan rates, one can observe a direct proportionality relationship between peak current and scan rates ($i \propto v$) for a capacitor and ($i \propto \sqrt{v}$) response for a battery. These features help us to distinguish the different categories for a new electrode material for energy storage.

Figure 2.7. Electrochemical evaluation of EDLCs, pseudocapacitors and batteries under cyclic voltammetry (a, b, d, e, g, h) and galvanostatic discharge measurements (c, f, i). Pseudocapacitive materials can have one or more mechanism of charge storage ranging from surface redox (b), intercalation (d) and intercalation with partial redox (e). Adapted from reference 99.

After identifying the category of electrode material used, the total charge can be calculated. For EDLCs or pseudocapacitors, the capacitance value should be determined. It is important to normalize these values to mass, volume or area of the electrode for a fair comparison with literature.⁹⁹

Doped graphene for supercapacitors

Several classes of layered materials have been presented in literature for supercapacitor applications. Compared to non-noble metals, carbon-based materials are generally cheaper, more abundant, and less susceptible to corrosion and oxidation. Moreover, graphene's high electrical conductivity and high theoretical specific surface area makes it a suitable candidate for electrode materials in supercapacitors.

Theoretically, pure graphene is calculated to achieve 550 F/g.¹⁰⁶ However, graphene sheets can easily restack between layers which greatly reduces its effective surface area and capacitive performance experimentally.^{107,108} Furthermore, there is great interest to enhance the capacitance of graphene to reach close to the energy density of batteries. Increase in capacitance can be achieved by increase in surface area, increase in electrode conductivity, and decrease in diffusion limitations of electrolyte ions.^{106,109} Several structural design strategies have been developed in this endeavour including the use of spacers to prevent re-stacking of graphene sheets, doping of heteroatoms, functionalization with redox molecules, synthesis of three-dimensional structures as well as synthesis of graphene composites.¹¹⁰⁻¹¹² Doping is chosen here for further exploration as it has been proven to be an effective approach in modulating properties across many materials and for different applications.¹¹³⁻¹¹⁷

Doping refers to the introduction of foreign elements into a material's structure. The doping process disrupts the planar structure of graphene thereby changing its electronic properties. Graphene can be doped with p-block elements such as nitrogen

(N), boron (B), sulfur (S), hydrogen (H), oxygen (O), fluorine (F), chlorine (Cl) etc.¹¹⁰ An illustration of various structures that could be formed by doping of graphene with p-block elements can be found in Figure 2.8. Alternatively, graphene can also be doped using d-block elements (metals) intentionally or unintentionally through impurities present during synthesis.^{118,119} Co-doping with more than one element is also possible and have been presented in literature.¹²⁰

Figure 2.8. Possible bonding configurations of graphene doped with different p-block elements. The different colours represent different elements: nitrogen-doped (red), boron-doped (pink), sulphur-doped (blue), fluorine-doped (green), hydrogenated (yellow) and hydroxylated (purple) graphene. Adapted from reference 110.

From semiconductor theory, there can be n- and p-type doping effects for materials. By substitution of a carbon atom in a graphene lattice with nitrogen, it is expected to behave as an n-type dopant due to additional electrons introduced into the network. Conversely, substituting a carbon atom in graphene with boron is expected to show p-type doping behaviour.¹²¹ Interestingly, in literature we find both p-type and n-type doping can enhance capacitance of graphene.¹²²⁻¹³⁰ Experimentally, doping of graphene can generally occur in two ways; either by (1) adsorption of moieties to the surface of graphene or (2) substitution of atoms into its lattice structure.¹³¹ Even though

the latter requires larger activation energy due to strong C-C bonds in graphene, DFT calculations have proven this to be possible by controlling reaction conditions.¹³²

Synthesis of doped graphene can be similar to that of undoped graphene but with an additional source of dopants introduced as reagents during the process. For instance, Poh et. al. prepared S-doped graphene by exfoliating graphite oxides using heat in sulphur dioxide, hydrogen sulphide and carbon disulfide atmospheres.¹³³ The authors found that varying the oxidation methods used in graphite oxide synthesis (i.e. Hummers, Staudenmaier and Hoffman), as well as using different sulphur precursors, give rise to different amounts of S doping. Alternatively, doped graphene can also be prepared via functionalization after graphene synthesis.¹³⁴ For example, Tang et. al. presented post-functionalization of mechanically exfoliated graphene using trimethylboron decomposed by microwave plasma in a CVD chamber.¹³⁵ This method had successfully incorporated 13.85 atomic percent of boron in the graphene sample.

While the mechanism behind the improved activity is still not entirely clear, several postulations have been made. A group reported that doping alters the electronic structure of N-doped graphene in a manner that increases its charge carrier density and improves its quantum capacitance to result in higher capacitance.¹³⁶ This was also supported by density functional theory (DFT) calculations.¹³⁷ Also, depending on the type of N-bonding in the graphene structure (i.e. pyridinic N, graphitic N, pyrrolic N), they can produce different electronic and magnetic effects.^{138,139} Interestingly, Ambrosi et. al. found that structural defects play a greater role in improving capacitance compared to the type and quantity of dopants.¹⁴⁰ Meanwhile for S-doping, it was reported that S species can reduce graphene's ability to absorb moisture thus improving adsorption of electrolyte ions.¹⁴¹

2.2.4. Hydrogen Evolution Reaction (HER)

Besides supercapacitors, another important area for energy research is developing electrocatalysts for hydrogen evolution reaction (HER). Hydrogen, an energy carrier with high energy density and potentially zero carbon emissions, is envisioned as the next fuel source. Promoted by efficient electrocatalysts, hydrogen can be produced cleanly using an electrochemical process known as HER ($2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$).^{142,143} This proposed solution, however, requires substantial technological improvements for its actual application. One important requirement is the development of efficient and cost-effective electrode materials to replace expensive and scarce Pt.^{142,144}

This reaction occurs at the cathodic side of electrolyser systems which provides a clean supply of hydrogen gas for fuel cells, without any carbon emissions. HER has thermodynamic and kinetic constraints; the kinetic constraints are typically resolved by using efficient platinum-based electrocatalysts. However, their high cost and limited abundance hinder their widespread practical implementation. To improve cost-competitiveness, it is critical to develop alternative efficient HER electrocatalysts based on earth-abundant elements.

HER occurs via a two-electron pathway ($2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$). It is generally accepted to involve two steps; namely electrochemical hydrogen adsorption (Volmer step) and desorption which can occur either electrochemically (Heyrovský step) or chemically (Tafel step).¹⁴⁵⁻¹⁴⁸ In the Volmer step, a proton and an electron combine to produce adsorbed hydrogen (H^*) on the active site of the electrocatalyst surface (M). Subsequently, the reaction can proceed by either the Heyrovský or Tafel step.¹⁴⁵ In the Heyrovský step, adsorbed hydrogen merges with a proton in the presence of an electron to produce hydrogen gas, whereas in the Tafel step, two adsorbed hydrogen atoms combine for hydrogen gas formation. Since H^* is present regardless of which route is followed, the Gibbs free energy of hydrogen adsorption (ΔG_{H^*}) greatly influences the overall rate of reaction and is often described as the main criterion for assessment of the process.

Optimal HER electrocatalysts, such as platinum, have ΔG_{H}^* close to zero which indicates that H^* binds neither too strongly (when ΔG_{H}^* is largely negative) nor too weakly (when ΔG_{H}^* is largely positive) onto the electrocatalyst surface.¹⁴⁹

In order to determine which pathway the HER process proceeds after the first step of electrochemical hydrogen adsorption, a Tafel plot obtained from the HER polarization curve can be used.¹⁵⁰ The Tafel plot relates the overpotential (Z) as a function of logarithm of current density (j). By fitting the linear portion of the graph to the Tafel equation ($Z = b \log j + a$), the Tafel slope value can be obtained from the value 'b'. Tafel slope values close to 40 mV per decade (mV dec^{-1}) indicate that the Heyrovský step is the rate-determining step (RDS), while values close to 30 mV dec^{-1} suggest that the Tafel step is the RDS. From here, we can propose whether the reaction pathway proceeded through the electrochemical desorption or the chemical desorption process. In fact, Tafel slope is an inherent property of a material. For Pt, its Tafel slope value is measured to be 30 mV dec^{-1} which shows that it catalyses HER via the Volmer–Tafel pathway, with the Tafel step being the RDS.¹⁴⁸

Besides acidic media, HER can also be performed under alkaline conditions ($2\text{H}_2\text{O} + 2\text{e}^- \rightarrow \text{H}_2 + 2\text{OH}^-$) in electrolyser systems. However, comparing acidic and alkaline electrolyzers, the former is generally more superior to the latter in terms of industrial operation.¹⁴² This could be due to an additional water dissociation step required for hydrogen generation under basic conditions, which increases the energy requirement of the process.¹⁴⁸ A summary and comparison of the HER mechanism in acidic and basic media is presented in Table 2.1.

Table 2.1 HER mechanism in acidic and basic media

Reaction pathway	Acidic media	Basic media	Tafel slope (mV dec ⁻¹)
Volmer step	$M + H^+ + e^- \leftrightarrow M-H^*$	$M + H_2O + e^- \leftrightarrow M-H^* + OH^-$	120
Heyrovský step	$M-H^* + H^+ + e^- \leftrightarrow M + H_2$	$M-H^* + H_2O + e^- \leftrightarrow M + H_2 + OH^-$	40
Tafel step	$2(M-H^*) \leftrightarrow H_2 + 2M$	$2(M-H^*) \leftrightarrow H_2 + 2M$	30
Overall:	$2H^+ + 2e^- \leftrightarrow H_2$	$2H_2O + 2e^- \leftrightarrow H_2 + 2OH^-$	-

To evaluate the performance of a material towards hydrogen evolution reaction, two parameters are typically used; overpotential at a certain current density (e.g. -10 mA/cm²) as well as Tafel slope.¹⁴² The former parameter (i.e. overpotential at a certain current density) is an indication of potential required to initiate the hydrogen evolution reaction, and allows for comparison of electrode activities across different materials easily, whereas the latter parameter (i.e. Tafel slope) presents a measure of additional voltage required to increase the resulting current by an order of magnitude, and is often reported in units of mV/decade.¹⁴² A low Tafel slope is desirable as it indicates a faster, more efficient HER catalysis. As mentioned earlier, Tafel slope can also be an indication of the reaction mechanism, however, it cannot be used ambiguously without further experimental evidence.¹⁵¹

MoS₂ and WS₂ as HER electrocatalysts

Among many different layered materials presented in literature, we are interested to further expand on the development of MoS₂ and WS₂ due to their promising HER activities reported.¹⁵² This was discovered through DFT calculations.⁴⁰ HER performances of various catalysts can be presented in a ‘volcano plot’ where exchange current density is plotted as a function of ΔG_H (Figure 2.9). It predicts that the edge sites on 2H MoS₂ can give high HER catalytic activity with ΔG_H close to zero. This was subsequently confirmed experimentally with the active edge sites of MoS₂ determined to be the sulfide-terminated Mo-edge.¹⁵³ The basal plane however was found to be electrocatalytically inert.¹⁵⁴⁻¹⁵⁶ Between the different MoS₂ polymorphs, 1T metallic phase was observed to be more favourable than 2H semi-conducting phase.¹⁵⁷ With initial promising performance of MoS₂ seen, the field further developed with many strategies to enhance its performance to approach closer to that of Pt. This includes activating the basal plane, increasing number of edge sites through engineering (i.e. nanowires, vertically aligned, defects formation, 3D structures, MoS_x, MoS clusters), controlling number of layers, control of polymorph structure, doping, functionalization, composite structures, etc.^{142,158-161} Neighbouring TMDs are also ventured into and WS₂ is another widely studied TMD as it displays favourable activities.^{162,163}

Figure 2.9. Volcano plot comparing exchange current density and Gibbs free energy of adsorbed proton (ΔG_H) for nanoparticulate MoS₂ and pure metals. Adapted from reference 153.

2.3. Layered Materials and their Safety Aspects

After looking at layered materials and their potential in energy-related fields, especially from an electrochemistry point of view, we shall move on to discuss their safety aspects in this section.

2.3.1. A Brief History

We have seen many advances and possibilities arising from research on layered materials, including benefits in alternative energy, environmental remediation, medical uses, electronics, photonics, optics, sensing and electrocatalysis. This is evident from the massive amount of attention and investment given for its development. This field of study is also often termed as nanotechnology as one of the dimensions for 2D materials lies in the nanoscale (between 1 to 100nm). Despite its hype and bright outlooks, there are concerns about the potential health hazards that these nanomaterials can pose.¹⁶⁴

The reason for its concern is not without basis. Historically, we have witnessed adverse effects of quartz, coal and asbestos which had resulted significant cases of deaths and respiratory illness.¹⁶⁵ For instance, in the Hawk's Nest tunnel incident in West Virginia in the 1920s, the construction of a tunnel to divert water for hydroelectric power generation had produced the state's worst industrial power. Silica rock dust produced from the process had resulted in 764 deaths out of an estimated 2500 workers and another 1500 employees later developed acute silicosis.¹⁶⁶ This tragedy had stirred recognition of occupational lung diseases from respiration of fine particles. These incidents brought about the birth of particle toxicology which aims to understand the causes, mechanisms and relationships behind fine particle inhalation. Research data developed from such studies led to agreement between government, employers as well as trade unions for guideline standards of respirable dust of 7 mg/m³ and 2 mg/m³ in the UK and USA respectively to protect workers.^{167,168}

Realizing the possible health risks associated with nanomaterials, there is a need for regulatory bodies, funding agencies and manufacturing industries to encourage investigation of toxicological profiles of these materials. Besides particle toxicology, there is also a need to investigate the ecological implications of nanomaterials (such as implications on aquatic life, soil quality etc) as these effects are not yet well understood.¹⁶⁹ As nanomaterials expand their application to the biomedical sector, toxicity studies beyond exposure to the respiratory systems are also explored. Through these assessments to address the risks and safety of nanomaterials, we can be better informed of the possible threats posed by these materials, and proper regulations can thus be developed to protect workers, consumers as well as the environment.¹⁷⁰

In this thesis, we will be focussing on inhalation studies of 2D nanomaterials as it is the primary route of entry for nanoobjects into the body. Our lungs transport air from the environment to the alveoli, via the respiratory tract, where gaseous oxygen and carbon dioxide are exchanged. Three hundred million alveoli with a surface area encompassing 140 m² are available to facilitate this diffusion process.¹⁷¹ Large particles would be trapped in the upper airways by mucociliary clearance mechanism while fine particles below 2.5 µm can be delivered into the alveoli with the air. At the alveoli, these foreign particles are likely to be removed by macrophages and transported back to the bronchus for removal by mucociliary clearance.¹⁷² This natural protection mechanism is extremely effective so long as they have not been overstressed by means of excessive smoking or prolonged exposure of dust in the environment. Literature have reported that high doses of nanomaterials can enter the bloodstream by overcoming the thin air-blood barrier at the alveoli or via nerve fibres in the olfactory epithelium but these quantities are significantly small.¹⁷¹

2.3.2. Current Progress

In the next section, we will look at current progress on cytotoxicity assessment of 2D nanomaterials by covering an overview of the general steps to conduct toxicity assessment.

Material characterization

As there can be a wide range of variations within a class of 2D material in terms of size, composition, thickness, etc, it is important to couple their cytotoxicity assessments with sufficient characterizations of the materials tested. Typical material characterizations include imaging techniques such as SEM, TEM, elemental composition analysis such as EDS, XPS, and size determination techniques as well as surface charge analysis using dynamic light scattering (DLS). Proper material characterization is imperative as it enables us to investigate how the observed toxicity effect can be linked to a material's properties.¹⁷³

In vitro studies

Typically, in vitro investigation is the starting point for nanotoxicity study due to its ease of execution and low cost.¹⁷⁴ A general methodology involves cell viability assessment after a certain period of cell exposure to the layered materials. Human lung carcinoma cancer epithelial cell (A549) is a common cell line used because the primary route of entry for fine materials into the body is the respiratory tract.¹⁷⁵ Besides A549 cells, human bronchial epithelial cells (BEAS-2B), alveolar macrophage (AM), human cervical cancer cell (HeLa), mouse fibroblast cells (NIH 3T3), human embryonic kidney cells (HEK293), mouse embryo fibroblast cells (NIH 3T3), human skin fibroblasts (CRL-2522), human lung fibroblast cells (HLF), human liver carcinoma cells (HepG2), human ovarian carcinoma cells (OVCAR-3), human breast carcinoma cells (MCF-7) and many

other cell lines have been used for cytotoxicity assessment and biocompatibility studies of 2D nanomaterials reported in literature.¹⁷⁶ Different cell lines have different sensitivities and can respond differently to the same nanomaterials.

Certain guidelines have been reported by organizations on how to conduct in vitro cytotoxicity assessment of nanomaterials.^{177,178} However, a standard approach on its implementation has yet to be agreed upon in literature. For example, a large variety of values with regards to the range/unit of test concentrations and duration of exposures have been reported. In literature, typically the test concentration ranges to fall between 0 to 100, 200 or 500 µg/mL over a period of 0 to 12, 24 or 48 hours of exposure for in vitro studies.^{173,176}

Cell viability assays

After cell exposure with the nanomaterials, two commonly used cell viability assays are water-soluble tetrazolium salt and methyl-thiazolyldiphenyl-tetrazolium bromide or WST-8 and MTT in short.¹⁷⁹ They are absorbance-based cell viability assays with active tetrazolium salts present which can produce a colour change with viable cells by dehydrogenase activity, thereby quantifying the amount of metabolically active cells.^{180,181} Normalization of data obtained with a negative control of cells unexposed with any test materials would enable us to determine the extent of cytotoxicity of the materials. The use of more than one assay is recommended due to conflicting responses observed in previous toxicity assessment of nanomaterials.¹⁸²

Due to reports of nanomaterial-induced interference in literature, control experiments between the assays and materials in the absence of cells are necessary.¹⁸³⁻¹⁸⁶ In the event of nanomaterial interfering with these cell viability assays, washing steps can be introduced, or a background subtraction of the assay and test materials can be performed for a more reliable assessment.¹⁸⁷

In vivo studies

Apart from in vitro studies, in vivo studies can also be conducted. In vivo studies can provide a more holistic assessment as animal models can account for complex interactions between the 2D test materials with biological components e.g. proteins, different cells and organs, as well as unique responses in terms of biodistribution, immune response and removal via excretion and egestion.¹⁸⁸ In spite of these advantages, there are differences between the respiratory systems of rodents and humans which can provide erroneous comparison.¹⁸⁸ Moreover, there are strict regulations to follow to ensure that animal rights are protected.

Exposure of the 2D materials to the test animals can be achieved by inhalation of aerosolized nanoparticles as well as intratracheal and intranasal instillation. An evaluation of literature reported on in vivo studies since 2000 revealed that instillation studies are significantly preferred over inhalation ones.¹⁸⁹ Even though inhalation-based analysis is closer to reality, its setup and maintenance is more complex and demanding as it requires a controlled aerosol environment over long durations (typically 6h per day of treatment for several days/weeks). Hence, instillation method is often favoured as an alternative.

In contrast to in vivo studies, in vitro studies can provide rapid high throughput screening of nanomaterials.¹⁷² With advancement of in vitro models, they are able to simulate multicellular systems and whole organs to enable a more realistic investigation.¹⁹⁰⁻¹⁹⁴ This can reduce the need for in vivo experiments in the future.

In silico studies

A recent advancement in the field of nanotoxicology is *in silico* studies. It is an alternative approach to predict toxicity of materials using computation and theoretical models, with support from experimental data and mathematical calculations for interpolation.¹⁹⁵⁻¹⁹⁸ This novel methodology is favoured for its efficiency in terms of saving time, money and resources.¹⁹⁶ Moreover, it does not entail ethical conflicts associated with animal usage. However, this technology is still under development and requires experimental work for verification.¹⁹⁹⁻²⁰²

References:

1. Geim, A. K.; Novoselov, K. S. *Nat. Mater.* **2007**, *6*, 183–191.
2. Pumera, M. *Chem. Soc. Rev.* **2010**, *39*, 4146–4157.
3. Cai, M.; Thorpe, D.; Adamson, D. H.; Schniepp, H. C. *J. Mater. Chem.* **2012**, *22*, 24992–25002.
4. Pumera, M. *J. Mater. Chem. C* **2014**, *2*, 6454–6461.
5. Morozov, S.V.; Novoselov, K. S.; Katsnelson, M. I.; Schedine, F.; Elias, D. C.; Jaszczak, J. A.; Geim, A. K. *Phys. Rev. Lett.* **2008**, *100*, 016602.
6. Withers, F. et. al. *Nat. Mater.* **2015**, *14*, 301–306.
7. Ren, W.; Cheng, H.-M. *Nat. Nanotechnol.* **2014**, *9*, 726–730.
8. Service, R. F. *Science* **2009**, *324*, 875–877.
9. Allen, M. J.; Tung, V. C.; Kaner, R. B. *Chem. Rev.* **2010**, *110*, 132–145.
10. Schedin, F. et. al. *Nat. Mater.* **2007**, *6*, 652–655.
11. Raccichini, R.; Varzi, A.; Passerini, S.; Scrosati, B. *Nat. Mater.* **2015**, *14*, 271–279.
12. Wang, X.; Shi, G. *Energy Environ. Sci.* **2015**, *8*, 790–823.
13. Wolfe, K. *Nanotechnology: Concepts, Methodologies, Tools, and Applications*; Information Resources Management Association: USA, 2015.
14. Sharon, M.; Sharon, M. *The Intriguing Electronic Properties (Dirac Point) Graphene: An Introduction to the Fundamentals and Industrial Applications*; Wiley: USA, 2015.
15. Edwards, R. S.; Coleman, K. S. *Nanoscale* **2013**, *5*, 38–51.
16. Sturala, J.; Luxa, J.; Pumera, M.; Sofer, Z. *Chem. - Eur. J.* **2018**, *24*, 5992–6006.
17. RSC Chemical Methods Ontology (CMO),
<http://www.rsc.org/publishing/journals/prospect/ontology.asp?id=CMO:0001314&MSID=c2nr32629a> (assessed August 4, 2018)
18. Reina, A. et. al. *Nano Lett.* **2009**, *9*, 30–35.
19. Rao, C. N. R.; Sood, A. K.; Subrahmanyam, K. S.; Govindaraj, A. *Angew. Chem.* **2009**, *48*, 7752–7777.
20. Chen, L. et. al. *Angew. Chem., Int. Ed.* **2012**, *51*, 7640.
21. Fitzer, E. et. al. *Pure Appl. Chem.* **1995**, *67*, 473.

22. Ambrosi, A.; Chua, C. K.; Bonanni, A.; Pumera, M. *Chem. Rev.* **2014**, *114*, 7150–7188.
23. Oreskes, N. *Science* **2004**, *306*, 1686.
24. National Academy of Sciences Committee on the Science of Climate Change, *Climate Change Science: An Analysis of Some Key Questions*; National Academy Press: Washington DC, 2001.
25. Intergovernmental Panel on Climate Change (IPCC)'s fifth assessment report 2014, http://ipcc.ch/pdf/assessment-report/ar5/syr/AR5_SYR_FINAL_SPM.pdf (assessed May 31, 2018)
26. Cambridge Centre for Advanced Research and Education in Singapore (CARES) in Campus for Research Excellence and Technological Enterprise (CREATE). <https://www.create.edu.sg/about-create/research-centres/cares> (assessed May 31, 2018)
27. Simon, P.; Gogotsi, Y. *Nat. Mater.* **2008**, *7*, 845–854.
28. Lee, S.; Lee, S. H.; Kim, T. H.; Cho, M.; Yoo, J. B.; Kim, T.; Lee, Y. *ACS Appl. Mater. Interfaces* **2015**, *7*, 8070–8075.
29. Nitta, N.; Wu, F.; Lee, J. T.; Yushin, G. *Mater. Today* **2015**, *18*, 252–264.
30. Li, Q.-F.; Duan, C.-G.; Wan, X. G.; Kuo, J.-L. *J. Phys. Chem. C* **2015**, *119*, 8662–8670.
31. Jiang, K. C.; Wu, X. L.; Yin, Y. X.; Lee, J.S.; Kim, J.; Guo, Y. G. *ACS Appl. Mater. Interfaces* **2012**, *4*, 4858–63.
32. Tahersima, M. H.; Sorger, V. J. *Nanotechnology* **2015**, *26*, 344005.
33. Tsuboi, Y. et. al. *Nanoscale* **2015**, *7*, 14476–14482
34. Tsai, M.-L.; Su, S.-H.; Chang, J.-K.; Tsai, D.-S.; Chen, C.-H.; Wu, C.-I.; Li, L.-J.; Chen, L.-J.; He, J.-H. *ACS Nano* **2014**, *8*, 8317–8322.
35. Yang, J.; Shin, H. S. *J. Mater. Chem. A* **2014**, *2*, 5979–5985.
36. Lv, R. et. al. *Acc. Chem. Res.* **2015**, *48*, 56–64.
37. Eng, A. Y. S. et. al. *ACS Nano* **2014**, *8*, 12185–12198.
38. Late, D. J.; Rout, C. S.; Chakravarty, D.; Ratha, S. *Can. Chem. Trans.* **2015**, *3*, 118-157.
39. Gibney, E. *Nature* **2015**, *522*, 274-276.
40. Chhowalla, M. et. al. *Nat. Chem.* **2013**, *5*, 263–275.

41. Pumera, M.; Ambrosi, A.; Bonanni, A.; Chng, E. L. K.; Poh, H. L. *TrAC, Trends Anal. Chem.* **2010**, *29*, 954–965.
42. Geim, A. K. *Science* **2009**, *324*, 1530–1534.
43. Chen, D.; Feng, H.; Li, J. *Chem. Rev.* **2012**, *112*, 6027–6053.
44. Chandrasekhar, P. Graphene Applications in Electronics, Electrical Conductors, and Related Uses. In *Conducting Polymers, Fundamentals and Applications*; Chandrasekhar, P. Ed.; Springer: Cham, 2018.
45. Garcia, J. H.; Vila, M.; Cummings, A. W.; Roche, S. *Chem. Soc. Rev.* **2018**, *47*, 3359–337.
46. Zhang, Q. et. al. *Mater. Sci. Engin. C* **2017**, *77*, 1363–1375.
47. Shahil, K. M. F.; Balandin, A. A. *Solid State Commun.* **2012**, *152*, 1331–1340.
48. Munuera, J. M. et. al. *ACS Appl. Mater. Interfaces* **2017**, *9*, 24085–24099.
49. Zhang, H.; Chhowalla, M.; Liu, Z. *Chem. Soc. Rev.* **2018**, *47*, 3015–3017.
50. Kuo, W.-S. et. al. *ACS Appl. Mater. Interfaces* **2018**, *10*, 14438–14446.
51. Chia, X. et. al. *Chem. Rev.* **2015**, *115*, 11941–11966.
52. Yang, E. et. al. *J. Phys. Chem. C* **2015**, *119*, 26374–26380.
53. Jariwala, D. et. al. *ACS Nano* **2014**, *8*, 1102–1120.
54. Pumera, M.; Sofer, Z.; Ambrosi, A. *J. Mater. Chem. A* **2014**, *2*, 8981–8987.
55. Wang, H.; Feng, H.; Li, J. *Small* **2014**, *10*, 2165–2181.
56. Tang, H.; Wang, J.; Yin, H.; Zhao, H.; Wang, D.; Tang, Z. *Adv. Mater.* **2015**, *27*, 1117–1123.
57. Acerce, M.; Voiry, D.; Chhowalla, M. *Nat. Nanotechnol.* **2015**, *10*, 313–318.
58. Chia, X.; Adriano, A.; Lazar, P.; Sofer, Z.; Luxa, J.; Pumera, M. *Adv. Funct. Mater.* **2016**, *26*, 4306–4318.
59. Radisavljevic, B.; Radenovic, A.; Brivio, J.; Giacometti, V.; Kis, A. *Nat. Nanotechnol.* **2011**, *6*, 147–150.
60. Kuc, A.; Heine, T. *Chem. Soc. Rev.* **2015**, *44*, 2603–2614.
61. Lin, Y.-C.; Dumcenco, D. O.; Huang, Y.-S.; Suenaga, K. *Nat. Nanotechnol.* **2014**, *9*, 391–396.
62. Toh, R. J.; Sofer, Z.; Luxa, J.; Sedmidubský, D.; Pumera, M. *Chem. Commun.* **2017**, *53*, 3054–3057.
63. Humbert, A.; Salvan, F.; Mouttet, C. *Surf. Sci.* **1987**, *181*, 307–312.

64. Adach, S. *The Handbook on Optical Constants of Semiconductors: In Tables and Figures*; World Scientific: Singapore, 2012.
65. Chikan, V.; Kelley, D. F. *J. Chem. Phys.* **2002**, *117*, 8944–8952.
66. Yu, P.; Cardona, M. *Fundamentals of Semiconductors: Physics and Materials Properties*, 2nd ed; Springer: New York, 1999.
67. Li, C.; Huang, L.; Snigdha, G. P.; Yu, Y.; Cao, L. *ACS Nano* **2012**, *6*, 8868–8877.
68. Antunez, P. D.; Buckley, J. J.; Brutchey, R. L. *Nanoscale* **2011**, *3*, 2399–2411.
69. Cho, Y. J. et. al. *Chem. Commun.* **2013**, *49*, 4661–4663.
70. Hu, P.; Wen, Z.; Wang, L.; Tan, P.; Xiao, K. *ACS Nano* **2012**, *6*, 5988–5994.
71. Vaughn II, D. D.; Patel, R. J.; Hickner, M. A.; Schaak, R. E. *J. Am. Chem. Soc.* **2010**, *132*, 15170–15172.
72. Shi, L.; Dai, Y. J. *J. Appl. Crystallogr.* **2014**, *47*, 527–531.
73. Peng, H.; Meister, S.; Chan, C. K.; Zhang, X. F.; Cui, Y. *Nano Lett.* **2007**, *7*, 199–203.
74. Du, K.-Z. et. al. *ACS Nano* **2016**, *10*, 1738–1743.
75. Frindt, R. F.; Yang, D.; Westreich, P. *J. Mater. Res.* **2005**, *20*, 1107–1112.
76. Kuo, C.-T. et. al. *Sci. Rep.* **2016**, *6*, 20904.
77. Takano, Y.; Arai, N.; Arai, A.; Takahashi, Y.; Takase, K.; Sekizawa, K. *J. Magn. Magn. Mater.* **2004**, *272–276*, E593–E595.
78. Li, X. et. al. *J. Am. Chem. Soc.* **2014**, *136*, 11065–11069.
79. Mayorga-Martinez, C. C. et. al. *ACS Appl. Mater. Interfaces* **2017**, *9*, 12563–12573.
80. Gusmao, R.; Sofer, Z.; Sedmidubský, D.; Huber, Š.; Pumera, M. *ACS Catal.* **2017**, *7*, 8159–8170.
81. Ling, X.; Wang, H.; Huang, S.; Xia, F.; Dresselhaus, M. S. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 4523–4530.
82. Wang, L.; Sofer, Z.; Pumera, M. *ChemElectroChem* **2015**, *2*, 324–327.
83. Li, L. et. al. *Nat. Nanotechnol.* **2014**, *9*, 372–377.
84. Qiao, J.; Kong, X.; Hu, Z.; Yang, F.; Ji, W. *Nat. Commun.* **2014**, *5*, 4475.
85. Das, S. et. al. *Nano Lett.* **2014**, *14*, 5733–5739.
86. Shen, Z. et. al. *J. Mater. Chem. A* **2015**, *3*, 3285–3288.
87. Park, C.-M.; Sohn, H.-J. *Adv. Mater.* **2007**, *19*, 2465–2468.
88. Wang, X. et. al. *Nat. Nanotechnol.* **2015**, *10*, 517.

89. M. Pumera, *Chem. Soc. Rev.* **2010**, 39, 4146–4157.
90. Forghani, M.; Donne, S. W. *J. Electrochem. Soc.* **2018**, 165, A664–A673.
91. Weidner, J. W. *Electrochem. Soc. Interface* **2018**, 27, 45.
92. Conway, B. E. *Electrochemical Supercapacitors: Scientific Fundamentals and Technological Applications*; Springer: New York, 1999.
93. Simon, P.; Gogotsi, Y. *Nat. Mater.* **2008**, 7, 845–854.
94. Yu, Z.; Tetard, L.; Zhai, L.; Thomas, J. *Energy Environ. Sci.* **2015**, 8, 702–730.
95. Simon, P.; Gogotsi, Y.; Dunn, B. *Science* **2014**, 343, 1210–1211.
96. Wang, Y.; Song, Y.; Xia, Y. *Chem. Soc. Rev.* **2016**, 45, 5925–5950.
97. Winter, M.; Brodd, R. J. *Chem. Rev.* **2004**, 104, 4245–4269.
98. Conway, B. E. *J. Electrochem. Soc.* **1991**, 138, 1539–1548.
99. Gogotsi, Y.; Penner, R. M. *ACS Nano* **2018**, 12, 2081–2083.
100. Armand, M.; Tarascon, J. M. *Nature* **2008**, 451, 652–657.
101. Islam, M. S. et. al. *Chem. Soc. Rev.* **2014**, 43, 185–204.
102. Choi, N.-S.; Chen, Z. H.; Freunberger, S. A.; Ji, X. L.; Sun, Y. K.; Amine, K.; Yushin, G.; Nazar, L. F.; Cho, J.; Bruce, P. G. *Angew. Chem., Int. Ed.* **2012**, 51, 9994–10024.
103. Ji, H.; Zhao, X.; Qiao, Z.; Jung, J.; Zhu, Y.; Lu, Y.; Zhang, L. L.; MacDonald, A. H.; Ruoff, R. S. *Nat. Commun.* **2014**, 5, 3317.
104. Ke, Q.; Wang, J. *J. Materiomics* **2016**, 2, 37–54.
105. Choi, W.; Choudhary, N.; Han, G. H.; Park, J.; Akinwande, D.; Lee, Y. H. *Mater. Today* **2017**, 20, 116–130.
106. Chen, K.; Song, S.; Liu, F.; Xue, D. *Chem. Soc. Rev.* **2015**, 44, 6230–6257.
107. Yang, W.; Ni, M.; Ren, X.; Tian, Y.; Li, N.; Su, Y.; Zhang, X. *Curr. Opin. Colloid Interface Sci.* **2015**, 20, 416–428.
108. Liu, C.; Yu, Z.; Neff, D.; Zhamu, A.; Jang, B. Z. *Nano Lett.* **2010**, 10, 4863–4868.
109. Anasori, B.; Beidaghi, M.; Gogotsi, Y. *Mater. Today* **2014**, 17, 253–254.
110. Ambrosi, A.; Chua, C. K.; Latiff, N. M.; Loo, A. H.; Wong, C. H. A.; Eng, A. Y. S.; Bonanni, A.; Pumera, M. *Chem. Soc. Rev.* **2016**, 45, 2458–2493.
111. Tan, Y. B.; Lee, J.-M. *J. Mater. Chem. A* **2013**, 1, 14814–14843.
112. Liu, Y.; Peng, X. *Appl. Mater. Today* **2017**, 8, 104–115.

113. Novoselov, K. S.; Fal'ko, V. I.; Colombo, L.; Gellert, P. R.; Schwab, M. G.; Kim, K. *Nature* **2012**, *490*, 192–200.
114. Pumera, M. *J. Mater. Chem. C* **2014**, *2*, 6454–6461.
115. Tian, H. et. al. *Nanoscale* **2017**, *9*, 3530–3536.
116. Thearle, R. A. et. al. *Electroanalysis* **2017**, *29*, 45–50.
117. Hui, K. H.; Ambrosi, A.; Sofer, Z.; Pumera, M.; Bonanni, A. *Nanoscale* **2015**, *7*, 9040–9045.
118. Pinto, H. et. al. *Phys. Status Solidi A* **2010**, *207*, 2131–2136.
119. Chua, C. K.; Ambrosi, A.; Sofer, Z.; Mackova, A.; Havranek, V.; Tomandl, I.; Pumera, M. *Chem. – Eur. J.* **2014**, *20*, 15760–15767.
120. Wang, T.; Wang, L.-X; Wu, D. L.; Xia, W.; Jia, D.-Z. *Sci Rep.* **2015**, *5*, 9591.
121. Lazar, P.; Zbořil, R.; Pumera, M.; Otyepka, M. *Phys. Chem. Chem. Phys.* **2014**, *16*, 14231–14235.
122. Śliwak, A.; Grzyb, B.; Díez, N.; Gryglewicz, G. *Appl. Surf. Sci.* **2017**, *399*, 265–271.
123. Lee, M. S.; Choi, H.-Y.; Baek, J.-B.; Chang, D. W. *Appl. Energy* **2017**, *195*, 1071–1078.
124. Xia, K.; Wang, G.; Zhang, H.; Yu, Y.; Liu, L.; Chen, A. *J. Nanopart. Res.* **2017**, *19*, 254.
125. Chen, Y.; Xiao, Z.; Liu, Y.; Fan, L.-Z. *J. Mater. Chem. A* **2017**, *5*, 24178–24184.
126. Wen, Z.; Wang, X.; Mao, S.; Bo, Z.; Kim, H.; Cui, S.; Lu, G.; Feng, X.; Chen, J. *Adv. Mater.* **2012**, *24*, 5610–5616.
127. Jeong, H. M.; Lee, J. W.; Shin, W. H.; Choi, Y. J.; Shin, H. J.; Kang, J. K.; Choi, J. W. *Nano Letters* **2011**, *11*, 2472–2477.
128. Li, S.; Wang, Z.; Jiang, H.; Zhang, L.; Ren, J.; Zheng, M.; Dong, L.; Sun, L. *Chem. Commun.* **2016**, *52*, 10988–10991.
129. Yeom, D.-Y.; Jeon, W.; Tu, N. D. K.; Yeo, S. Y.; Lee, S.-S.; Sung, B. J.; Chang, H.; Lim, J. A.; Kim, H. *Sci. Rep.* **2015**, *5*, 9817.
130. Thirumal, V.; Pandurangan, A.; Jayavel, R.; Ilango vand, R. *Synth. Met.* **2016**, *220*, 524–532.
131. Wang, H.; Maiyalagan, T.; Wang, X. *ACS Catal.* **2012**, *2*, 781–794.
132. Tsetseris, L. et. al. *Carbon* **2014**, *67*, 58–63.
133. Poh, H.L.; Šimek, P.; Sofer, Z.; Pumera, M. *ACS Nano* **2013**, *7*, 5262–5272.
134. Agnoli, S.; Favaro, M. *J. Mater. Chem. A* **2016**, *4*, 5002–5025.

135. Tang, Y.-B. et. al. *ACS Nano* **2012**, 2, 1970–1978.
136. Zhang, L. L.; Zhao, X.; Ji, H.; Stoller, M. D.; Lai, L.; Murali, S.; McDonnell, S.; Cleveger, B.; Wallace, R. M.; Ruoff, R. S. *Energy Environ. Sci.* **2012**, 5, 9618–9625.
137. Paek, E.; Pak, A. J.; Kweon, K. E.; Hwang, G. S. *J. Phys. Chem. C* **2013**, 117, 5610–5616.
138. Ma, C.; Liao, Q.; Sun, H.; Lei, S.; Zheng, Y.; Yin, R.; Zhao, A.; Li, Q.; Wang, B. *Nano Lett.* **2018**, 18, 386–394.
139. Błoński, P.; Tuček, J.; Sofer, Z.; Mazánek, V.; Petr, M.; Pumera, M.; Otyepka, M.; Zbořil, R. *J. Am. Chem. Soc.* **2017**, 139, 3171–3180.
140. Ambrosi, A.; Poh, H. L.; Wang, L.; Sofer, Z.; Pumera, M. *ChemSusChem* **2014**, 7, 1102–1106.
141. Seredych, M.; Bandoz, T. J. *J. Mater. Chem. A* **2013**, 1, 11717–11727.
142. Benck, J. D.; Hellstern, T. R.; Kibsgaard, J.; Chakthranont, P.; Jaramillo, T. F. *ACS Catal.* **2014**, 4, 3957–3971.
143. Zheng, Y.; Jiao, Y.; Jaroniec, M.; Qiao, S. Z. *Angew. Chem., Int. Ed.* **2015**, 54, 52–65.
144. Safizadeh, F.; Ghali, E.; Houlachi, G. *J. Int. Hydrogen Energy* **2015**, 40, 256–274.
145. Jiao, Y.; Zheng, Y.; Jaroniec, M.; Qiao, S. Z. *Chem. Soc. Rev.* **2015**, 44, 2060–2086.
146. Benck, J. D.; Hellstern, T. R.; Kibsgaard, J.; Chakthranont, P.; Jaramillo, T. F. *ACS Catal.* **2014**, 4, 3957–3971.
147. Zou, X.; Zhang, Y. *Chem. Soc. Rev.* **2015**, 44, 5148–5180.
148. Duan, J.; Chen, S.; Jaroniec, M.; Qiao, S. Z. *ACS Catal.* **2015**, 5, 5207–5234.
149. Zheng, Y.; Jiao, Y.; Jaroniec, M.; Qiao, S. Z. *Angew. Chem., Int. Ed.* **2015**, 54, 52–65.
150. Conway, B. E.; Tilak, B. V. *Electrochim. Acta* **2002**, 47, 3571–3594.
151. Chen, Z.; Cummins, D.; Reinecke, B. N.; Clark, E.; Sunkara, M. K.; Jaramillo, T. F. *Nano Lett.* **2011**, 11, 4168–4175.
152. Pumera, M.; Sofer, Z.; Ambrosi, A. *J. Mater. Chem. A* **2014**, 2, 8981–8987.
153. Jaramillo, T. F. et. al. *Science* **2007**, 317, 100–102.
154. Bollinger, M. V.; Lauritsen, J. V.; Jacobsen, K. W.; Nørskov, J. K.; Helveg, S.; Besenbacher, F. *Phys. Rev. Lett.* **2001**, 87, 196803.
155. Bollinger, M. V.; Jacobsen, K. W.; Nørskov, J. K. *Phys. Rev. B: Condens. Matter Mater. Phys.* **2003**, 67, 085410.

156. Lauritsen, J. V.; Bollinger, M. V.; Lægsgaard, E.; Jacobsen, K. W.; Nørskov, J. K.; Clausen, B. S.; Topsøe, H.; Besenbacher, F. *J. Catal.* **2004**, *221*, 510–522.
157. Yu, Y. et. al. *Nat. Chem.* **2018**, *10*, 638–643.
158. Yan, Y.; Xia, B. Y.; Xu, Z.; Wang, X. *ACS Catal.* **2014**, *4*, 1693–1705.
159. Yu, Y.; Huang, S.-Y.; Li, Y.; Steinmann, S. N.; Yang, W.; Cao, L. *Nano Lett.* **2014**, *14*, 553–558.
160. Ouyang, Y. et. al. *Chem. Mater.* **2016**, *28*, 4390–4396.
161. Cummins, D. R. et. al. *Nat. Commun.* **2016**, *7*, 11857.
162. Mayorga-Martinez, C. C.; Ambrosi, A.; Eng, A. Y. S.; Sofer, Z.; Pumera, M. *Electrochem. Commun.* **2015**, *56*, 24–28.
163. Leong, S. X.; Mayorga-Martinez, C. C.; Chia, X.; Luxa, J.; Sofer, Z.; Pumera, M. *ACS Appl. Mater. Interfaces* **2017**, *9*, 26350–26356.
164. Pumera, M. *Chem.–Asian J.* **2011**, *6*, 340.
165. Donaldson, K.; Seaton, A. *Part. Fibre Toxicol.* **2012**, *9*, 13.
166. Thomas, C. R.; Kelley, T. R. *Environ. Health Insights* **2010**, *4*, 21–26.
167. Jacobsen, M.; Rae, S.; Walton, W. H.; Rogan, J. M. *Nature* **1970**, *227*, 445–447.
168. Attfield, M. D.; Kuempel, E. D. *Ann. Occup. Hyg.* **2003**, *47*, 525–529.
169. Fojtů, M. et. al. *Environ. Sci.: Nano* **2017**, *4*, 1617–1633.
170. Pietroiusti, A.; Stockmann-Juvala, H.; Lucaroni, F.; Savolainen, K. *WIREs Nanomed. Nanobiotechnol.* **2018**, *e1513*, DOI: 10.1002/wnan.1513.
171. Krug, H. F.; Wick, P. *Angew. Chem. Int. Ed.* **2011**, *50*, 1260–1278.
172. Fröhlich, E.; Salar-Behzadi, S. *Int. J. Mol. Sci.* **2014**, *15*, 4795–4822.
173. Armstead, A. L.; Li, B. *Int. J. Nanomedicine* **2016**, *11*, 6421–6433.
174. Takhar, P.; Mahant, S. *Arch Appl Sci Res.* **2011**, *3*, 389–403.
175. Kong, B.; Seog, J. H.; Graham, L. M.; Lee, S. B. *Nanomedicine* **2011**, *6*, 929–941.
176. Ou, L.; Song, B.; Liang, H.; Liu, J.; Feng, X.; Deng, B.; Sun, T.; Shao, L. *Part. Fibre Toxicol.* **2016**, *13*, 57.

177. Ponti, J. et. al. European Commission's Joint Research Centre (JRC). Interlaboratory comparison study of the Colony Forming Efficiency assay for assessing cytotoxicity of nanomaterials. Publications Office of the European Union. 2014. <https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/interlaboratory-comparison-study-colony-forming-efficiency-assay-assessing-cytotoxicity> (assessed on 16 July 2018), DOI: 10.2788/406937.
178. Cattaneo, A. G.; Gornati, R.; Bernardini, G.; Sabbioni, E.; Manzo, L.; Di Gioacchino, M. Testing Nanotoxicity: An Update of New and Traditional Methods. In Handbook of Nanotoxicology, Nanomedicine and Stem Cell Use in Toxicology [Online]; Sahu, S. C.; Casciano, D. A., Eds.; Wiley Online Library, 2014; Chapter 1. <https://onlinelibrary.wiley.com/doi/abs/10.1002/9781118856017.ch1> (assessed on 24 July 2018).
179. Riss, T. L.; Moravec, R. A.; Niles, A. L.; Duellman, S.; Benink, H. A.; Worzella, T. J.; L. Minor. Cell viability assays. In *Assay Guidance Manual [Internet]*; Sittampalam, G. S.; Coussens, N. P.; Brimacombe, K.; et al., Eds.; Assay Guidance Manual Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2004. <https://www.ncbi.nlm.nih.gov/books/NBK144065/?report=printable> (assessed May 31, 2018)
180. Richards, D.; Ivanisevic, A. Inorganic material coatings and their effect on cytotoxicity. *Chem. Soc. Rev.* **2012**, *41*, 2052–2060.
181. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Chem.-Eur. J.* **2014**, *20*, 9627–9632.
182. Monteiro-Riviere, N. A.; Inman, A. O. *Carbon* **2006**, *44*, 1070–1078.
183. Wörle-Knirsch, J. M.; Pulskamp, K.; Krug, H. F. *Nano Lett.* **2006**, *6*, 1261–1268.
184. Ciofani, G. et. al. *Biochem. Biophys. Res. Commun.* **2010**, *394*, 405–411.
185. Latiff, N.; Teo, W. Z.; Sofer, Z.; Huber, Š.; Fisher, A. C.; Pumera, M. *RSC Adv.* **2015**, *5*, 67485–67492.
186. Chng, E. L. K.; Pumera, M. *Chem.-Eur. J.* **2013**, *19*, 8227–8235.
187. Rosli, N. F. et. al. *ACS Sustainable Chem. Eng.* **2018**, *6*, 7432–7441.
188. Fischer, H. C.; Chan, W. C. W. *Curr. Opin. Biotechnol.* **2007**, *18*, 565–571.
189. Krug, H. F. *Angew. Chem.* **2014**, *53*, 12304–12319.
190. Wottrich, R.; Diabaté, S.; Krug, H. F. *Int. J. Hyg. Environ. Health* **2004**, *207*, 353–361.

191. Rothen-Rutishauser, B.; Blank, F.; Mühlfeld, C.; Gehr, P. *Expert Opin. Drug Metab. Toxicol.* **2008**, *4*, 1075–1089.
192. Mallampati, R.; Patlolla, R. R.; Agarwal, S.; Babu, R. J.; Hayden, P.; Klausner, M.; Singh, M. S. *Toxicol. in Vitro* **2010**, *24*, 669–676.
193. Wick, P. et. al. *Environ. Health Perspect.* **2010**, *118*, 432–436.
194. Lee, J.; Lilly, G. D.; Doty, R. C.; Podsiadlo, P.; Kotov, N. A. *Small* **2009**, *5*, 1213–1221.
195. Ganguly, P.; Breen, A.; Pillai, S. C. *ACS Biomater. Sci. Eng.* **2018**, *4*, 2237–2275.
196. Gleeson, M. P. et. al. *Curr. Drug Metab.* **2012**, *18*, 1266–1291.
197. Aires, A.; Cadenas, J. F.; Guantes, R.; Cortajarena, A. L. *Nanoscale* **2017**, *9*, 13760–13771.
198. Sizochenko, N.; Mikolajczyk, A.; Jagiello, K.; Puzyn, T.; Leszczynski, J.; Rasulev, B. *Nanoscale* **2018**, *10*, 582–591.
199. George, S.; Xia, T.; Rallo, R.; Zhao, Y.; Ji, Z.; Lin, S.; Wang, X.; Zhang, H.; France, B.; Schoenfeld, D. *ACS Nano* **2011**, *5*, 1805–1817.
200. Laomettachit, T.; Puri, I.; Liangruksa, M. *Toxicol. Appl. Pharmacol.* **2017**, *334*, 47–54.
201. Gao, Y.; Feng, J.; Kang, L.; Xu, X.; Zhu, L. *Sci. Total Environ.* **2018**, *610*, 442–450.
202. Bell, D. R.; Kang, S.-G.; Huynh, T.; Zhou, R. *Nanoscale* **2018**, *10*, 351–358.

Part I: Electrochemical Studies of Layered Materials for Energy Applications

Chapter 3 –

Effect of Doping Graphene on their Capacitance

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Microwave irradiated N- and B,Cl-doped graphene: Oxidation method has strong influence on capacitive behavior

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3.1 Introduction

With increasing demand for energy worldwide and decreasing supply of limited fossil fuels, technological advances to utilize renewable sources of energy are underway. However, their intermittent nature would require the storage of excess energy generated during low-power demands. This can be achieved through energy storage systems such as capacitors. To meet the higher requirements of future systems, their performances need to be improved substantially through development of better materials.¹

Graphene shows great potential in this regard due to its high conductivity and large surface area.^{2–9} Doping has been explored as a strategy to further enhance its capacitance.^{2–4} There have been numerous examples in literature reporting N-doped graphene showing improved capacitive performance compared to its undoped counterpart.^{10–15} Apart from nitrogen, boron, fluorine, chlorine, sulphur, and phosphorus have also been introduced to graphene as effective dopants for capacitor applications and more.^{16–32} Current methods used for the synthesis of doped graphene materials include chemical vapor deposition (CVD) method,³³ arc-discharge method,³⁴ as well as thermal processes.^{10,35,36}

Recently, microwave irradiation was presented as an alternative method of exfoliation and/or doping for the top-down synthesis of graphene.^{37–41} Advantages of this approach include shorter preparation time and lower cost, while being simple and scalable.

Previously, our group had demonstrated that microwave-exfoliated graphene exhibit fast heterogeneous electron transfer (HET) rates and share similar electrochemical properties to thermally reduced graphene oxides (TRGOs).³⁸ In the top-down synthesis of graphene materials, graphite is typically first oxidized to graphite oxide (GO) to increase the interlayer distance between the graphene layers prior to

exfoliation.^{42,43} Three well-known classical methods have been reported for this oxidation process,⁴⁴ namely Staudenmaier (ST),⁴⁵ Hummers (HU),⁴⁶ and Hofmann (HO)⁴⁷ methods, which differ in the types of oxidizing agents used (Scheme 3.1). Due to their similar functions, these different oxidation methods might be assumed to be interchangeable. However, there have been reports, which showed that the type of GO precursor used can largely influence the electrochemical properties and doping amounts in TRGOs.^{48–50}

Here, we wish to extend this investigation further by introducing dopants into graphene through the microwave irradiation method, studying how the different oxidation methods would affect the doping levels, as well as explore how the resultant properties would affect their capacitance behavior. To achieve this, GOs prepared by ST, HU, and HO oxidation methods were reduced, exfoliated, and simultaneously doped using microwave plasma treatment in the presence of gaseous boron trichloride and ammonia for the synthesis of B,Cl-doped and N-doped microwave reduced graphene, respectively (see Scheme 3.1). These materials were then extensively characterized by a series of methods to obtain a comprehensive understanding of the specific structural and chemical nature of the materials under discussion. This is important, as there is large variability of graphene materials prepared using different approaches.⁴² After combining several characterization techniques to obtain a clear idea of their properties, we examined the role of B,Cl and N dopants on graphene electrochemistry in terms of their capacitive behaviors. For a fairer study, we also compared our results with undoped graphene prepared via microwave irradiation of the respective GO precursors under vacuum as controls. This study sheds light for optimizing the synthesis method of heteroatom-doped graphene for capacitor applications.

Scheme 3.1. Scheme for the synthesis of doped graphene materials using microwave plasma treatment. The inset photos (A) and (B) show the formation of heteroatom-doped graphene materials under BCl_3 and NH_3 plasma sources.

3.2. Results and discussion

Material characterization

The materials discussed here are labelled in accordance with the type of parent GO precursor preparation method used (i.e., ST, HU, HO) as well as the type of dopants introduced (i.e., B, Cl, or N). For example, ST-N signifies N-doped graphene prepared via Staudenmaier oxidation route whereas HU-B,Cl indicates B, Cl-doped graphene prepared via the Hummers oxidation route. By looking at the morphology of the materials under scanning electron microscopy (SEM), we were able to verify the successful exfoliation of GO precursors after microwave plasma treatment. Figure 3.1 shows the SEM images obtained for the doped graphene materials. All of them showed typical exfoliated structures with wrinkles and highly porous structures similar to previous studies, thereby confirming the successful exfoliation of graphite oxide conducted through microwave approach in BCl_3 and NH_3 atmospheres.^{37,48,51}

Figure 3.1. SEM images of B,Cl-doped and N-doped microwave-exfoliated graphene prepared by different graphite oxidation methods (ST, HU, and HO), taken at 20,000 $\times$ (left) and 3000 $\times$ (right) magnifications. The scale bars represent 1 μm .

After ascertaining the exfoliation of graphite oxide by microwave treatment, we proceeded to characterize the samples using Raman spectroscopy. The Raman spectra for the different microwave-exfoliated samples under study are shown in Figure 3.2.

Figure 3.2. Raman spectra for B,Cl-doped and N-doped microwave-exfoliated graphene prepared by Staudenmaier (ST), Hummers (HU) and Hofmann (HO) oxidation methods.

An intense band at around 1580 cm^{-1} (also known as the G band) is characteristic for in-plane vibrations of sp^2 -bonded atoms, whereas the D band at about 1360 cm^{-1} is produced by the presence of defects such as edges, sp^3 carbons, and vacancies.⁴² By analysing the ratio of intensities between the D and G bands (I_D/I_G), we can measure the degree of structural defects in a carbon structure.⁴² The data obtained are tabulated in Table 3.1.

Table 3.1. Calculated values for the ratio of intensities between the D and G bands (I_D/I_G) of the graphene materials under study.

	ST	HU	HO
B,Cl-doped	0.56	0.87	0.77
N-doped	0.65	0.79	0.76

From Table 3.1, we observed slight differences in values when different GO preparation methods were used. Hummers oxidation method produced graphene with the largest degree of disorder, followed closely by Hoffman for both B,Cl- and N-doped microwave-exfoliated graphene. The doped graphene materials prepared via Staudenmaier method showed generally lower ratios of structural defects among the three oxidation methods studied. In comparison with the undoped samples, which had previously been reported (I_D/I_G values between 0.76 and 0.93),³⁸ we see that the I_D/I_G ratios remained relatively unchanged with or without the doping process during microwave irradiation for the graphene prepared via the Hummers and Hoffman method, thereby preserving their general graphitic network. However, in the case of graphene prepared via Staudenmaier method, a decrease in I_D/I_G ratio was observed. Since a higher degree of structural defects has been reported to be favorable for better capacitance, varying capacitive behavior is anticipated for these materials.⁵²

Next, we investigated the elemental compositions and bonding arrangements of B,Cl- and N-doped graphene using X-ray photoelectron spectroscopy (XPS). The data are presented in Figure 3.3.

Figure 3.3. XPS analyses for the various heteroatom-doped graphene materials under study: wide scan spectra (left column), high-resolution C1s spectra (middle column), and high-resolution O1s spectra (right column).

The wide scan survey spectra (between 0 and 1200 eV) as shown in the figure (left column) revealed the overall composition of elements present in the microwave-exfoliated graphene samples. From the area composition of C 1s and O 1s, we can determine the C/O ratio for the materials. These values are as summarized in Table 3.2.

Table 3.2. Comparison of C/O ratios as measured from XPS.

	ST	HU	HO
B,Cl-doped	18.19	12.09	15.50
N-doped	16.15	18.25	17.29

Since the parent GOs contain significantly higher quantities of oxygen (with C/O ratios reported previously to be between 2.05 and 2.71), the C/O ratios obtained here verify that the graphene samples have indeed been successfully reduced.⁵³ In comparison to the C/O ratios from undoped samples as reported in a previous study (between 6 and 11),³⁸ the presence of doping gases was observed to aid the reduction process as seen from the increase in C/O ratios here.

Small amounts of Cl (~200 eV) were detected in the B,Cl-doped samples (0.24% for ST-B,Cl, 2.14% for HU-B,Cl, and 1.48% for HO-B,Cl). In the case of B (~190 eV) and N (~400 eV), these elements were not detectable in most of the materials by XPS surface analysis likely due to low doping levels as well as insufficient sensitivity of the XPS technique. Nevertheless, XPS was able to shed some light on the composition of residual O-containing groups through deconvolution of the XPS high-resolution signals for C 1s and O 1s.

All the high-resolution C 1s spectra (Figure 3.3, middle column) show a dominant peak at ca. 284 eV with many smaller components at higher binding energies. From previous studies, the possible carbon bonding present in the C 1s signal are C=C (284.5 eV), C-C (~285.6 eV), C-O (~286.6 eV), C=O (~287.6 eV), O-C=O (~289.6 eV), and π - π^* (~290 eV).^{37,48,49} If we compare the composition for the oxygen-containing carbon bonds (i.e. C-O, C=O, and O-C=O) in the high-resolution C1s spectra, we can observe that there is a consistent decreasing trend in their composition with increasing oxidation state of the bond arrangements across the six materials (i.e. C-O > C=O > O-C=O). More details on

the deconvolution analysis of C 1s are available in the Supporting Information (SI, Table 3.S1).

Similarly, if we analyze the different carbon–oxygen bond arrangements and compositions obtained from the O 1s high-resolution XPS spectra (between 523 and 538 eV), we find a similar trend. With reference to the deconvolution high-resolution O 1s analysis from a previous study,⁵⁴ the possible carbon–oxygen bonding present in the O 1s signal in reduced graphene are C-OH (~533 eV), C=O (~531 eV), and O-C=OH (~530 eV). Unlike the identical peak shapes seen for high-resolution C 1s, varying peak shapes are seen in the high-resolution O 1s spectra for the different graphene samples (Figure 3.3, right column). In spite of this, from the O 1s deconvolution analysis, a similar consistent decreasing trend in composition with increasing oxidation states of the carbon–oxygen bond arrangements was observed (i.e. C-OH > C=O > O-C=OH). The relative amount of components from the deconvolution analysis of high-resolution O1s is tabulated in the SI, Table 3.S2. This agreement reflects a consistency in the microwave exfoliation method in reducing GO to graphene.

The amounts of boron, chlorine, and nitrogen were determined using combustible elemental analysis (Table 3.3). From here, we can highlight several points about this new approach of using microwave irradiation to dope B, Cl, and N atoms into graphene. The GO precursor (i.e., ST, HU, or HO) used can greatly influence the level of doping of the resulting graphene. Even though trends in doping levels observed for B/Cl and N are different across the various GO preparation methods, both agree that ST oxidation method introduces the least amount of dopants into graphene. Besides the influence of GO precursor used, we find that the type of heteroatoms used can also influence the doping levels. It appears that N and Cl atoms are more easily doped into graphene as compared to B atoms using microwave irradiation. In comparison with the thermal exfoliation method, we found that B-doping using microwave exfoliation in BCl₃ allows introduction of boron atoms in levels of approximately two orders of magnitude higher than that observed for B-doping by thermal exfoliation in BH₃.³⁷ On the other hand, a

study on N-doped graphene synthesized using thermal exfoliation of various GO precursors in ammonia reported about two orders of magnitude higher amounts of nitrogen introduced compared to the microwave exfoliation method used here.⁴⁹ These findings suggest that different doping methods may be more favorable for different types of dopants. Furthermore, we can vary the doping levels by selecting the type of GO oxidation method used.

Table 3.3. Elemental compositions (in at. %) from combustible elemental analysis of B,Cl- and N-doped microwave exfoliated graphene.

Elements	B,Cl-doped			N-doped		
	ST	HU	HO	ST	HU	HO
C	85.74	80.07	82.8	88.43	81.63	83.90
H	7.71	6.49	6.11	2.75	6.99	6.47
O	5.53	11.91	9.15	8.16	8.82	7.54
N	-	-	-	0.66	2.56	2.09
B	0.08	0.12	0.34	-	-	-
Cl	0.93	1.42	1.60	-	-	-

Besides elemental compositions, we also studied the level of metallic impurities in the samples as they have been reported to alter the electrochemical properties of graphene materials.^{44,55} This was measured using inductively-coupled plasma oxygen emission spectroscopy (ICP-OES). The data obtained are available in the SI, Tables 3.S3-S5. The data generally show a decrease in metallic impurities when dopants were introduced into graphene compared to the control samples. This implies that the microwave irradiation process can reduce the amount of metallic impurities from carbon samples.

Capacitance studies

Two methods were used to probe into the capacitance behaviour of the graphene samples, namely cyclic voltammetry (CV) as well as electrochemical charge–discharge studies. The CV analysis was performed between –0.2 and 0.5 V (vs. Ag/AgCl) at various scan rates (i.e. 25, 50, 75, 100, 200, and 400 mV/s) in 0.1 M potassium hydroxide solution. The results obtained from the CV measurements are shown in Figure 3.4A and 3.4B. Figure 3.4A shows the CV recorded at 400 mV/s whereas Figure 3.4B shows a plot of current values at 0.15 V at the different scan rates tested.

The following relationship was used to determine the capacitance of the materials:

$$C = \frac{dQ}{dE} = \frac{I}{dE/dt}$$

where, C represents capacitance (in F), Q represents charge (in C), I represents current (in A) and dE/dt represents scan rate (in mV/s).^{51,56–58}

Figure 3.4. Comparison of capacitance studies between B,Cl- and N-doped microwave-irradiated graphene prepared from various GO starting precursors (Staudenmaier, Hummers, and Hoffman). (A) CV measurements at scan rate (v) of 400 mV/s, (B) anodic current readings for CV measurements at 0.15 V (vs. Ag/AgCl) across different scan rates, and (C) profiles for charge/discharge conducted at 0.7 A/g current density. These electrochemical measurements were performed in 0.1 M KOH (aq).

The results from the calculations are summarized in Figure 3.5A. From this figure, we see that capacitance improves with increments in doping levels. For B,Cl-doped samples, the capacitance enhances in the following manner: HO > HU > ST. This trend correlates well with the doping levels of B and Cl as seen in Table 3.3. Likewise, for N-doped samples, a similar relationship is observed between capacitance measured and amount of N doping (HU > HO > ST). In comparison with the undoped samples, we find that doping can improve the capacitance of graphene materials prepared from Hoffman and Hummers methods. However, in the case of using Staudenmaier oxidation method, we observe a decrease in the capacitive behavior upon doping with B,Cl and N. This could be due to the lower degree of structural defects produced after the doping process as

seen in Table 3.1 previously. It has been reported that this property can dominate the capacitance of doped graphene materials.⁵²

To confirm the capacitive behavior of our samples, we also conducted charge and discharge studies (Figure 3.4C). This was performed at a current density of 0.7 A/g in the same potential window and electrolyte as conducted for the CV measurements. The capacitances can be calculated by:

$$C = i \frac{\Delta t}{\Delta E}$$

where i represents the applied current (in A), Δt represents the discharging time (in s) and ΔE represents the range of the applied potential (in V).^{52,58,59}

The results from the calculations are presented in Figure 3.5B. Even though the weight-specific capacitance obtained are slightly higher than those calculated from the CV measurements,⁵⁸ both results agree on the general trend observed. The capacitance correlates closely to the doping levels introduced during the microwave irradiation process, demonstrating that doping can be an effective method to improve capacitance. Additionally, our results show that using Staudenmaier oxidation method to prepare doped graphene through microwave irradiation process seems ineffective to improve its capacitance. This could be linked to a lower degree of structural defects produced from the process.

Figure 3.5. Gravimetric capacitances calculated for various B,Cl- and N-doped graphene prepared from different starting precursors (Staudenmaier, Hummers, and Hoffman) gathered from (A) CV measurements and (B) charge and discharge analysis.

3.3. Conclusion

In summary, we had successfully synthesized B,Cl- and N-doped graphene using microwave irradiation of various GO oxidation methods (Staudenmaier, Hummers and Hoffman) in BCl_3 and NH_3 atmospheres. We found that the type of GO precursor used can produce varying amount of dopants. Also, we show that capacitance enhances with increments of doping levels achieved. Hummers and Hoffman oxidation methods prove to be more effective routes for doping graphene relative to the Staudenmaier method. This microwave approach provides a scalable, simple yet cost-effective method for doping graphene. Furthermore, we can tune the doping levels by varying the type of GO starting precursors used. This study paves the way in optimizing the synthesis method of graphene materials for supercapacitor application.

3.4. Experimental section

Graphite oxide (GO) synthesis

The procedures for GO synthesis via Staudenmaier, Hummers, and Hoffman oxidation methods are as reported in literature 38.

Exfoliation/doping by microwave plasma treatment

To produce boron-doped graphene, GO (250 mg) was added in 500 mL quartz glass reactor with magnetron of 1000 W and 2.45 GHz. Prior to introducing boron trichloride (99.99%, Koch-Light Laboratories) as the doping agent, the reactor was flushed three times with inert nitrogen gas (99.9999%, Siad). Following the repeated evacuation of reactor with BCl_3 , this gas was allowed to flow continuously at approximately 50 mL per min for the microwave exfoliation process at 10 mbar for 3 min. Boron–chlorine plasma generated soon after plasma formation accelerated the reaction. Lastly, the reactor was purged and allowed to cool under inert N_2 environment. To produce nitrogen-doped graphene, similar equipment and steps were implemented. However, in this case, ammonia (99.9995%, Siad) was used as the doping agent instead. Ammonia plasma, which was produced soon after the plasma formation, accelerated the exfoliation process.

Material characterization

Images by Scanning Electron Microscopy (JEOL 7600F, Japan) were taken at 5.00 kV accelerating voltage and 5.4 mm working distance under SEI mode. Raman spectroscopy (LabRam HR instrument, Horiba Scientific) was performed using a 514.5 nm Argon laser. Calibration was done with reference to silicon peak at 520 cm^{-1} . X-ray photoelectron spectroscopy (XPS) measurements (SPECS, Germany) were performed using a monochromatic Magnesium X-ray radiation source. Combustible elemental analysis (CHNS-O) was carried out with a CHNS/O Analyzer (Perkin Elmer, USA). N-phenyl urea was used for internal calibration of the instrument. Electro Thermal Vaporization

Inductively Coupled Plasma Optical Emission Spectrometry (ETV ICP-OES) was used to measure the elemental composition of Al, Ba, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn (SPECTRO Analytical Instruments, Germany). The system was used together with calibration standards (Analytica, Czech Republic).

All electrochemical measurements were carried out using an Autolab PGSTAT101 (Eco Chemie, The Netherlands) in a three-electrode configuration system at ambient conditions (using glassy carbon as the working electrode, platinum electrode as the counter electrode, and Ag/AgCl as the reference electrode). Suspensions of the graphene materials were prepared in dimethylformamide (DMF, Sigma Aldrich) to achieve a 5 mg mL⁻¹ concentration, followed by a 20-min sonication. After that, 1 µL of the appropriate suspension (5 µg) was dropcasted onto the working electrode surface and left to dry under ambient conditions. Prior to the next measurement, the working electrode surface was polished with alumina particles (0.05mm) and rinsed with ultrapure water.

3.5. Supporting Information

Table 3.S1. Relative amount of components from deconvoluted analysis of XPS high resolution C1s core spectra of B,Cl- and N-doped microwave exfoliated graphenes.

Bond	B,Cl-doped			N-doped		
	ST	HU	HO	ST	HU	HO
C=C	62.32	57.97	58.16	65.06	60.74	59.31
C-C	14.09	11.30	10.11	14.40	12.26	12.68
C-O	11.39	14.70	15.27	9.98	12.63	12.28
C=O	4.28	6.83	6.16	4.60	5.75	8.88
O-C=O	3.99	5.00	3.05	2.80	3.45	1.77
π - π^*	3.93	4.19	7.25	3.17	5.17	5.09

Table 3.S2. Relative amount of components from deconvoluted analysis of XPS high resolution O 1s core spectra of B,Cl- and N-doped microwave exfoliated graphenes.

Bond	B,Cl-doped			N-doped		
	ST	HU	HO	ST	HU	HO
C-OH	55.99	66.5	69.35	66.54	59.35	69.65
C=O	34.70	25.35	27.35	27.70	36.24	25.89
O=C-OH	9.30	8.15	3.30	5.76	4.41	4.47

Table 3.S3. Amount of metallic impurities (in ppm) found in microwave exfoliated graphene oxide materials prepared by Staudenmaier oxidation method.

	ST	ST-B,Cl	ST-N
Al	2401	30.6	8.6
Ba	9.5	3.9	3.0
Cd	0.31	4.4	3.6
Co	1.13	1.9	1.6
Cr	24.1	5.7	5.5
Cu	1.43	5.2	4.7
Fe	1271	15.2	13.8
Mn	22.3	2.2	1232
Ni	1.9	2.9	2.8
Pb	0.45	1.9	7.8
Zn	7.58	3.1	6.6

Table 3.S4. Amount of metallic impurities (in ppm) found in microwave exfoliated graphene oxide materials prepared by Hummers oxidation method.

	HU	HU-B,Cl	HU-N
Al	3002	41.1	36.9
Ba	837.8	3.7	4.2
Cd	3.12	3.7	3.7
Co	39	2.1	2.5
Cr	15.1	9.1	12.7
Cu	10.6	7.6	62.1
Fe	543.4	43.4	46.6
Mn	5408	2047	2779
Ni	7.04	4.7	34
Pb	148.5	5.8	16.9
Zn	30.9	8.1	59.8

Table 3.S5. Amount of metallic impurities (in ppm) found in microwave exfoliated graphene oxide materials prepared by Hoffman oxidation method.

	HO	HO-B,Cl	HO-N
Al	2615	20.6	7.9
Ba	10.5	4.3	5.4
Cd	1.00	5.0	5.4
Co	2.55	2.6	2.8
Cr	96.2	23.8	16.9
Cu	5.98	7.6	7.3
Fe	1088	127.2	61.2
Mn	31.8	1.7	1.6
Ni	28.9	8.7	5.7
Pb	0.52	6.8	9.1
Zn	39.6	5.1	9.5

References:

1. Simon, P.; Gogotsi, Y. *Nat. Mater.* **2008**, *7*, 845–854.
2. Ambrosi, A.; Chua, C. K.; Latiff, N. M.; Loo, A. H.; Wong, C. H. A.; Eng, A. Y. S.; Bonanni, A.; Pumera, M. *Chem. Soc. Rev.* **2016**, *45*, 2458–2493.
3. El-Kady, M. F.; Shao, Y.; Kaner, R. B. *Nat. Rev. Mater.* **2016**, *1*, 16033.
4. Ke, Q.; Wang, J. *J. Materiomics* **2016**, *2*, 37–54.
5. Wang, X.; Sun, G.; Routh, P.; Kim, D.-H.; Huang, W.; Chen, P. *Chem. Soc. Rev.* **2014**, *43*, 7067–7098.
6. Xiong, Z.; Liao, C.; Han, W.; Wang, X. *Adv. Mater.* **2015**, *27*, 4469–4475.
7. Huang, J.; Wang, J.; Wang, C.; Zhang, H.; Lu, C.; Wang, J. *Chem. Mater.* **2015**, *27*, 2107–2113.
8. Sun, G.; Liu, J.; Zhang, X.; Wang, X.; Li, H.; Yu, Y.; Huang, W.; Zhang, H.; Chen, P. *Angew. Chem. Int. Ed.* **2014**, *53*, 12576–12580.
9. Eng, A. Y. S.; Chua C. K.; Pumera, M. *Phys. Chem. Chem. Phys.* **2016**, *18*, 9673–9681.
10. Wang, H.; Maiyalagan, T.; Wang, X. *ACS Catal.* **2012**, *2*, 781–794.
11. Balaji, S. S.; Elavarasan, A.; Sathish, M. *Electrochim. Acta* **2016**, *200*, 37–45.
12. Wang, J.; Ding, B.; Xu, Y.; Shen, L.; Dou, H.; Zhang, X. *ACS Appl. Mater. Interfaces* **2015**, *7*, 22284–22291.
13. Jeong, H. M.; Lee, J. W.; Shin, W. H.; Choi, Y. J.; Shin, H. J.; Kang, J. K.; Choi, J. W. *Nano Lett.* **2011**, *11*, 2472–2477.
14. Wang, K.; Li, L.; Zhang, T.; Liu, Z. *Energy* **2014**, *70*, 612–617.
15. Luo, G.; Liu, L.; Zhang, J.; Li, G.; Wang, B.; Zhao, J. *ACS Appl. Mater. Interfaces* **2013**, *5*, 11184–11193.
16. Wang, T.; Wang, L.-X.; Wu, D.-L.; Xia, W.; Jia, D.-Z. *Sci. Rep.* **2015**, *5*, 9591.

17. Peng, Z.; Ye, R.; Mann, J. A.; Zakhidov, D.; Li, Y.; Smalley, P. R.; Lin, J.; Tour, J. M. *ACS Nano* **2015**, *9*, 5868–5875.
18. Yeom, D.-Y.; Jeon, W.; Tu, N. D. K.; Yeo, S. Y.; Lee, S.-S.; Sung, B. J.; Chang, H.; Lim, J. A.; Kim, H. *Sci. Rep.* **2015**, *5*, 9817.
19. Thirumal, V.; Pandurangan, A.; Jayavel, R.; Ilangoan, R. *Synth. Met.* **2016**, *220*, 524–532.
20. Wu, Z.-S.; Winter, A.; Chen, L.; Sun, Y.; Turchanin, A.; Feng, X.; Müllen, K. *Adv. Mater.* **2012**, *24*, 5130–5135.
21. Kakaei, K.; Hamidi, M.; Husseindoost, S. *J. Colloid Interface Sci.* **2016**, *479*, 121–126.
22. Parveen, N.; Ansari, M. O.; Ansari, S. A.; Cho, M. H. *J. Mater. Chem. A* **2016**, *4*, 233–240.
23. Chen, X.; Chen, X.; Xu, X.; Yang, Z.; Liu, Z.; Zhang, L.; Xu, X.; Chen, Y.; Huang, S. *Nanoscale* **2014**, *6*, 13740–13747.
24. Akhter, T.; Islam, M. M.; Faisal, S. N.; Haque, E.; Minett, A. I.; Liu, H. K.; Konstantinov, K.; Dou, S. X. *ACS Appl. Mater. Interfaces* **2016**, *8*, 2078–2087.
25. Yu, X.; Kang, Y.; Park, H. S. *Carbon* **2016**, *10*, 49–56.
26. Seredych, M.; Singh, K.; Bandoz, T. J. *Electroanalysis* **2014**, *26*, 109–120.
27. Wen, Y.; Wang, B.; Huang, C.; Wang, L.; Hulicova-Jurcakova, D. *Chem. –Eur. J.* **2015**, *21*, 80–85.
28. Karthika, P.; Rajalakshmi, N.; Dhathathreyan, K. S. *J. Nanosci. Nanotechnol.* **2013**, *13*, 1746–1751.
29. Vineesh, T. V.; Nazrulla, M. A.; Krishnamoorthy, S.; Narayanan, T. N.; Alwarappan, S. *Appl. Mater. Today* **2015**, *1*, 74–79.
30. Ma, X.-X.; He, X.-Q. *Appl. Mater. Today* **2016**, *4*, 1–8.
31. Hu, P.; Meng, D.; Ren, G.; Yan, R.; Peng, X. *Appl. Mater. Today* **2016**, *5*, 1–8.

32. Otyepková, E.; Lazar, P.; Čépe, K.; Tomanec, O.; Otyepka, M. *Appl. Mater. Today* **2016**, *5*, 142–149.
33. Reddy, A. L. M.; Srivastava, A.; Gowda, S. R.; Gullapalli, H.; Dubey M.; Ajayan, P. M. *ACS Nano* **2010**, *4*, 6337–6342.
34. Li, N.; Wang, Z.; Zhao, K.; Shi, Z.; Gu Z.; Xu, S. *Carbon* **2010**, *48*, 255–259.
35. Li, X.; Wang, H.; Robinson, J. T.; Sanchez, H.; Diankov G.; Dai, H. *J. Am. Chem. Soc.* **2009**, *131*, 15939–15944.
36. Wang, H.; Maiyalagan T.; Wang, X. *ACS Catal.* **2012**, *2*, 781–794.
37. Poh, H. L.; Šimek, P.; Sofer, Z.; Pumera, M. *Chem. –Eur. J.* **2013**, *19*, 2655–2662.
38. Eng, A. Y. S.; Sofer, Z.; Šimek, P.; Kosina, J.; Pumera, M. *Chem. –Eur. J.* **2013**, *19*, 15583–15592.
39. Wong, C. H. A.; Jankovský, O.; Sofer, Z.; Pumera, M. *Carbon* **2014**, *77*, 508–517.
40. Economopoulos, S. P.; Rotas, G.; Miyata, Y.; Shinohara, H.; Tagmatarchis, N. *ACS Nano* **2010**, *4*, 7499–7507.
41. Han, H. J.; Chen, Y. N.; Wang, Z. J. *RSC Adv.* **2015**, *5*, 92940–92946.
42. Matsumoto, M.; Saito, Y.; Park, C.; Fukushima, T.; Aida, T. *Nat. Chem.* **2015**, *7*, 730–736.
43. Ambrosi, A.; Chua, C. K.; Bonanni, A.; Pumera, M. *Chem. Rev.* **2014**, *114*, 7150–7188.
44. Joshi, R. K.; Alwarappan, S.; Yoshimura, M.; Sahajwalla, V.; Nishina, Y. *Appl. Mater. Today* **2015**, *1*, 1–12.
45. Wang, L.; Pumera, M. *Appl. Mater. Today* **2016**, *5*, 134–141.
46. Staudenmaier, L. *Ber. Dtsch. Chem. Ges.* **1898**, *31*, 1481–1487.
47. Hummers, W. S.; Offeman, R. E. *J. Am. Chem. Soc.* **1958**, *80*, 1339–1339.
48. Hofmann, U.; König, E. *Z. Anorg. Allg. Chem.* **1937**, *234*, 311–336.
49. Poh, H. L.; Simek, P.; Sofer, Z.; M. Pumera. *ACS Nano* **2013**, *7*, 5262–5272.

50. Wang, L.; Sofer, Z.; Luxa, J.; Pumera, M. *J. Mater. Chem. C* **2014**, *2*, 2887–2893.
51. Poh, H. L.; Šaněk, F.; Ambrosi, A.; Zhao, G.; Sofer, Z.; Pumera, M. *Nanoscale* **2012**, *4*, 3515–3522.
52. Wang, L.; Sofer, Z.; Simek, P.; Tomandl, I.; Pumera, M. *J. Phys. Chem. C* **2013**, *117*, 23251–23257.
53. Ambrosi, A.; Poh, H. L.; Wang, L.; Sofer, Z.; Pumera, M. *ChemSusChem* **2014**, *7*, 1102–1106.
54. Chua, C. K.; Sofer, Z.; Pumera, M. *Chem. –Eur. J.* **2012**, *18*, 13453–13459.
55. Yang, D.; Velemakanni, A.; Bozoklu, G.; Park, S.; Stoller, M.; Piner, R. D.; Stankovich, S.; Jung, I.; Field, D. A.; Ventrice Jr., C. A.; Ruoff, R. S. *Carbon* **2009**, *47*, 145–152.
56. Wong, C. H. A.; Sofer, Z.; Kubešová, M.; Kučera, J.; Matějková, S.; Pumera, M. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 13774–13779.
57. Buglione, L.; Khim Chng, E. L.; Ambrosi, A.; Sofer, Z.; Pumera, M. *Electrochem. Commun.* **2012**, *14*, 5–8.
58. Buglione, L.; Bonanni, A.; Ambrosi, A.; Pumera, M. *ChemPlusChem* **2012**, *77*, 71–73.
59. Mayorga-Martinez, C. C.; Ambrosi, A.; Eng, A. Y. S.; Sofer, Z.; Pumera, M. *Electrochem. Commun.* **2015**, *56*, 24–28.
60. Ratha, S.; Rout, S. R. *ACS Appl. Mater. Interfaces* **2013**, *5*, 11427–11433.
61. Ambrosi, A.; Sofer, Z.; Pumera, M. *Small* **2015**, *11*, 605–612.
62. Feng, J.; Sun, X.; Wu, C.; Peng, L.; Lin, C.; Hu, S.; Yang, J.; Xie, Y. *J. Am. Chem. Soc.* **2011**, *133*, 17832–17838.
63. Sun, G.; Liu, J.; Zhang, X.; Wang, X.; Li, H.; Yu, Y.; Huang, W.; Zhang, H.; Chen, P. *Angew. Chem. Int. Ed.* **2014**, *53*, 12576–12580.

Chapter 4 –

Effect of Valence and Oxide Impurities in MoS₂ and WS₂ on HER

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Valence and oxide impurities in MoS₂ and WS₂ dramatically change their electrocatalytic activity towards proton reduction

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4.1 Introduction

With global warming, climate change and the depleting supply of limited fossil fuels, there is an urgent need for us to develop clean (low CO₂ emissions), sustainable and renewable sources of energy. However, the intermittent and uncontrolled nature of renewable sources of energy prompts energy storage systems to play a greater role in our lives. One attractive solution is the conversion of excess electrons to hydrogen gas, a high energy density carrier gas, in a process known as the hydrogen evolution reaction (HER). However, this method typically requires expensive and scarce platinum-based electrocatalysts to drive the reaction efficiently, thereby imposing economic constraints for its wide spread practical implementation. As such, there is intensive research for alternative materials to replace platinum as HER electrocatalysts.^{1–3}

In this quest, layered molybdenum disulfide (MoS₂), tungsten disulfide (WS₂) and their related materials have demonstrated promising HER activities that are close to those of Pt.^{2,4–8} Through a variety of synthetic routes and strategies, a wide range of performances for MoS₂ and WS₂ has been reported in terms of overpotential at –10 mA cm^{–2} current density and Tafel slope results.^{2,8} Recently, there were reports that amorphous molybdenum sulfides (MoS_x), with compositions typically closer to MoS₃ compared to MoS₂, display excellent HER activities.^{9–13} The same is true about WS₂ and the corresponding WS_x compounds.¹⁴ MoS₃ and the corresponding Mo(IV) and Mo(VI) oxides are typical impurities in MoS₂ materials; similar to the case of WS₂.^{2,14–18} Such findings have triggered us to explore the possible effects of MoS₃, MoO₂/MoO₃ impurities (and their tungsten counterparts), which may be formed during synthesis or storage, in affecting the HER performances of MoS₂ and WS₂ materials.¹⁷ Here, we investigate the effects of different possible valence and oxide impurities in MoS₂ and WS₂ on their HER catalysis, in terms of affecting their overpotentials, Tafel slope values as well as the shape of the polarization curves. Following that, we identify the Mo- and W-based impurities which show a highly catalytic effect towards the hydrogen evolution reaction and

therefore may be responsible for the large disparity of reported potentials of catalytic hydrogen evolution on MoS₂ (and WS₂) surfaces.

To achieve this aim, we first characterized MoS₂, WS₂ and their possible synthetic impurities using scanning electron microscopy (SEM), energy-dispersive spectroscopy (EDS) and X-ray photoelectron spectroscopy (XPS). We have identified the possible synthetic impurities of MoS₂ and WS₂ as their oxidized moieties (i.e. MoS₃, MoO₂, MoO₃, WS₃, WO₂, WO₃) due to the use of oxides as starting precursors, or possible oxidation under reaction conditions or prolonged exposure.^{18–22}

After material characterization, their HER performances were compared. Subsequently, we prepared physical mixtures of various impurities with MoS₂ and WS₂ to study the effect of different impurities on the overpotential, Tafel slope and shape of HER polarization curves.

4.2. Results and Discussion

To verify the materials used for study, we first conducted material characterization using several techniques. SEM images of MoS₂, WS₂ and their possible synthetic impurities are shown in Figures 4.1 and 4.2. Their SEM images display the typical layered structures as expected. The remaining materials (i.e. MoS₃, MoO₂, MoO₃, WS₃, WO₂, WO₃) show amorphous structures, with MoS₃, WS₃ and WO₃ displaying distinctly non-crystalline structures amongst them.

Figure 4.1. SEM images of MoS₂ and its possible impurities during synthesis (MoS₃, MoO₂, MoO₃) at 2000× (left) and 15 000× (right) magnifications. Scale bars representing 10 μm and 1 μm are shown in the bottom images.

Figure 4.2. SEM images of WS₂ and its possible impurities during synthesis (WS₃, WO₂, WO₃) at 2000× (left) and 15 000× (right) magnifications. Scale bars representing 10 µm and 1 µm are shown in the bottom images.

We used MoS₂ and WS₂ in their bulk forms to minimize the variation in properties seen upon exfoliation.^{23,24} For example, in the case of bulk MoS₂ exfoliation via Li intercalation, a phase change from semiconducting to metallic MoS₂ has been reported.²⁵ This would also allow a better comparison between the different materials as their valence and oxide forms were not exposed to exfoliation conditions.

EDS analysis (Figures 4.S1 and 4.S2) is further used to verify the composition of the materials. In addition, XPS (Figure 4.3) was performed to probe into their detailed bonding information. For Mo 3d deconvolution,²⁶ Mo⁴⁺ and Mo⁶⁺ doublet peaks were observed to be around 229.5 eV, 232.6 eV and 232.3 eV, 235.4 eV respectively whereas for W 4f deconvolution,²⁶ W⁴⁺ and W⁶⁺ doublet peaks were found at approximately 32.9 eV, 35.0 eV and 35.8 eV, 37.9 eV respectively. The deconvolution analysis for the high resolution S 2p spectra^{27,28} shows the presence of S²⁻ terminal doublet peaks at 162.3 eV, 163.4 eV to be dominant in compounds with the metal 6+ oxidation state (i.e. MoS₃, WS₃) while those with the metal 4+ oxidation state (i.e. MoS₂ and WS₂) have prevalent S²⁻ bridging double peaks at 163.0 eV and 164.2 eV. The XPS characterization shows that whilst MoS₂ is pure, WS₂ contains slight W(VI) impurities.

Figure 4.3. X-ray photoelectron spectroscopy (XPS) spectra for the materials under study: (A) high-resolution Mo 3d spectra for Mo compounds (MoS₂, MoS₃, MoO₂ and MoO₃), (B) high-resolution W 4f spectra for W compounds (WS₂, WS₃, WO₂ and WO₃), and (C) high-resolution S 2p spectra for S-containing compounds (MoS₂, MoS₃, WS₂ and WS₃).

The as-prepared MoS₂, WS₂ and their mixtures were also characterized to probe into the morphology and composition of the prepared samples. SEM images of the as-prepared mixtures reveal the presence of amorphous structures of MoS₃ and WS₃ dispersed randomly over surfaces of bulk layered MoS₂ and WS₂ sheets (Figure 4.S3), while their EDS mapping confirms the presence of transition metal and chalcogen elements in the prepared samples (Figures 4.S4-S5).

After material characterization, the hydrogen evolution activity of the materials was evaluated using linear sweep voltammetry (LSV) in 0.5 M H₂SO₄ (aq). Figure 4.4 presents the results obtained from the electrochemical analysis. From this figure, MoS₃ and WS₃ show better catalytic performances compared to metal oxides, with 370 mV and 704 mV overpotentials achieved at -10 mA cm^{-2} current density respectively. It appears that compounds with a higher metal oxidation state (6+) and higher S content (i.e. MoS₃ and WS₃) offer better HER catalytic performance compared to those of the lower metal oxidation state and lower S content. This observation is in line with other previous reports which found that MoS₃ and WS₃ give better HER activities compared to MoS₂ and WS₂.^{11,17} With reference to the XPS results gathered, comparison between XS₃ and XS₂ compounds (where X represents the metal Mo or W) suggests that higher HER performances could be linked to the presence of S²⁻ terminal bonds (see high resolution XPS S 2p spectra in Figure 4.3).

Figure 4.4. Hydrogen evolution for MoS₂, WS₂ and their possible impurities during synthesis (MoS₃, MoO₂, MoO₃, WS₃, WO₂, WO₃) tested in 0.5 M H₂SO₄ at a scan rate of 2 mV s⁻¹: (A) HER polarization curves of the various compounds tested, (B) bar charts comparing their overpotentials at -10 mA cm⁻² current density, (C) their corresponding Tafel plots, and (D) bar charts comparing their calculated Tafel slope values.

To elucidate the HER mechanisms for the various materials tested, we calculated the Tafel slope values from their corresponding HER polarization curves. The Tafel slope values can allow us to discern the mechanism and rate-determining step (RDS) for the reaction as different ranges of values would indicate different RDS steps. For bulk MoS₂ and WS₂, their Tafel slope values were determined to be 179 and 174 mV dec⁻¹ respectively, similar to those reported in the literature.^{23,24} These results show that in their bulk states, the HER process for MoS₂ and WS₂ is limited by the Volmer step (electrochemical adsorption step). In contrast, MoS₃ and WS₃ have lower Tafel slope values of 75 mV dec⁻¹ and 109 mV dec⁻¹ respectively, suggesting a different HER mechanism is involved. The lower Tafel slopes achieved by the metal trisulfides could be attributed to their lower crystallinity as reported by a study by Li et al.²⁹ This is also observed in this work by WO₃ that yields a remarkably low Tafel slope value of 38 mV dec⁻¹ which also shows an amorphous structure. This Tafel slope value is interesting as it is close to that reported for the best well known performing HER electrocatalyst (Pt); 30 mV dec⁻¹.³⁰ This could be the reason for its increasing exploration for HER applications.^{31–35} However, its overpotential at –10 mA cm⁻² current density is relatively high at 561 mV in comparison with Pt which has almost zero overpotential.⁸

After identifying MoS₃ and WS₃ to be catalytically active towards the HER, we proceeded to systematically study the potential effect of heterogeneous valence impurities. We created physical mixtures of MoS₂ and WS₂ with a wide variety of metal(VI) sulfide impurities (i.e. 1, 2.5, 5, 7.5, 10, 12.5, 15, 20, 25, 50, and 75%) to investigate their electrocatalytic effect on hydrogen evolution. The data gathered in Figure 4.5 show that increasing amounts of MoS₃ and WS₃ impurities can decrease the overpotential at -10 mA cm^{-2} current density. In other words, different amounts of MoS₃ and WS₃ can cause variation in the HER performance of MoS₂ and WS₂ materials in the positive direction, that is towards lower overpotentials. This provides an explanation for the wide range of overpotentials reported for molybdenum and tungsten disulfides across different synthesis methods. From the wide range of concentrations tested, we are able to identify 12.5% MoS₃ and 25% WS₃ as the optimal proportions of impurities in the transition metal disulfides, where the effect of additional impurities in lowering the overpotential of the overall catalyst becomes less significant beyond these concentrations (Figure 4.5B).

Figure 4.5. Linear scan voltammetry (LSV) measurements for MoS₂ with different amounts of MoS₃ impurities (left), and WS₂ with different amounts of WS₃ impurities (right): (A) HER polarization curves of the various compounds tested, (B) bar charts comparing their overpotentials at -10 mA cm^{-2} current density, (C) their corresponding Tafel plots, and (D) bar charts comparing their calculated Tafel slope values. For better clarity, only five concentrations of mixtures are presented for comparison of LSVs in graph (A).

In terms of the Tafel slope, we observe an interesting decrease in value with a mere 1% of MoS₃ introduced (Figure 4.5D). With further increase in the metal trisulfide content, however, the Tafel slope values remain relatively constant. This can also be seen for its tungsten counterpart. This suggests that composition does not influence the Tafel slope values as much as a low degree of crystallinity, in agreement with another study.²⁹ Most importantly, our findings show that small traces of amorphous MoS₃/WS₃ are sufficient to significantly alter the HER mechanism of MoS₂ and WS₂.

Besides studying the effects of MoS₃ and WS₃ impurities on overpotential and Tafel slope values, the effect of these impurities on the shape of HER polarization curves was also explored. Figure 4.5 shows that small amounts of MoS₃ in MoS₂ can give rise to pre-waves in their LSV curves. Interestingly for WS₂, the unmixed sample itself showed a slight dip in LSV which significantly lowers the onset potential (defined as overpotential recorded at -0.1 mA cm^{-2}) to be close to that measured for WS₃ (WS₂ onset: 322 mV, WS₃ onset: 305 mV). This early dip in LSV could be attributed to the slight W(VI) impurities as detected in WS₂ by XPS analysis (Figure 4.3). Similarly, mixtures of WS₃ in WS₂ also showed pre-waves in their LSVs. This observation provides evidence that physical mixtures of catalytic impurities can alter the shape of the HER polarization curves. In addition, different amounts of catalytic impurities are required to produce alterations in the shape of LSVs for MoS₂ and WS₂ materials. For MoS₂, the pre-waves are observed with the presence of 1 to 10% MoS₃ whereas for its W counterpart, the concentration range was found to be between 0 and 25% WS₃.

Pre-waves observed in LSVs in published studies had previously been attributed to the inherent electrochemistry or different catalytic sites of a material²³ as well as a reduction of structural defects such as oxidized metal clusters.³⁶ In this study, we provide evidence that these pre-waves may arise due to the presence of valence impurities. Apart from valence impurities, we had also tested mixtures with oxide impurities. For WO₂ and WO₃, which have intermediate HER activities between that of WS₃ and bulk WS₂, slight pre-waves were observed from their LSVs (Figure 4.S6). However, for MoO₂ and MoO₃ which were found to be less catalytically active than their metal disulfides, no pre-waves in the LSVs were seen (Figure 4.S7). This suggests that pre-waves in the LSVs could be due to the presence of more catalytic impurities instead of less active ones.

To understand the origin behind the better HER performances seen for MoS₃ and WS₃, as well as their mixtures with the metal disulfides, we performed electrochemical impedance spectroscopy (EIS) to compare their charge transfer resistances (R_{ct}). Nyquist plots (Figure 4.6) revealed that the metal trisulfides show marked improvements in conductivities as seen from their significantly lower R_{ct} values (MoS₃: 0.2 k Ω , WS₃: 0.2 k Ω) compared to the metal disulfides (MoS₂: 48.8 k Ω , WS₂: 8.4 k Ω).

Figure 4.6. Electrochemical impedance spectroscopy (EIS) measurements conducted for (A) MoS₂, 1% MoS₃ in MoS₂, MoS₃ as well as their (B) W counterparts. Bar charts (C) and (D) compares the resistances (R_{ct} values) for the various samples tested. R_{ct} represents charge transfer resistance, R_b is short for bulk resistance while CPE stands for the constant phase element. The Nyquist plots were measured at frequencies between 10 000 kHz and 0.1 Hz and at an overpotential of -500 mV for MoS₂/MoS₃ and -750 mV for WS₂/WS₃.

Since the electrocatalytic reaction involves charge transfer processes between electrons from the glassy carbon surface to the surface of the catalyst, and subsequently from the catalyst surface to protons in the electrolyte solution, an enhancement in electron conductivity would greatly improve the HER catalysis. Similarly, small amounts of MoS₃/WS₃ present in their metal disulfides result in low R_{ct} values (10% MoS₃: 1.1 k Ω , 10% WS₃: 1.3 k Ω). This suggests that the presence of small traces of such metal trisulfides (MoS₃/WS₃) can noticeably lower charge transfer resistance of molybdenum and tungsten disulfides, thereby dominating their electrochemistry. This noticeable lowering

R_{ct} thus means a facile electron transfer for proton reduction at MoS₂/MoS₃ and WS₂/WS₃ mixtures and influences the rate determining step of the reaction. Consequently, this is translated to a decrease in the Tafel slope values as observed in Figure 4.5D. The Tafel slope values seen in Figure 4.5D could also be explained by these EIS data. With similar R_{ct} values between mixtures containing small traces of metal trisulfides and the pure metal trisulfides, they are all likely to have a similar RDS and therefore similar Tafel slope values as observed.

For the charge transfer between the catalyst surface and protons in the electrolyte solution, it is facilitated by catalyst active sites. For layered transition metal dichalcogenides (TMDs) MoS₂ and WS₂, it has been well reported that the unsaturated S and/or metal (Mo, W) edge sites are the catalytic sites for their HER activity, while the basal planes are inert.¹⁷ In contrast, for amorphous transition metal trisulfides, their structures and HER active sites have not been well understood yet. Previously, several groups have suggested that the active sites for amorphous molybdenum sulfides (MoS_x, MoS₃) are similar to that for amorphous molybdenum sulfide clusters; that is the S edge sites (in this case the bridging S₂²⁻ and terminal S²⁻).²⁹ In a more recent study, Tran et al. reported amorphous molybdenum sulfides as molecular-based coordination polymers built up from discrete [Mo₃S₁₃]²⁻ units that are linked together by two terminal disulfide (S₂²⁻) ligands with a third free terminal disulfide ligand available to generate HER active molybdenum hydride moieties.³⁶ Meanwhile, their tungsten counterpart is even less studied but similar inferences have been made due to their resemblances.¹⁷ In any case, the amorphous nature of the metal trisulfides is likely to expose more of these catalytic edge sites, to facilitate better HER activities.¹⁷

From the numerous successful catalyst design strategies seen in the literature, it has been well established that exposing more active sites for reaction is an effective concept to improve the HER performance of MoS₂ and WS₂ materials. This includes disorder engineering,^{37,38} vertically aligned TMDs,^{39,40} and synthesis of nanoflowers,⁴¹ nanoflakes,⁴² nanoparticles^{5,43} as well as mesoporous structures.⁴⁴ Therefore, it is reasonable to deduce that the amorphous structures of MoS₃ and WS₃ which give rise to larger specific areas of the metal trisulfides can expose more catalytic edge sites thereby resulting in enhanced HER activities. These pieces of evidence highlight the importance of structure and conductivity in the design of HER electrocatalysts.

Previously, Xie et al. had successfully modulated these two factors in molybdenum disulfide nanosheets through varying different synthesis temperatures.³⁷ The authors achieved an optimum balance between the degree of structural defects and conductivity through incorporation of conductive Mo(IV)–O species at different synthesis temperatures. In comparison with their work, our results may seem contradictory as we found that physical mixtures of MoO₂ in bulk MoS₂ did not improve the HER performance of the molybdenum disulphide (Figure 4.12). However, this could be due to different effects arising from the bulk nature of TMDs in our study. Nevertheless, both our findings agree that structural and electronic effects play important roles in efficient HER electrocatalysts. While this was achieved through finding a balance between the degree of structural defects and incorporation of conductive Mo(IV)–O species in their work, here we showed that physical mixtures of amorphous and conductive MoS₃ can also enhance the HER activity of the molybdenum disulfides.

4.3. Conclusions

In summary, we have demonstrated that even small amounts of catalytic impurities of Mo or W trisulfides can cause prewaves in the voltammogram and that only 10% of such trisulfides can completely dominate the electrochemistry of the corresponding transition metal dichalcogenides. These findings highlight the effects of impurities during material synthesis of MoS₂ and WS₂ materials for hydrogen evolution application and aid our understanding of the observed HER behaviour of the material. In addition, these findings can serve as a platform for us to further develop the catalytic activities of these materials.

4.4. Experimental section

Materials

Bulk molybdenum (IV) sulfide powder (<2 μm , 99%), molybdenum (IV) oxide powder (99%), molybdenum (VI) oxide powder (99.97%), tungsten(IV) oxide powder (–100 mesh, 99.99%), and tungsten(VI) oxide powder (99.9%) were purchased from Sigma-Aldrich, Singapore while bulk tungsten(IV) sulfide (99.8%) and molybdenum(VI) sulfide dehydrate were obtained from Alfa Aesar (Germany). Ammonium heptamolybdate tetrahydrate (99.5%), sodium tungstate dihydrate (99.5%), hydrochloric acid (35%), acetone (99.9%) and carbon disulphide (99.99%) were obtained from Penta, Czech Republic. Selenium (99.5%) and aluminium (99.7%) were obtained from STREM, Germany. H₂S (99.5%) and argon (99.999%) were obtained from SIAD, Czech Republic.

Synthesis of WS₃

Synthesis was performed by acidic decomposition of ammonium tetrathiotungstate. 25 g of sodium tungstate dihydrate was dissolved in 250 mL of water. Subsequently, 100 mL of 1 M hydrochloric acid was slowly added to the solution. The tungstic acid that was formed was purified by repeated decantation and centrifugation. Thereafter, the tungstic acid was separated by suction filtration, washed with water and dried in a vacuum oven at 50 °C for 48 hours. Following that, 10 g of tungstic acid was dissolved in 100 mL of concentrated ammonia and filtered using a 450 nm nylon membrane. The solution was then bubbled with H₂S gas for 10 hours (about 200 mL min⁻¹). After which, the red crystals of ammonium tetrathiotungstate formed were separated by suction filtration and washed with cold water. To 5 g of (NH₄)₂WS₄ in 5 wt% of ammonia solution, 1 M solution of hydrochloric acid was slowly added to the mixture with vigorous stirring and under an argon atmosphere. WS₃ formed was then separated by suction filtration and washed with water followed by acetone. WS₃ was subsequently dried under vacuum at 50 °C for 24 hours. Later, WS₃ was dispersed in carbon disulfide and repeatedly decanted to remove

excess of sulphur. Finally, WS₃ was separated by suction filtration, washed with carbon disulfide and dried in a vacuum oven at 50 °C for 48 hours.

Material characterization

Scanning electron microscopy (SEM) was performed using a field-emission scanning electron microscope (JOEL, Japan) in gentle-beam high mode at 2 kV (for MoS₂, MoO₂, WS₂) and 1 kV (for the remaining materials: MoS₃, MoO₃, WS₃, WO₂, WO₃). For the SEM of the as-prepared samples, SEM mode was used at 2 kV. Energy dispersive X-ray spectroscopy (EDS) data were obtained at an accelerating voltage of 15 kV. X-ray photoelectron spectroscopy (XPS) analyses were performed using a Phoibos 100 spectrometer and a monochromatic Mg X-ray radiation source (SPECS, Germany).

Electrochemical measurement

All electrochemical hydrogen evolution reaction (HER) measurements were performed using Linear Sweep Voltammetry (LSV) with an Autolab PGSTAT101 electrochemical analyzer (Eco Chemie, The Netherlands). A three-electrode configuration electrode system at room temperature was used to measure the HER activity of the materials in aq. H₂SO₄ (0.5 M) using a 5 mL electrochemical cell at a scan rate of 2 mV s⁻¹. A glassy carbon (GC) electrode was used as the working electrode, a platinum electrode served as the auxiliary electrode and an Ag/AgCl one served as the reference electrode. All electrochemical potentials herein were reported versus the Ag/AgCl reference electrode. Prior to immobilization of the test samples onto the working electrode, suspensions of the materials were prepared in N,N-dimethylformamide to achieve a 5 mg mL⁻¹ concentration, followed by a 20 min sonication. For mixtures, the prepared suspensions of various materials were physically mixed and sonicated for another 20 min to obtain a homogeneous solution. Subsequently, 1 µL aliquot of the appropriate suspension (5 µg) was deposited onto the GC electrode surface. Upon evaporation of the solvent at room temperature, the LSV measurement was conducted. The GC electrode surfaces were renewed by polishing with alumina particles (0.05 µm) on a polishing pad and washed

with distilled water of 18.2 MΩ cm resistivity. The Nyquist plots were measured at frequencies between 10 000 kHz and 0.1 Hz and at an overpotential of –500 mV for MoS₂/MoS₃ and –750 mV for WS₂/WS₃. The impedance data were fitted to a simplified Randles circuit (where constant phase element (CPE) replace the double layer capacitance) to obtain the series and charge-transfer resistances.

4.5. Supporting Information

Figure 4.S1. Energy Dispersive Spectroscopy (EDS) electron images, elemental mapping and spectrum for MoS₂, MoS₃, MoO₂ and MoO₃, taken at 1000X magnification.

Figure 4.S2. Energy Dispersive Spectroscopy (EDS) electron images, elemental mapping and spectrum for WS₂, WS₃, WO₂ and WO₃ at 1000X magnification.

Figure 4.S3. Scanning electron microscopy (SEM) images of MoS₂, MoS₃, WS₂, WS₃ and their 25% as-prepared mixtures. Only one mixture is shown here to represent the various concentrations tested as the remaining mixtures prepared would show very similar EDS mapping data. 25% MoS₃ is chosen as this was found to be representative proportion for good HER performance across the different MoS₂-MoS₃ mixtures tested.

Figure 4.S4. Energy Dispersive Spectroscopy (EDS) electron images, elemental mapping and spectrum for MoS₂, 25% MoS₃ and MoS₃ as-prepared samples drop-casted on SiO₂ wafer. Only one mixture is shown here to represent the various concentrations tested as the remaining mixtures prepared would show very similar EDS mapping data. 25% MoS₃ is chosen as this was found to be representative proportion for good HER performance across the different MoS₂-MoS₃ mixtures tested.

Figure 4.S5. Energy Dispersive Spectroscopy (EDS) electron images, elemental mapping and spectrum for WS_2 , 25% WS_3 and WS_3 as-prepared samples drop-casted on SiO_2 wafer. Only one mixture is shown here to represent the various concentrations tested as the remaining mixtures prepared would indicate very similar EDS mapping data. 25% WS_3 is chosen as this was found to be representative proportion for good HER performance across the different WS_2 - WS_3 mixtures tested.

Figure 4.S6. Results from hydrogen evolution reaction (HER) experiments for WS₂ with different amounts of WO₂ impurities (left), and WO₃ impurities (right) in 0.5 M H₂SO₄ (aq) at a scan rate of 2 mV/s: (A) HER polarization curves of the various compounds tested, and (B) bar charts comparing their overpotentials at -10 mA/cm² current density.

Figure 4.S7. Results from HER experiments for MoS₂ with different amounts of MoO₂ impurities (left), and MoO₃ impurities (right) in 0.5 M H₂SO₄ (aq) at a scan rate of 2 mV/s: (A) HER polarization curves of the various compounds tested, and (B) bar charts comparing their overpotentials at -10 mA/cm² current density.

References:

1. Zou, X.; Zhang, Y. *Chem. Soc. Rev.* **2015**, *44*, 5148.
2. Benck, J. D.; Hellstern, T. R.; Kibsgaard, J.; Chakthranont, P.; Jaramillo, T. F. *ACS Catal.* **2014**, *4*, 3957.
3. Zeng, M.; Li, Y. *J. Mater. Chem. A* **2015**, *3*, 14942.
4. Chhowalla, M.; Shin, H. S.; Eda, G.; Li, L.-J.; Loh, K. P.; Zhang, H. *Nat. Chem.* **2013**, *5*, 263.
5. Hinnemann, B.; Moses, P. G.; Bonde, J.; Jørgensen, K. P.; Nielsen, J. H.; Horch, S.; Chorkendorff, I.; Nørskov, J. K. *J. Am. Chem. Soc.* **2005**, *127*, 5308.
6. Voiry, D.; Yamaguchi, H.; Li, J.; Silva, R.; Alves, D. C. B.; Fujita, T.; Chen, M.; Asefa, T.; Shenoy, V. B.; Eda, G.; Chhowalla, M. *Nat. Mater.* **2013**, *12*, 850.
7. Yang, J.; Shin, H. S.; *J. Mater. Chem. A* **2014**, *2*, 5979.
8. (a) Vesborg, P. C. K.; Seger, B.; Chorkendorf, I. *J. Phys. Chem. Lett.* **2015**, *6*, 951; (b) He, Z.; Que, W. *Appl. Mater. Today* **2016**, *3*, 23.
9. Benck, J. D.; Chen, Z.; Kuritzky, L. Y.; Forman, A. J.; Jaramillo, T. F. *ACS Catal.* **2012**, *2*, 1916.
10. Merki, D.; Fierro, S.; Vrubela, H.; Hu, X. *Chem. Sci.* **2011**, *2*, 1262.
11. Vrubel, H.; Merkia, D.; Hu, X. *Energy Environ. Sci.* **2012**, *5*, 6136.
12. Vrubel, H.; Moehl, T.; Grätzel, M.; Hu, X. *Chem. Commun.* **2013**, *49*, 8985.
13. Ge, X.; Chen, L.; Zhang, L.; Wen, Y.; Hirata, A.; Chen, M. *Adv. Mater.* **2014**, *26*, 3100.
14. Tan, S. M.; Pumera, M. *ACS Appl. Mater. Interfaces* **2016**, *8*, 3948.
15. Chen, S.; Duan, J.; Tang, Y.; Jin, B.; Qiao, S. Z. *Nano Energy* **2015**, *11*, 11.
16. Murugesan, S.; Akkineni, A.; Chou, B. P.; Glaz, M. S.; Bout, D. A. V.; Stevenson, K. J. *ACS Nano* **2013**, *7*, 8199.

17. Huang, Z.; Wang, C.; Chen, Z.; Meng, H.; Lv, C.; Chen, Z.; Han, R.; Zhang, C. *ACS Appl. Mater. Interfaces* **2014**, *6*, 10408.
18. (a) Sobczynski, A.; Yildiz, A.; Bard, A. J.; Campion, A.; Fox, M. A.; Mallouk, T.; Webber, S. E.; White, J. M. *J. Phys. Chem.* **1988**, *92*, 2311; (b) Li, S.; Wang, S.; Tang, D.-M.; Zhao, W.; Xu, H.; Chu, L.; Bando, Y.; Golberg, D.; Eda, G. *Appl. Mater. Today*, **2015**, *1*, 60.
19. Gao, J.; Li, B.; Tan, J.; Chow, P.; Lu, T.-M.; Koratkar, N. *ACS Nano* **2016**, *10*, 2628.
20. Lin, Y.-C.; Zhang, W.; Huang, J.-K.; Liu, K.-K.; Lee, Y.-H.; Liang, C.-T.; Chu, C.-W.; Li, L.-J. *Nanoscale* **2012**, *4*, 6637.
21. Ennaoui, A.; Fiechter, S.; Ellmer, K.; Scheer, R.; Diesner, K. *Thin Solid Films* **1995**, *261*, 124.
22. Liu, H.; Han, N.; Zhao, J. *RSC Adv.* **2015**, *5*, 17572.
23. Eng, A. Y. S.; Ambrosi, A.; Sofer, Z.; Simek, P.; Pumera, M. *ACS Nano* **2014**, *8*, 12185.
24. Ambrosi, A.; Sofer, Z.; Pumera, M. *Small* **2015**, *11*, 605.
25. Eda, G.; Yamaguchi, H.; Voiry, D.; Fujita, T.; Chen, M.; Chhowalla, M. *Nano Lett.* **2011**, *11*, 5111.
26. Wang, L.; Sofer, Z.; Luxa, J.; Pumera, M. *Adv. Mater. Interfaces* **2015**, *2*, 1500041.
27. Weber, T.; Muijsers, J. C.; Niemantsverdriet, J. W. *J. Phys. Chem.* **1995**, *99*, 9194.
28. Kibsgaard, J.; Jaramillo, T. F.; Besenbacher, F. *Nat. Chem.* **2014**, *6*, 248.
29. Li, Y.; Yu, Y.; Huang, Y.; Nielsen, R. A.; Goddard, W. A.; Li, Y.; Cao, L. *ACS Catal.* **2015**, *5*, 448.
30. Duan, J.; Chen, S.; Jaroniec, M.; Qiao, S. Z. *ACS Catal.* **2015**, *5*, 5207.
31. Li, Y. H.; Liu, P. F.; Pan, L. F.; Wang, H. F.; Yang, Z. Z.; Zheng, L. R.; Hu, P.; Zhao, H. J.; Gu, L.; Yang, H. G. *Nat. Commun.* **2015**, *6*, 8064.
32. Choi, S.-J.; Chattopadhyay, S.; Kim, J. J.; Kim, S.-J.; Tuller, H. L.; Rutledge, G. C.; Kim, I.-D. *Nanoscale* **2016**, *8*, 9159.

33. Pihosh, Y.; Turkevych, I.; Mawatari, K.; Uemura, J.; Kazoe, Y.; Kosar, S.; Makita, K.; Sugaya, T.; Matsui, T.; Fujita, D.; Tosa, M.; Kondo, M.; Kitamori, T. *Sci. Rep.* **2015**, *5*, 11141.
34. Yang, L.; Zhu, X.; Xiong, S.; Wu, X.; Shan, Y.; Chu, P. K. *ACS Appl. Mater. Interfaces* **2016**, *8*, 13966.
35. Song, J.; Huang, Z.-F.; Pan, L.; Zou, J.-J.; Zhang, X.; Wang, L. *ACS Catal.* **2015**, *5*, 6594.
36. Tran, P. D.; Tran, T. V.; Orio, M.; Torelli, S.; Truong, Q. D.; Nayuki, K.; Sasaki, Y.; Chiam, S. Y.; Honma, R. Yi, I.; Barber, J.; Artero, V. *Nat. Mater.* **2016**, *15*, 640.
37. Xie, J.; Zhang, J.; Li, S.; Grote, F.; Zhang, X.; Zhang, H.; Wang, R.; Lei, Y.; Pan, B.; Xie, Y. *J. Am. Chem. Soc.* **2013**, *135*, 17881.
38. Li, H.; Tsai, C.; Koh, A. L.; Cai, L.; Contryman, A. W.; Fragapane, A. H.; Zhao, J.; Han, H. S.; Manoharan, H. C.; Abild-Pedersen, F.; Nørskov, J. K.; Zheng, X. *Nat. Mater.* **2015**, *15*, 48.
39. Wang, H.; Lu, Z.; Xu, S.; Kong, D.; Cha, J. J.; Zheng, G.; Hsu, P.-C.; Yan, K.; Bradshaw, D.; Prinz, F. B.; Cui, Y. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 19701.
40. Yan, Y.; Xia, B.; Li, N.; Xu, Z.; Fisher, A.; Wang, X. *J. Mater. Chem. A* **2015**, *3*, 131.
41. Wang, D.; Pan, Z.; Wu, Z.; Wang, Z.; Liu, Z. *J. Power Sources* **2014**, *264*, 229.
42. Choi, C. L.; Feng, J.; Li, Y. G.; Wu, J.; Zak, A.; Tenne, R.; Dai, H. J. *Nano Res.* **2013**, *6*, 921.
43. Li, Y.; Wang, H.; Xie, L.; Liang, Y.; Hong, G.; Dai, H. J. *J. Am. Chem. Soc.* **2011**, *133*, 7296.
44. Kibsgaard, J.; Chen, Z.; Reinecke, B. N.; Jaramillo, T. F. *Nat. Mater.* **2012**, *11*, 963.

Part II: Layered Materials and their Safety Aspects

Chapter 5 –

Cytotoxicity of Semiconductor Chalcogenides

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Toxicity of layered semiconductor chalcogenides: beware of interferences

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5.1. Introduction

Two-dimensional (2D) layered nanomaterials have received much attention since the successful isolation of graphene from graphite.^{1,2} They typically possess unique and distinct properties from their three-dimensional (3D) forms. As one of the first prototypes of layered structures, research on 2D materials has been predominated by graphene. Graphene is a single layer of sp^2 carbon atoms bonded hexagonally together, with high surface area, high mechanical strength, high elasticity, and fast heterogeneous electron transfer. However, the absence of an intrinsic bandgap has limited its application in nanoelectric and optoelectronic devices. To overcome this, alternative 2D materials have been explored to find new possible opportunities for specific applications.

Recently, a new stable class of 2D materials; semiconductor Group III and Group IV chalcogenides have been studied. They contain an element from either Group III or Group IV element (e.g. Ga, Ge, In, Sn, Tl) and a chalcogen (e.g. S, Se or Te). These materials have opened a new fascinating chapter in nanoelectronics applications. For example, gallium selenide (GaSe) and germanium sulfide (GeS) have been reported to have suitable properties, such as thermal, mechanical and photostability, for a wide range of potential applications ranging from solar cells, field effect transistors, photodetectors, infrared light-emitting diodes, cut-off devices, enhanced lithium ion batteries, fiber optics, sensors and optical lenses for infrared transmission.¹⁻¹⁴ Furthermore, their suitability as optical fibres as well as photodetectors, have triggered efforts to extend these applications towards the biomedical field such as medical diagnosis.^{15,16} With the rise in research and possible commercialization of this group of materials in the future, it is therefore necessary to investigate their toxicological effects in order to be informed of any potential health hazards that they may pose.¹⁷

Currently, to the best of our knowledge, even though there have been toxicity reports found for Ga- and Ge-containing compounds,^{18–21} no toxicity assessments were performed on GaSe and GeS specifically. For instance, copper gallium diselenide (CGS), used in photovoltaic and semiconductor industries, was reported to leach gallium and selenide ions in the lung tissues of rats after 24 h intratracheal instillation of the material, causing mild transient inflammatory response in the lung.¹⁹ For Ge, some reactive germanium intermediate compounds have been reported to be poisonous, while some germanium compounds showed low toxicity.^{20,21} Since toxicity data on GaSe and GeS is unexplored, we aim to investigate the toxicological effects of GaSe and GeS, for the first time, on the human lung carcinoma epithelial cell line (A549) through in vitro studies of cell viability after 24 h exposure to these semiconductor chalcogenides. A549 cells were chosen for the cytotoxicity assessment as the lungs are likely to be the first point of contact with the body when these nanomaterials enter through inhalation.

Various concentrations of the nanomaterials were tested to study the effect of concentration on their cytotoxicity. After a 24 h incubation with GaSe and GeS, the viability of cells was analyzed using two assays: water-soluble tetrazolium salt (WST-8) and methyl-thiazolyldiphenyl-tetrazolium bromide (MTT). These two well-established cell viability assays produce coloured formazan dyes in the presence of metabolically active cells.²² The amount of cells that remained viable after exposure to the nanomaterials can then be determined using absorbance spectroscopy. With the utilization of two cell viability assays that work on similar principles, misinterpretation of the absorbance data could be avoided.²³

5.2. Results and Discussion

Material characterization

Characterization of GaSe and GeS nanomaterials were carried out by scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), X-ray diffraction (XRD) and scanning transmission electron microscopy (STEM) to determine the morphology and elemental composition of the nanomaterials. This is necessary as toxicity of a nanomaterial have been reported to be associated to its physicochemical properties.²⁴ SEM images in Figure 5.1 show that both GaSe and GeS nanomaterials are platelets of various dimensions but are fairly similar in morphologies. In addition, STEM confirms that the platelets are few- to multi-layered. Both nanomaterials also exhibit similar diversity in their particle size distributions, with a large spectrum of platelet sizes ranging from approximately 20 nm and 1 μm . Since the particle size distributions of GaSe and GeS in this study are relatively comparable from the SEM and STEM images, we did not study the influence of particle sizes on the cytotoxicity of the materials.

Figure 5.1. Size characterization of GaSe (left) and GeS (right). (A) SEM and (B) STEM images of the nanomaterials at 5000x and 20 000x magnification respectively. The white scale bars represent 5 μm for SEM and 500 nm for STEM images.

Elemental composition analysis revealed that the ratio of M : X in GaSe and GeS was 1 : 1.0 and 1 : 1.2 respectively. In addition, trace amounts of magnesium (4.8 at%) was found in GeS nanosheets, whereas GaSe was free from metal impurities. Figure 5.2 shows the results of X-ray diffraction measurement. The samples were prepared for the measurement by mechanical grinding in agate mortar from bulk material. On both diffractograms, it is clearly visible that there is preferential orientation along (00/) direction originating from highly anisotropic mechanical properties of layered materials. Both materials are single phase with GaSe having hexagonal structure (space group $P6_3/mmc$) and GeS exhibiting orthorhombic structure (space group $Pbnm$). The corresponding structures are shown on Figure 5.2.

Figure 5.2. X-ray diffractogram of GeS (top) and GaSe (bottom) and corresponding structures (S, Se – yellow, Ge – red, Ga – blue).

Cytotoxicity assessment of GaSe and GeS

Following 24 h incubation of various concentrations of the GaSe and GeS (between 3.125 to 400 mg mL⁻¹) with A549 cells, WST-8 and MTT cell viability assays were performed to investigate the cytotoxicity of these nanomaterials. Prior to these assessments, the nanomaterials were removed through two washing steps with phosphate buffer solution (PBS), pH 7.2, so as to reduce any possible interferences of the nanomaterials with the cell viability assays. The active reagent in WST-8 and MTT assays produce orange and purple formazan products respectively after receiving two electrons from dehydrogenases present in metabolically active cells.²² In the case of WST-8 assay, the formazan product formed is water-soluble, whereas for MTT assay, insoluble formazan crystals were generated. The insoluble formazan crystals can then be dissolved using organic solvents such as dimethyl sulfoxide (DMSO) for spectroscopic analysis. In both WST-8 and MTT assays, the amount of formazan produced is directly proportional to the number of metabolically active cells present.²⁴ By normalizing the colour intensity of the formazan products formed from cells exposed to varying concentrations of GaSe and GeS with that from a negative control where cells were incubated without any nanomaterials, the extent of cytotoxicity of the nanomaterials can be determined. Due to the different sensitivities of the cell viability reagents in the two assays, cells tested with the WST-8 reagent were incubated for 1 h at 37 °C and 5% CO₂, whereas cells with the MTT reagent were incubated for 3 h at the same conditions.

WST-8 assay

The cytotoxicity of GaSe and GeS was first investigated using the WST-8 assay. Figure 5.3 shows that both nanomaterials induced concentration-dependent effect on the cell viability of A549 cells. However, they resulted in opposite trends of cell viabilities with increasing dosage of the nanomaterial. While GaSe caused an overall decreasing cell viability with increasing concentration, suggesting that the nanomaterial is toxic, the percentage cell viability derived from the WST-8 assay indicated an increasing cell viability with increasing dosage of GeS nanomaterial. The latter was unexpected of toxic nanomaterials and thus control experiments were conducted to determine if this peculiar observation was due to the presence of nanomaterial-induced interference on the absorbance measurements.

Figure 5.3. Percentage cell viability of A549 cells as measured by WST-8 assay, following 24 h exposure to varying amounts of nanomaterials (GaSe and GeS). The percentages are normalized to data obtained from the negative control that are not exposed to any nanomaterials. These results are mean values with $\pm$ standard deviations of a minimum of three repeat experiments, each consisting of four wells per treatment for every concentration.

In order to examine for nanomaterials-induced artifacts on WST-8 assay measurements, GaSe and GeS nanomaterials were incubated separately with WST-8 reagent in the absence of cells. In cell-free conditions, an increase in the relative percentage of formazan generated will indicate that the nanomaterials could reduce the WST-8 reagent and interfere with the absorbance measurements, contributing to an overestimation of cell viability. The relative percentages of formazan formation from GaSe and GeS in cell-free condition are shown in Figure 5.4.

Figure 5.4. Percentage of formazan generated from GaSe and GeS under cell-free conditions, normalized to the amount of formazan generated in the absence of nanomaterials. The graph illustrates the degree of reduction of WST-8 reagent to formazan product with different GaSe and GeS concentrations. The black dotted line represents the blank control where there is no nanomaterial incubated with the WST-8 assay. The green dotted line represents 150% control formazan concentration limit set, below which interference is considered as insignificant.

From Figure 5.4, it was observed that both nanomaterials reacted with the WST-8 reagent significantly, with GaSe and GeS resulting in the production of 1062% and 2350% control formazan concentration respectively at the highest concentration (400 mg mL⁻¹) of nanomaterial tested. A plateau in the absorbance intensity was observed for GeS above 100 mg mL⁻¹, possibly because WST-8 reagent was the limiting reagent for the reduction reaction. Between the two nanomaterials, GeS imparted a greater interference as compared to GaSe. This differing extent of artifact-induced interference could have resulted in the opposite cell viability trends observed between GaSe and GeS. Hypothetically, we might assume any nanomaterial to induce significant artifacts on the absorbance measurements if the calculated formazan concentration is beyond $\pm 50\%$ of the blank control which is free from cells and the nanomaterial of interest. As such, we could deduce from the WST-8 assay control experiments that GaSe and GeS would distort the cell viability data if they existed at concentrations above ~ 30 mg mL⁻¹ and ~ 3 mg mL⁻¹ (inset in Figure 6.4) in the cell cultures respectively when the WST-8 assay was added. Through careful repeated washings prior to the addition of WST-8 assay for our cell viability assessments, the amount of nanomaterial left in the cell culture that could react with the WST-8 assay should be reduced drastically, to around 25–50 mg mL⁻¹ for the highest concentration of nanomaterial introduced (400 mg mL⁻¹). For GaSe, these residual amounts are unlikely to cause interferences for concentrations tested between 3.125 to 200 mg mL⁻¹. However, for GeS, similar residual amounts after performing the same washing procedure can still impart significant interference effects for all concentrations tested due to its higher reducing activity with the WST-8 assay reagent as compared to GaSe. Therefore, it is highly probable that the cell viability measurements of GeS was affected by interference from the nanomaterial, leading to the perceived trend of higher cell viability with increased dosage of GeS incubated.

MTT assay

In order to verify the validity of the cell viability data obtained from the WST-8 assay, a second cell viability assay (MTT) was used to assess the cytotoxicity of GaSe and GeS (Figure 5.5). Similar to the results obtained for WST-8 assay, GaSe displayed dose-dependent toxicological effect on A549 cells while GeS continued to give high cell viability values at the highest concentration (400 mg mL⁻¹) exposed.

Figure 5.5. Percentage cell viability of A549 cells as measured by MTT assay, following 24 h exposure to varying amounts of GaSe and GeS. The percentages are normalized to data obtained from the negative control that are not exposed to any nanomaterials. These results are mean values with $\pm$ standard deviations of a minimum of three repeat experiments, each consisting of four wells per treatment for every concentration.

Two control experiments were carried out to identify possible nanomaterial-induced interferences on the MTT cell viability measurements. The first control experiment was to test for possible binding of the nanomaterials to the insoluble formazan crystals and subsequent removal of these crystals after centrifugation. Ascorbic acid, a substance known to reduce tetrazolium compounds to formazan,²⁵ was added to the MTT-nanomaterial mixtures after 3 h incubation to preferentially generate the insoluble formazan crystals for this investigation. In the event that the relative percentage control formazan concentration of the MTT-nanomaterial–ascorbic acid mixture is significantly lower than 100%, it could be inferred that there is sufficient binding between the formazan crystals and the nanomaterials.

The second control experiment was similar to that conducted for WST-8, which examined the possibility of the reduction of MTT reagent by the nanomaterial to form formazan products in the absence of cells. The results from these MTT control experiments are illustrated in Figure 5.6.

Figure 5.6. Percentage of formazan generated from GaSe and GeS under cell-free conditions, normalized to the amount of formazan generated in the absence of nanomaterials. Graph (A) shows the level of binding between MTT formazan products with the nanomaterials. Graph (B) shows the degree of reduction of MTT reagent to formazan product with varying GaSe and GeS concentrations. The black dotted line represents the blank control where there is no nanomaterial incubated with the MTT assay. The green dotted line in graph (B) represents 150% control formazan concentration limit set, below which interference is considered as insignificant.

Binding effects between the nanomaterials and the MTT formazan crystals has been reported to prevent the dissolution of the formazan formed by the organic solvent used, thereby giving an inflated cytotoxicity response.²⁵ Since the relative percentage control formazan concentration values in Figure 5.6A did not show any significant reduction below 100% for both nanomaterials, it was deduced that there was negligible interference due to this binding effect.

However, both nanomaterials were found to cause significant reduction of the MTT reagent (Figure 5.6B), similar to that for WST-8 reagent. GaSe and GeS resulted in the production of 1548% and 2054% control formazan concentration respectively, at the highest dosage of 400 mg mL⁻¹ tested. Figure 5.6B also showed that between the two nanomaterials, GeS would cause greater interference to the MTT cell viability measurements as compared to GaSe. In the same manner, we could infer from the data attained in Figure 5.6B that GeS and GaSe would distort the MTT cell viability results at concentrations above ~15 mg mL⁻¹ and ~55 mg mL⁻¹ respectively, if we regard 150% percentage formazan concentration to be the threshold for significant interference (inset in Figure 5.6B).

For GaSe, the thorough washing steps performed on the cell culture to remove the nanomaterials before the addition of the MTT assay should render little nanomaterial-induced artefact for all concentrations tested. In the case of GeS, despite the same washing steps performed, its cytotoxicity data as derived from the MTT assay measurements could still be affected by the generation of excess MTT formazan crystals from the reaction between GeS and the MTT reagent, especially for the higher amounts of GeS, as evident from the percentage cell viability values in Figure 5.5. Thus, the cytotoxicity of GeS in both WST-8 and MTT assays is underestimated and erroneous.

Based on the percentage cell viability values in both WST-8 and MTT assays measurements, it was observed that GaSe is relatively toxic, with 46% (WST-8) and 54% (MTT) viable cells remaining after 24 h exposure to the lowest dosage (3.125 mg mL^{-1}) of nanomaterial treated, where the interference effect on the absorbance measurements by residual amounts of nanomaterial following careful washings is minimal. In addition, it was found that GaSe exhibited much higher toxicity when compared to many other well-known chalcogenides such as MoS_2 , WS_2 and WSe_2 (Figure 5.7).²⁴ This difference in toxicities may be attributed to the higher in vitro solubility of gallium ions compared to transition metals at physiological pH. This is supported by previous studies where compounds with higher in vitro solubility showed greater toxicity.²⁶ Besides higher in vitro solubility, involvement of a variety of other factors such as different synthesis methods, particle characteristics and physicochemical properties can also affect the toxicity of the nanomaterial.^{27–31}

Figure 5.7. Comparison of cell viability measurements using (A) WST-8 and (B) MTT assays between GaSe and various transition metal chalcogenides (TMDs; MoS₂, WS₂, WSe₂) across different nanomaterial concentrations, after 24 h exposure with A549 cells. Data presented for TMDs are obtained ref. 24. Note that these TMDs can also reduce the MTT reactive agent, however, in a much lesser extent compared to GaSe. The same washing procedures were conducted for the TMDs, ensuring comparable and almost interference-free cell viability data.

5.3. Conclusion

In view of the rising research on the applications of GaSe and GeS, we conducted an in vitro cytotoxicity assessment of these semiconductor chalcogenides to determine if these materials may have an adverse effect on our wellbeing if they were to be commercialized in the future. GaSe and GeS were incubated with A549 cells for 24 h and cell viability measurements were performed thereafter using WST-8 and MTT assays. The cytotoxicity results obtained showed that GaSe is significantly more toxic than transition metal dichalcogenides (MoS_2 , WS_2 , WSe_2). GeS, on the other hand, appeared to be non-toxic, with

concentration having a positive correlation with cell viability. Control experiments under cell-free conditions revealed significant interferences between both nanomaterials and the assay reagents, with GeS showing greater interferences than GaSe. In view of this, other cell viability assays should be explored to study the cytotoxicity effects of GaSe and GeS more accurately. As toxicity studies of GaSe and GeS are still in its infancy, more research can be done to determine their effects on our health to ensure our safety before their actual commercial application.

5.4. Experimental section

Apparatus

The SEM images were obtained from a JEOL 7600 field-emission scanning electron microscopy (JEOL, Japan) with an accelerating voltage of 2.00 kV, and a working distance of 5.9 mm under gentle beam mode, while the STEM images were obtained from the same equipment using an accelerating voltage of 30.00 kV, and a working distance of 8.3 mm under SEM mode. 0.1 mg mL⁻¹ samples in dimethylformamide (DMF) were prepared for the STEM imaging. EDS was obtained using Oxford instruments x-stream2 and micsf+ with an accelerating voltage of 30.00 kV. X-ray powder diffraction data were collected at room temperature with an X'Pert PRO θ – θ powder diffractometer with parafocusing Bragg–Brentano geometry using Cu K α radiation (λ = 0.15418 nm, U = 40 kV, I = 30 mA). Data were scanned with an ultrafast detector X'Celerator over the angular range 5–80° 2 θ with a step size of 0.016° 2 θ and a counting time of 20.32 s per step. Data evaluation was performed in the software package HighScore Plus.

Chemicals

Germanium (99.999%), gallium (99.999%), selenium (99.999%) and sulfur (99.999%) were obtained from STREM, USA. Quartz glass ampoules were washed with hydrofluoric acid, deionized water and acetone before use. Subsequently, the ampoules were heated by hydrogen/oxygen torch under high vacuum (below 5 x 10³ Pa) to remove any contamination. Methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Water-soluble tetrazolium salt (WST-8) was purchased from Dojindo. Dimethyl sulfoxide (DMSO) was purchased from Tedia. Dulbecco's Modified Eagle medium and phosphate buffer solution (PBS), pH 7.2 were purchased from Gibco. Fetal bovine serum and 1% penicillin–streptomycin liquid were purchased from PAA Laboratories.

Synthesis of GaSe and GeS

For the GaSe synthesis, 15 g stoichiometric mixture of granulated Ga and Se was placed in quartz glass ampoules (25 mm x 150 mm) and evacuated below 5×10^{-3} Pa using diffusion pump. Evacuated ampoule was sealed using oxygen–hydrogen torch. The ampoule was heated at a rate of $1\text{ }^{\circ}\text{C min}^{-1}$ on $850\text{ }^{\circ}\text{C}$. After 48 h the ampoule was cooled on room temperature. Subsequently the ampoule heated on $970\text{ }^{\circ}\text{C}$ at a rate of $1\text{ }^{\circ}\text{C min}^{-1}$. After 1 h the ampoule was cooled on room at a rate of $1\text{ }^{\circ}\text{C min}^{-1}$.

For GeS synthesis, 10 g stoichiometric mixture of granulated Ge and S was placed in quartz glass ampoules (25 mm x 150mm) and evacuated below 5×10^{-3} Pa using diffusion pump. Evacuated ampoule was sealed using oxygen–hydrogen torch. The ampoule was heated on $700\text{ }^{\circ}\text{C}$ for 5 hours. The heating and cooling rate was $1\text{ }^{\circ}\text{C min}^{-1}$.

Cell culture

The human lung carcinoma epithelial cell line A549, a popular cell line in nanotoxicological studies, was used to determine the nanotoxicity of the GaSe and GeS. A459 cells have a typical cell cycle of 22 h and were purchased from Bio-REV Singapore. Cells were cultured with Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin liquid in an incubator maintained at $37\text{ }^{\circ}\text{C}$ under 5% CO_2 . The cells were seeded in 24-well plates (570 mL per well) with a cell density of 8.8×10^4 cells per mL for 24 h before introducing the nanomaterials.

Cell exposure to GaSe and GeS

After seeding, the culture medium was first removed and each well was rinsed with PBS (pH 7.2). The cells were then incubated with different concentrations (3.125, 6.25, 12.5, 25, 50, 100, 200, 400 mg mL^{-1}) of nanomaterial dispersions (570 mL per well) for another 24 h. Cells incubated without nanomaterials were used as a negative control.

WST-8 assay

After 24 h incubation with the nanomaterials, the cells were washed twice with PBS (pH 7.2) and incubated with 10% diluted stock WST solution (300 μ L per well) at 37 °C and 5% CO₂ for 1 h. The solutions were subsequently centrifuged at 8000 rpm for 10 min to remove any traces of GaSe and GeS, before transferring 100 μ L of the supernatant to a 96-well plate to measure their absorbance at 450 nm and 800 nm (background absorbance).

MTT assay

After 24 h incubation with GaSe and GeS, the cells were washed twice with PBS (pH 7.2) and incubated with 1 mg mL⁻¹ of MTT solution (300 μ L per well) at 37 °C and 5% CO₂ for 3 h. Thereafter, the MTT solution was removed and an equal volume of DMSO was added to dissolve the purple formazan crystals formed by the viable cells. The plates were gently agitated at 500 rpm for 5 min, and then the solutions were centrifuged at 8000 rpm for 10 min to remove any traces of the nanomaterials. Following centrifugation, 100 μ L of the supernatant were transferred to a 96-well plate and their absorbance were measured at 570 nm and 690 nm (background absorbance).

WST-8 assay control experiment

The different concentrations of GaSe and GeS in culture media were prepared and incubated with 10% diluted stock WST solution (300 μ L per well) in the absence of viable cells at 37 °C and 5% CO₂ for 1 h. Subsequently, the solutions were centrifuged at 8000 rpm for 10 min to remove any traces of nanomaterials, before transferring 100 μ L of the supernatant to a 96-well plate to measure their absorbance at 450 nm and 800 nm (background absorbance).

MTT assay control experiments

Two control experiments were conducted. For the first control experiment, different concentrations of GaSe and GeS in culture media were prepared and incubated with 1 mg mL⁻¹ MTT solution (500 μ L per well) in the absence of viable cells at 37 °C and 5 % CO₂ for 3 h. Thereafter, the MTT solutions were removed and an equal volume of DMSO were added to dissolve the purple formazan crystals formed by living cells. The plates were gently agitated at 500 rpm for 5 min, and then the solutions were centrifuged at 8000 rpm for 10 min to remove any traces of the nanomaterials. Following centrifugation, 100 μ L of the supernatant were transferred to a 96-well plate and their absorbance were measured at 570 nm and 690 nm (background absorbance).

For the second MTT control experiment, a set of 24-well plates containing 200 μ L of the above-mentioned cell-free MTT-nanomaterial solution (subjected to 3 h incubation at 37 °C and 5% CO₂) was prepared. 160 μ L of 4 mM of ascorbic acid was added to each well, and the solutions were mixed gently by agitation at 500 rpm for 5 min. Subsequently, the MTT-nanomaterial–ascorbic acid mixtures were incubated at 37 °C and 5 % CO₂ for 1 h. DMSO was then added to the mixtures at a ratio of 2 : 1 before incubating them for another 10 min. Finally, the solutions were centrifuged at 8000 rpm for 10 min, and their absorbance were measured at 570 nm and 690 nm (background absorbance).

References:

1. Jie, W.; Chen, X.; Li, D.; Xie, L.; Hui, Y. Y.; Lau, S. P.; Cui, X.; Hao, J. *Angew. Chem. Int. Ed. Engl.* **2015**, *54*, 1185–1189.
2. Lei, S.; Ge, L.; Liu, Z.; Najmaei, S.; Shi, G.; You, G.; Lou, J.; Vajtai, R.; Ajayan, P. M. *Nano Lett.* **2013**, *13*, 2777–2781.
3. Ma, Y.; Dai, Y.; Guo, M.; Yu, L.; Huang, B. *Phys. Chem. Chem. Phys.* **2013**, *15*, 7098–7105.
4. Rybkovskiy, D. V.; Arutyunyan, N. R.; Orekhov, A. S.; Gromchenko, I. A.; Vorobiev, I. V.; Osadchy, A. V.; Salaev, E. Y.; Baykara, T. K.; Allakhverdiev, K. R.; Obratsova, E. D. *Phys. Rev. B: Condens. Matter Mater. Phys.* **2011**, *84*, 085314.
5. Hu, P.; Wen, Z.; Wang, L.; Tan, P.; Xiao, K. *ACS Nano* **2012**, *6*, 5988–5994.
6. Aksimentyeva, O. I.; Demchenko, P. Y.; Savchyn, V. P.; Balitskii, O. A. *Nanoscale Res. Lett.* **2013**, *8*, 29–33.
7. Murugesan, S.; Kearns, P.; Stevenson, K. J. *Langmuir* **2012**, *28*, 5513–5517.
8. Makinistian, L.; Albanesi, E. A. *Phys. Rev. B: Condens. Matter Mater. Phys.* **2006**, *74*, 045206.
9. Cho, Y. J.; Im, H. S.; Myung, Y.; Kim, C. H.; Kim, H. S.; Back, S. H.; Lim, Y. R.; Jung, C. S.; Jang, D. M.; Park, J.; Cha, E. H.; Choo, S. H.; Song, M. S.; Cho II, W. *Chem. Commun.* **2013**, *49*, 4661–4663.
10. Li, C.; Huang, L.; Snigdha, G. P.; Yu, Y.; Cao, L. *ACS Nano* **2012**, *6*, 8868–8877.
11. Vaughn II, D. D.; Patel, R. J.; Hickner, M. A.; Schaak, R. E. *J. Am. Chem. Soc.* **2010**, *132*, 15170–15172.
12. Shi, L.; Dai, Y. J. *J. Appl. Crystallogr.* **2014**, *47*, 527–531.
13. Peng, H.; Meister, S.; Chan, C. K.; Zhang, X. F.; Cui, Y. *Nano Lett.* **2007**, *7*, 199–203.
14. Late, D. J.; Liu, B.; Luo, J.; Yan, A.; Matte, H. S. S. R.; Grayson, M.; Rao, C. N. R.; David, V. P. *Adv. Mater.* **2012**, *24*, 3549–3554.
15. Ndangili, P. M.; Jijana, A. N.; Olowu, R. A.; Mailu, S. N.; Ngece, F. R.; Williams, A.; Waryo, T. T.; Baker, P. G. L.; Iwuoha, E. I. *Int. J. Electrochem. Sci.* **2011**, *6*, 1438–1453.

16. Cui, S.; Chahal, R.; Boussard-Plédel, C.; Nazabal, V.; Doualan, J.; Troles, J.; Lucas, J.; Bureau, B. *Molecules* **2013**, *18*, 5373–5388.
17. Pumera, M. *Chem.–Asian J.* **2011**, *6*, 340.
18. Tanaka, A.; Hirata, M.; Shiratani, M.; Koga, K.; Kiyohara, Y. *J. Occup. Health* **2012**, *54*, 187–195.
19. Morgan, D. L.; Shines, C. J.; Jeter, S. P.; Blazka, M. E.; Elwell, M. R.; Wilson, R. E.; Ward, S. M.; Price, H. C.; Moskowitz, P. D. *Toxicol. Appl. Pharmacol.* **1997**, *147*, 399–410.
20. Gerber, G. B.; L'éonard, A. *Mutat. Res.* **1997**, *387*, 141–146.
21. Tao, S. H.; Bolger, P. M. *Regul. Toxicol. Pharmacol.* **1997**, *25*, 211–219.
22. Richards, D.; Ivanisevic, A. *Chem. Soc. Rev.* **2012**, *41*, 2052–2060.
23. Kong, B.; Seog, J. H.; Graham, L. M.; Lee, S. B. *Nanomedicine* **2011**, *6*, 929–941.
24. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Chem.–Eur. J.* **2014**, *20*, 9627–9632.
25. Belyanskaya, L.; Manser, P.; Spohn, P.; Bruinink, A.; Wick, P. *Carbon* **2007**, *45*, 2643–2648.
26. Morgan, D. L.; Shines, C. J.; Jeter, S. P.; Wilson, R. E.; Elwell, M. P.; Price, H. C.; Moskowitz, P. D. *Environ. Res.* **1995**, *71*, 16–24.
27. Huang, Y.-W.; Wu, C.-H.; Aronstam, R. S. *Materials* **2010**, *3*, 4842–4859.
28. Chng, E. L. K.; Pumera, M. *RSC Adv.* **2015**, *5*, 3074–3080.
29. Chng, E. L. K.; Sofer, Z.; Pumera, M. *Nanoscale* **2014**, *6*, 14412–14418.
30. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Nanoscale* **2014**, *6*, 1173–1180.
31. Chng, E. L. K.; Pumera, M. *Chem.–Eur. J.* **2013**, *19*, 8227–8235.

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Chapter 6 –

Cytotoxicity of Black Phosphorus

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Part of the writing presented in this chapter was published in the following journal article:

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6.1. Introduction

Recently, black phosphorus (BP) has been rediscovered as a 2D layered material since its first successful synthesis in 1914 in the bulk form.¹ As the latest addition to the family of 2D layered materials, it has attracted tremendous interest within the research community in a variety of fields, ranging from chemists, material scientists, semiconductor device engineers, to physicists.²

In a similar way to graphite, BP is made up of only one element, with sheets of six-membered rings held together by van der Waals interaction.^{1–4} However, unlike in graphite, each sheet of BP is not flat but is uniquely puckered with two chemically bonded double layers, and the elemental atoms are not connected by sp^2 -type bonding.³ Recent exploration of this phosphorus allotrope revealed promising properties, that is, high carrier mobility⁵ and tunable electronic band gap,^{3, 6} which had opened up its potential in optoelectronics,^{2,7,8} nanoelectronics,^{2,7,9} biosensing,^{2,3} photocatalysis,¹⁰ rechargeable batteries,¹¹ and biomedical imaging.^{2,3} With the rapid advancement in exploring this novel material, there is increasing possibility of its commercial application in the future. However, very little is known about its toxicity, and this research gap needs to be filled in before we can extend the use of black phosphorus in the biomedical field.

Previous studies on the toxicity of phosphorus mainly revolved around its other known allotropes; namely white and red phosphorus. White phosphorus has been reported to be nearly as toxic as cyanide, with chronic exposure capable of causing an awful condition of bone necrosis.^{12–14} Another work indicated that inhalation of 185–592 mg m^{-3} white phosphorus smoke between 5 to 15 min can cause headache, upper respiratory tract irritation, and nasal discharge.^{13,14} Red phosphorus, on the other hand, was found to be non-toxic.^{13,15,16} Surprisingly, for black phosphorus, no conclusive data pertaining to its toxicity has yet been established, with only one article mentioning BP to be non-toxic.¹⁰ However, no supporting reference was provided as

convincing evidence for this statement. Therefore, we aim to address this fundamental issue herein by investigating the in vitro toxicological effects of black phosphorus, for the first time, on human lung carcinoma cancer epithelial cells (A549). This cell line was chosen because the primary route of entry for 2D nanomaterials is most likely to be the respiratory tract.¹⁷ The cytotoxicity effects of BP was studied by assessing the cell viabilities following a 24 h exposure of various BP concentrations with A549 cells through the utilization of two well-established dye-based cell viability assays, water-soluble tetrazolium salt (WST-8) and methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) assays. The use of two similar assays that worked on similar principles could avoid errors in the cytotoxicity findings.¹⁷

6.2. Results and Discussion

Material characterization

BP had previously been characterized using scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), X-ray photoelectron spectroscopy (XPS), X-ray diffraction (XRD) and voltammetry.³ Its morphology as seen from SEM revealed the plate-like structure of BP (Figure 6.1).

Figure 6.1. SEM images of black phosphorus obtained at 1000x magnification (left) and 5000x magnification (right).

Cytotoxicity assessments

To investigate the cytotoxicity of black phosphorus, WST-8 and MTT cell viability assays were conducted after a 24 h incubation of A549 cells with varying concentrations of BP (ranging from 3.125 to 400 mg mL⁻¹). Since there have been reports of nanomaterials interfering with these dye-based cell viability assays,^{17,18} BP was removed prior to these assessments through two washing steps with phosphate buffer solution (PBS, pH 7.2). In the presence of metabolically active cells, the active tetrazolium reagents in WST-8 and MTT assays are reduced, producing orange water-soluble and purple insoluble formazan products, respectively.^{17,19} The insoluble MTT formazan crystals can be dissolved using organic solvents such as dimethyl sulfoxide (DMSO) for absorbance measurement.¹⁷ The degree of cytotoxicity can then be easily determined by normalizing the colour intensity of the formazan products formed from cells exposed to different BP concentrations with a negative control where cells were incubated with no nanomaterials.²⁰ Figure 6.2 illustrates the results obtained from WST-8 and MTT assays. As depicted in the figure, there is a similar trend observed for both cell viability assays conducted. For BP concentrations between 3.125 to 25 mg mL⁻¹, the percentage cell viability decreases gradually, and then declines sharply at 50 mg mL⁻¹. The initial decreasing trend suggests that BP is a toxic material, with the lowest cell viabilities reaching 48% (WST-8) and 34% (MTT) following 50 mg mL⁻¹ of BP exposure for 24 h. However, the increasing cell viability trend for BP concentrations beyond 100 mg mL⁻¹ is unexpected for toxic nanomaterials and control experiments were performed to investigate whether this observation could be due to nanomaterial-induced interferences with the cell viability assay reagents.

Figure 6.2. Percentage cell viability of human lung carcinoma epithelial cells (A549) as measured by WST-8 and MTT assays following 24 h exposure to varying amounts of BP. Data is normalized to the negative control, which was not exposed to any BP. The data are mean values with $\pm$ standard deviation of a minimum of three repeat experiments. Each repeat contained four wells per treatment for every concentration.

BP-induced interference

To investigate the possibility of any BP-induced interference on WST-8 and MTT assay measurements, different BP concentrations were incubated with the assay reagents under cell-free conditions.^{20–24} In the event that there is an increase in the relative percentage of formazan generated, it would indicate that the nanomaterial could reduce the assay reagents, thereby interfering with the absorbance measurements, and result in an overestimation of cell viability. In the case of MTT, where the formazan product is insoluble crystals, there is another possibility of the nanomaterial interfering

with the assay. There have been reports of nanomaterials binding with the insoluble MTT formazan crystals formed, which stabilizes the nanomaterial-MTT formazan crystals complex so that it will no longer be soluble in the organic solvents used to dissolve the MTT formazan crystals.¹⁷ Therefore, for the MTT assay, another control experiment was carried out to test this possibility of a binding interaction between BP and insoluble formazan crystals. For this investigation, ascorbic acid, which is known to reduce tetrazolium compounds to formazan,¹⁷ was introduced to BP-MTT mixtures after 3 h incubation at 37 °C to favour the formation of insoluble formazan crystals. Subsequently, the BP-MTT-ascorbic acid mixtures were incubated for another hour before measuring their absorbances. If there is sufficient binding between BP with the insoluble formazan crystals, we can hypothesize that the relative percentage control formazan concentration of the BP-MTT-ascorbic acid mixture would decline significantly below 100 %.

From the data collected through the control experiments (Figure 6.3), we found that BP interfered significantly with both the WST-8 and MTT cell-viability assessments. This is evident from the steep positive correlation between control formazan concentration and BP concentration for both assays (Figure 6.3A), with relative formazan concentrations produced reaching 818 % (WST-8) and 776 % (MTT) at the highest dosage of 400 mg mL⁻¹ BP tested. This efficient reductive capability observed in BP is probably related to its strong inherent electrochemical oxidation, as suggested from a previous study.³ Even though careful repeated washings were carried out prior to the addition of assay reagents, the amount of BP remaining in the cell cultures may exceed 25 mg mL⁻¹ for those which were incubated with more than 100 mg mL⁻¹ of BP. If we assume that interference generated by any nanomaterial to occur when the relative formazan concentration exceeds ± 50 % of the blank control where no nanomaterials are present, we could infer that BP would distort the cytotoxicity results if present above 25 mg mL⁻¹ (for WST-8) and 50 mg mL⁻¹ (for MTT) in the cell cultures when the assay reagents are introduced. Consequently, for concentrations exceeding 100 mg mL⁻¹, the cell viability assessments were prone to inflation owing to the BP-induced interferences with the WST-8 and MTT reagents. Besides BP-induced interference through reduction of assay

reagents, interference that is due to binding interactions between BP and insoluble MTT formazan crystals was also studied. Even though Figure 6.3B showed a declining trend of control formazan concentration of the MTT–BP–ascorbic acid mixture with increasing BP concentrations, the thorough washing steps adopted should render BP-induced artifacts owing to this binding interaction insignificant.

Figure 6.3. Percentage of formazan produced from varying amounts of BP in the absence of cells, normalized to the amount of formazan generated by the blank control where no BP is incubated with the assay reagents. (A) The extent of reduction of WST-8 and MTT reagents to formazan product by different concentrations of BP. The black dotted line represents the blank control, whereas the green dotted line indicates 150 % control formazan concentration limit set, above which interference is considered as significant. (B) The degree of binding between MTT formazan with different BP concentrations.

Based on the results from control experiments above, we can deduce that the cell viability data depicted in Figure 6.2 were likely to be overestimated for BP concentrations above 100 mg mL⁻¹ owing to reduction of the assay reagents by the BP nanomaterial that remained in the cell culture after the washing steps. For concentrations below 100 mg mL⁻¹, BP exhibited a dose-dependent toxicological effect on A549 cells, lowering cell viabilities to 48% and 34% for WST-8 and MTT assays respectively after a 50 mg mL⁻¹ exposure for 24 h. Between 3.125 to 6.25 mg mL⁻¹, BP showed moderate toxicity with cell viabilities between 92 % and 82 % for WST-8 and MTT, respectively.

Apart from investigating the cytotoxicity of BP, we can compare the results with other 2D layered materials studied previously to have a better understanding of their relative toxicities. Table 6.1 compares the cytotoxicity data gathered from our group using A549 cell line under similar conditions, among three different types of 2D materials, namely graphene oxide (GO) under different synthesis methods, various exfoliated transition-metal dichalcogenides (TMDs; MoS₂, WS₂, WSe₂) as well as black phosphorus. From the cell viability values shown, it is observed that, generally, the cytotoxicity of BP is intermediate between that of graphene oxides and TMDs.

Table 6.1. Comparison of normalized percentage of viable cells obtained through WST-8 and MTT assays, following 24 h exposure with 20 mg mL⁻¹ of graphene oxide prepared using Staudenmaier, Hoffman, Hummers, and Tour preparation methods (GO-ST, GO-HO, GO-HU, GO-TO), and 25 mg mL⁻¹ of various exfoliated transition metal dichalcogenides (MoS₂, WS₂, WSe₂) as well as 25 mg mL⁻¹ of black phosphorus (BP).^a

	Cell viability (%)							
Materials	GO-ST	GO-HO	GO-HU	GO-TO	MoS ₂	WS ₂	WSe ₂	BP
WST-8 assay	48	60	72	96	96	95	88	81
MTT assay	70	82	52	63	82	97	78	68

^aData presented are approximated from the graphs shown in Figure 6.2 and Reference 20–22.

6.3. Conclusion

With black phosphorus showing promise in electrical and optical sensing applications for biomedical research, we need to first examine its toxicity. Herein, we studied the cytotoxicity effects of black phosphorus by incubating various concentrations of BP with human lung epithelial cells for 24 h and subsequently assessed their cell viabilities using WST-8 and MTT assays. BP exhibited a dose-dependent response on A549 cells, with a generally intermediate toxicity between graphene oxides and TMDs. Analysis from control experiments revealed presence of nanomaterial-induced interferences, primarily through the reduction of assay reagents by the material. Despite careful repeated washing steps of cell cultures prior to the addition of the assay reagents, interference effects remained apparent for BP concentrations above 100 mg mL⁻¹. Nevertheless, this initial investigation has shed some light on the cytotoxicity of this new emerging 2D material. Further investigation of toxicity on single-layer black phosphorus is of very high importance, although this may be challenging due to its low stability in the ambient conditions.²⁵ Other cell viability assays and more toxicity assessments should be explored to better understand the potential impacts of black phosphorus on our health before its real application in the future.²⁶

6.4. Experimental section

Characterization

The SEM images were obtained from a JEOL 7600F field-emission scanning electron microscopy (JEOL, Japan) with an accelerating voltage of 5.00 kV, and a working distance of 7.6 mm under SEI mode. Sulfuric acid (98 %), potassium hydroxide (ACS p.a. purity), chloroform (99.9%), and iodine (99.8 %) were purchased from PENTA, Czech Republic. Gold wire of 99.99% purity was obtained from Safina, Czech Republic. Red phosphorus of 99.999 % purity and tin of 99.999 % purity was obtained from Sigma-Aldrich, Czech Republic. Human lung carcinoma epithelial cell line A549 was purchased from Bio-REV Singapore. Methylthiazolyldiphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Tedia. Water-soluble tetrazolium salt (WST-8) was purchased from Dojindo. Dulbecco's modified eagle medium and phosphate buffer solution (PBS), pH 7.2 were purchased from Gibco. Fetal bovine serum and 1% penicillin-streptomycin liquid were purchased from PAA Laboratories.

Synthesis of black phosphorus

Black phosphorus (BP) crystals were synthesized based on a procedure reported previously that used an Au/Sn alloy-like solvent for red phosphorus and SnI_4 as a vapour transport medium in a sealed ampoule.²⁷ 500 mg of AuSn alloy was prepared by melting stoichiometric amounts of Au and Sn under high vacuum directly into a quartz ampoule. 720 mg of red phosphorus and 15 mg of SnI_4 were added to the ampoule and were subsequently sealed by an oxygen/hydrogen torch. The ampoule was placed in a muffle furnace and heated to 400 °C for 1 hour. After 2 h at 400 °C, the ampoule was heated to 600 °C for 24 h. The furnace was then cooled to room temperature overnight. The formed crystals of black phosphorus in the form of plates with size up to 5 mm x 2 mm were removed from the ampoule and washed with CS_2 to remove white phosphorus formed as a by-product. The temperature was maintained for 2 h before the furnace was gradually

cooled down to room temperature. SnI_4 was prepared by direct synthesis using tin and iodine in chloroform under reflux, and subsequently purified by recrystallization using chloroform.

Cell culture

The human lung carcinoma epithelial cell line A549 was used to determine the nanotoxicity of BP. It is a popular cell line in nanotoxicological studies, with a typical cell cycle of 22 h. Cells were cultured with Dulbecco's modified eagle medium (DMEM) supplemented with 1% penicillin-streptomycin liquid and 10% fetal bovine serum (FBS) in an incubator maintained at 37 °C under 5 % CO_2 . The cells were seeded in 24-well plates (570 μL per well) with a cell density of 8.8×10^4 cells per mL for 24 h before exposing them to black phosphorus.

Cell exposure to black phosphorus

The culture medium was first removed after seeding and each well was rinsed with PBS (pH 7.2). The cells were then incubated with various concentrations (3.125, 6.25, 12.5, 25, 50, 100, 200, 400 mg mL^{-1}) of BP dispersions (570 μL per well) for another 24 h. Cells incubated without BP were used as a negative control.

WST-8 assay

After 24 h incubation with BP, the cells were washed twice with PBS (pH 7.2) and incubated with 10 % diluted stock WST solution (300 μL per well) at 37 °C and 5 % CO_2 for 1 h. The solutions were subsequently centrifuged at 8000 rpm for 10 min to remove any traces of BP, before transferring 100 μL of the supernatant to a 96-well plate to measure their absorbance at 450 nm and 800 nm (background absorbance).

MTT assay

After 24 h incubation with BP, the cells were washed twice with PBS (pH 7.2) and incubated with 1 mg mL⁻¹ of MTT solution (300 µL per well) at 37 °C and 5 % CO₂ for 3 h. Subsequently, the MTT solution was removed and an equal volume of DMSO was added to dissolve the purple formazan crystals formed by the viable cells. The plates were then gently agitated at 500 rpm for 5 min, and the solutions were centrifuged at 8000 rpm for 10 min. To remove any traces of BP. After centrifugation, 100 µL of the supernatant were transferred to a 96-well plate and their absorbance was measured at 570 nm and 690 nm (background absorbance).

WST-8 assay control experiment

Varying concentrations of BP prepared in culture media were incubated with 10% diluted stock WST solution (300 µL per well) in the absence of viable cells at 37°C and 5 % CO₂ for 1 h. Subsequently, the solutions were transferred to eppendorf tubes and centrifuged at 8000 rpm for 10 min to remove any traces of BP. 100 µL of the supernatant were then pipetted to a 96-well plate to measure their absorbance at 450 nm and 800 nm (background absorbance).

MTT assay control experiments

Two control experiments were performed to test for particle interferences with the MTT assay. For the first control experiment, different concentrations of BP prepared in culture media were incubated with 1 mg mL⁻¹ MTT solution (500 µL per well) at 37°C and 5 % CO₂ for 3 h in the absence of viable cells. Subsequently, the MTT solutions were removed and equal volume of DMSO were added to dissolve any purple formazan crystals formed by reduction of the MTT reagent by BP. The plates were gently agitated at 500 rpm for 5 min, and then the solutions were centrifuged at 8000 rpm for 10 min to remove any traces of BP. Following centrifugation, 100 µL of the supernatant were pipetted to a 96-well plate and their absorbances were measured at 570 nm and 690 nm (background absorbance).

For the second MTT control experiment, a set of 24-well plates containing 200 μ L of the above-mentioned cell-free MTT-BP solution (subjected to 3 h incubation at 37°C and 5 % CO₂) was prepared. 4 mM of ascorbic acid (160 μ L) was added to each well, and the solutions were mixed gently by agitation at 500 rpm for 5 min. Thereafter, the MTT–BP–ascorbic acid mixtures were incubated for 1 h at 37 °C and 5% CO₂. DMSO was then added to the mixtures at a ratio of 2:1. Subsequently, the mixtures were incubated for another 10 min and then centrifuged at 8000 rpm for 10 min. Finally, their absorbance was measured at 570 nm and 690 nm (background absorbance).

References:

1. Bridgman, P. W. *J. Am. Chem. Soc.* **1914**, *36*, 1344–1363.
2. Ling, X.; Wang, H.; Huang, S.; Xia, F.; Dresselhaus, M. S. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 4523–4530.
3. Wang, L.; Sofer, Z.; Pumera, M. *ChemElectroChem*. **2015**, *2*, 324–327.
4. Li, L.; Yu, Y.; Ye, G. J.; Ge, Q.; Ou, X.; Wu, H.; Feng, D.; Chen, X. H.; Zhang, Y. *Nat. Nanotechnol.* **2014**, *9*, 372–377.
5. Qiao, J.; Kong, X.; Hu, Z.; Yang, F.; Ji, W. *Nat. Commun.* **2014**, *5*, 4475.
6. Das, S.; Zhang, W.; Demarteau, M.; Hoffmann, A.; Dubey, M.; Roelofs, A. *Nano Lett.* **2014**, *14*, 5733–5739.
7. Liu, H.; Du, Y.; Deng, Y.; Ye, P. D. *Chem. Soc. Rev.* **2015**, *44*, 2732–2743.
8. Youngblood, N.; Chen, C.; Koester, S. J.; Li, M. *Nat. Photonics* **2015**, *9*, 247–252.
9. Hong, T.; Chamlagain, B.; Lin, W.; Chuang, H.-J.; Pan, M.; Zhou, Z.; Xu, Y.-Q. *Nanoscale* **2014**, *6*, 8978–8983.
10. Shen, Z.; Sun, S.; Wang, W.; Liu, J.; Liu, Z.; Yu, J. C. *J. Mater. Chem. A* **2015**, *3*, 3285–3288.
11. Park, C.-M.; Sohn, H.-J. *Adv. Mater.* **2007**, *19*, 2465–2468.
12. Nitschke, J. R. *Nat. Chem.* **2011**, *3*, 90.
13. Bradberry, S. M.; Vale, J. A. in *Oxford Desk Reference Toxicology*, Edn. 1 (Eds.: R. Bateman, S. Jefferson, J. Thomas, J. Thompson, A. Vale), Oxford University Press, London UK, 2014, pp. 284 –285.
14. US Environmental Protection Agency
<http://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=30001IQN.txt> (Accessed on 6 May 2015).
15. Xia, D.; Shen, Z.; Huang, G.; Wang, W.; Yu, J. C.; Wong, P. K. *Environ. Sci. Technol.* **2015**, *49*, 6264–6273.

16. Young, J. A. *J. Chem. Educ.* **2004**, *81*, 945.
17. Kong, B.; Seog, J. H.; Graham, L. M.; Lee, S. B. *Nanomedicine* **2011**, *6*, 929–941.
18. Belyanskaya, L.; Manser, P.; Spohn, P.; Bruinink, A.; Wick, P. *Carbon* **2007**, *45*, 2643–648.
19. Richards, D.; Ivanisevic, A. *Chem. Soc. Rev.* **2012**, *41*, 2052–2060.
20. Chng, E. L. K.; Pumera, M. *Chem. –Eur. J.* **2013**, *19*, 8227–8235.
21. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Chem. –Eur. J.* **2014**, *20*, 9627–632.
22. Chng, E. L. K.; Pumera, M. *RSC Adv.* **2015**, *5*, 3074–3080.
23. Chng, E. L. K.; Sofer, Z.; Pumera, M. *Nanoscale* **2014**, *6*, 14412–14418.
24. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Nanoscale* **2014**, *6*, 1173–1180.
25. Hersam, M. C. *ACS Nano* **2015**, *9*, 4661–4663.
26. Pumera, M. *Chem. Asian J.* **2011**, *6*, 340–348.
27. Lange, S.; Schmidt, P.; Nilges, T. *Inorg. Chem.* **2007**, *46*, 4028–4035.

Chapter 7 –

Cytotoxicity of Vanadium Dichalcogenides

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Cytotoxicity of exfoliated layered vanadium dichalcogenides

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7.1. Introduction

Since the first isolation of graphene from graphite in 2004, there have been many fascinating discoveries made about this novel two-dimensional (2D) material as they get exfoliated from their bulk states.^{1–3} This had spurred exploration on other types of layered materials, from graphene derivatives to black phosphorus, to a wide spectrum of inorganic 2D materials.^{4–6} Amongst them, layered transition-metal dichalcogenides (TMDs) exhibit one of the most favourable electrochemical properties.⁴

TMDs constitute a family of layered materials made up of a transition metal (e.g., Mo, W, V) and a chalcogen (i.e., S, Se, Te) in the formula MX_2 . Each layer consists of a metal sandwiched between two chalcogen atoms in the form of X-M-X. The highly researched members of the family are the Group 6 dichalcogenides, namely MoS_2 , $MoSe_2$ and WS_2 , owing to their promising potential in many energy-related applications,^{4,7} particularly for electrochemical hydrogen production,^{8–13} batteries^{14–17} and capacitors.^{18–20} Additionally, these materials also offer technological advances in a broader spectrum of applications ranging from electronic,^{21–23} optoelectronic,^{24–26} spintronics,^{27,28} solar cells,^{29,30} sensors,^{31,32} biological,^{33,34} biomedical^{35,36} and even lubrication.^{37, 38} In search of more exciting discoveries and better performances, other transition metals were also explored; notably the neighbouring Group 5 vanadium dichalcogenides. They have shown competitive performances for similar applications as their Group 6 counterparts (i.e. electrochemical hydrogen production,^{39–44} batteries^{45–50} and capacitors^{51, 52}).

In spite of their increasing research for possible implementation in the future, little is known about their toxicological effects due to their recent introduction. Even though a number of toxicological studies have been performed on Group 6 TMDs,^{53–55} these studies only account for a small proportion in comparison to the large body of reports dedicated towards actual commercialisation of TMD materials. There is still much to address in understanding the toxicological behaviour of TMD materials especially for Group 5 TMDs, which, to the best of our knowledge, has been unexplored. This gap in

literature needs to be filled to be informed of the potential health implications posed by them in view of their possible realisation in industry.

As such, we set out to investigate the toxicological response of exfoliated vanadium dichalcogenides (VX_2 ; VS_2 , VSe_2 , VTe_2) towards A549 human lung carcinoma epithelial cells. This cell line is commonly used for determining the toxicity of nanomaterials as the respiratory tract is likely to be their first point of entry to the human body. Moreover, being a popular cell line, we can easily compare our results obtained here with many previous reports.⁵⁶ Upon incubation of the VX_2 materials with A549 cells for 24 h, we measured the remaining cell viabilities using two cell viability assays; methylthiazolyldiphenyl tetrazoliumbromide (MTT) and water-soluble tetrazolium assay (WST-8). These two methods were selected to avoid misinterpretation of data and ensure reliability of results if they are in agreement.⁵⁷ Besides that, control experiments were conducted in the absence of cells to check for possible sources of error arising between the test materials and assays used. This is important as there have been several reports of particle-induced interferences between engineered nanomaterials with cell viability assay markers.^{58,59} In view of such interferences, we rinsed the treated cells with phosphate buffer solution (PBS) twice prior to cell viability assay measurements.

Even though toxicity studies pertaining to vanadium dichalcogenides specifically had been elusive before this study, a search on the toxicity of vanadium and its compounds can provide us with clues on their toxicological effects. It has been reported that inhalation of vanadium compounds may result in inflammation of the pharynx, mucous membrane inside the nose, trachea, lungs and bronchi as well as causing chronic productive cough.^{60, 61} Besides that, the workplace exposure limit for vanadium pentoxide had previously been set as 0.05 mg m^{-3} per 8 h exposure by the Health and Safety Executive (HSE) due to many adverse signs pertaining to its toxicity through inhalation.^{62–}
⁶⁴ These findings seem to allude to an inherent toxicity for vanadium compounds.

7.2. Results and Discussion

Exfoliated TMDs can have a wide range of physical and electrochemical properties through different synthesis method. These properties are likely to influence their toxicological behaviour.⁶⁵ As such, it is imperative to characterise the materials under study to properly correlate the toxicity data obtained with possible determining properties of the material.

Material characterisation

Here, we have characterised our test materials using various techniques. As seen in Figure 7.1, the scanning electron microscopy (SEM) and scanning transmission electron microscopy (STEM) images reveal similar layered structures of the three materials (VS_2 , VSe_2 and VTe_2). Additionally, the STEM images provide us a comparison on their extent of exfoliation. From the different shades of black/white observed, we find VS_2 to undergo the highest degree of exfoliation among the three materials, followed by VSe_2 , while VTe_2 shows the least degree of exfoliation. This is further supported by a recent report from Wang et al.⁶⁶ Even though the bulk vanadium dichalcogenides were treated using the same n-butyllithium liquid exfoliation method, they produce varying degrees of exfoliation. This difference in the number of layers could influence their toxicological behaviour as suggested by Chng et al. in a comparative study of various MoS_2 materials exfoliated by different lithium intercalating agents.⁶⁷ In addition, the SEM and STEM images reveal a wide variety of particle sizes ranging from 10 nm to over 1 μm in lateral dimension.

Figure 7.1. SEM (left) and STEM (right) images of exfoliated vanadium dichalcogenides (VS₂, VSe₂ and VTe₂). The scale bars represent 1 μm.

Energy dispersive X-ray spectroscopy (EDS), on the other hand, provides an elemental mapping of the elements present as well as their distribution over the whole material. The EDS images in Figure 7.2 show that the vanadium and chalcogen elements are well distributed on the sheets and that the primary difference between the materials is the chalcogen element. This difference of chalcogen type could be a determining factor in influencing their toxicological effects. Besides that, X-ray diffraction (XRD) conducted for the materials reported that they are free from impurities.⁶⁶

Figure 7.2. EDS images of exfoliated vanadium dichalcogenides (VS₂, VSe₂ and VTe₂). The scale bars represent 10 μm.

Cytotoxicity assessments

After material characterisation, we proceeded to evaluate the toxicological response of these exfoliated vanadium dichalcogenides towards human lung epithelial carcinoma A549 cells. To study this, we exposed A549 cells with the test materials and then measured the remaining cell viabilities. MTT and WST-8 assays were selected as they have been commonly used for in vitro nanotoxicity assessments; this allows easy comparison with other studies.^{68,69} Both assays have similar working principles, whereby they contain active tetrazolium reagents that become reduced in the presence of viable cells to coloured formazan dyes.^{70,71} For the MTT assay, purple insoluble crystals of formazan dye are produced that require an organic solvent, such as dimethyl sulfoxide, for dissolution prior to absorbance measurement.⁶⁸ On the other hand, for WST-8, the

formazan dye is water soluble and therefore its absorbance reading can be measured directly. Since the amount of formazan dye generated can be correlated to cell viability, cytotoxicity data can thus be determined spectroscopically by measuring absorbance.⁶⁸ By normalising the absorbance readings collected with a reference blank control that is not treated with any nanomaterial, we can obtain a measure of cell viability in percentages.

For the MTT assay, the data obtained is shown in Figure 7.3. Here, we observe a dose-dependent toxicological profile in which the cell viability decreases with increasing concentration of the vanadium dichalcogenides tested. This suggests that the materials are toxic. Among the three vanadium dichalcogenides, VS_2 gave the highest cell viability measurements for all the concentrations tested. VSe_2 and VTe_2 , on the other hand, show profiles of relatively similar extents of reduction in cell viabilities. This implies similar toxicities between VSe_2 and VTe_2 . To illustrate, after a treatment of 50 mg mL^{-1} with the exfoliated vanadium dichalcogenides, the cell viability decreases to about 43% for VS_2 , whereas for VSe_2 and VTe_2 , the cell viability reduces to about 9% and 8%, respectively.

Figure 7.3. Cell viability measurements using MTT assay upon 24 h treatment of A549 cells to different concentrations of exfoliated VX_2 nanomaterials.

In addition to MTT, we also assessed the remaining cell viability after nanomaterial exposure using the WST-8 assay. Figure 7.4 presents the data obtained from this measurement. Similar to the toxicological profile seen for MTT, we noted a dose-dependent trend for all the three vanadium chalcogenide materials with VS_2 generally showing higher cell viabilities compared to VSe_2 and VTe_2 . Furthermore, VSe_2 and VTe_2 also displayed similar toxicities as also observed from the MTT cytotoxicity measurements. The general agreement between the two assays ensures reliable interpretation of the cytotoxicity data obtained. This is important as there have been reports of nanomaterial-induced interferences masking cell viability measurements gathered from MTT and WST-8 assays.^{72–76}

Figure 7.4. Cell viability measurements using WST-8 assay upon 24 h treatment of A549 cells to different concentrations of exfoliated VX_2 nanomaterials.

Particle-induced interference investigations

If we take a closer look at the results obtained between the two assays, slight differences can be observed. For example, at the highest concentration of material tested (i.e. 200 mgmL⁻¹) there are almost no A549 cells that remained viable after the 24 h exposure with the test material seen from the WST-8 measurement, whereas MTT still detected a noticeable amount of viable cells. For better clarification of the cell viability data gathered, we set out to conduct control experiments to test the possibility of nanomaterial-induced interferences between the assay reagents and the test materials. This was conducted in the absence of cells.

Figure 7.5 presents data collected from different concentrations of VS₂, VSe₂ and VTe₂ incubation with the MTT assay reagent, normalised to a blank control that does not contain any nanomaterial. For MTT, two kinds of nanomaterial-induced interference effect have been reported.⁷⁵ One possibility is that the nanomaterial itself can reduce the active tetrazolium reagent to form formazan. This can be investigated by incubating different concentrations of the test material with the MTT assay without A549 cells. The results from this experiment, as shown in Figure 7.5A, reveal an increasing amount of formazan generated with increasing dosages of VSe₂ and VTe₂, up to approximately 200% of formazan produced at the highest concentration tested (i.e. 200 mgmL⁻¹). Interestingly, for VS₂, an increasing and decreasing trend is observed instead, with a maximum amount of formazan produced of about 350% at 50 mgmL⁻¹ VS₂ exposure. These results indicate that all the three test materials under study here (i.e., VS₂, VSe₂ and VTe₂) can interact with the MTT assay by reducing its active tetrazolium reagent without the presence of A549 cells.

Figure 7.5. Results from MTT control experiments: (A) Formazan produced upon incubation with varying concentrations of exfoliated VX_2 materials. (B) The extent of binding between insoluble MTT formazan produced with varying concentrations of exfoliated vanadium dichalcogenides.

A second possible nanomaterial-induced interference effect towards MTT assay arises from binding of the test material to the insoluble formazan crystal generated, which can subsequently alter the structure of the complex formed and prevent the dissolution of the formazan crystals produced.⁷⁶ This would result in a false response showing a greater toxicity of the nanomaterial tested. To investigate for this possibility, ascorbic acid was incubated with various concentration of nanomaterial to induce the formation of formazan crystals from MTT reagent. If the nanomaterial can interfere with the MTT assay through the binding effect mentioned above, the percentage of formazan generated should show a decrease with increasing amounts of the material. However, our result (as depicted in Figure 7.5B) shows that the vanadium dichalcogenides tested produce a relatively constant amount of formazan dye thereby indicating insignificant binding effect of the materials.

In the case of WST-8, a similar control experiment was also conducted. However, unlike MTT which produces insoluble formazan crystals, the formazan dye generated by WST-8 is water soluble. As such, there was no need to test for possibility of binding effect of the test material with WST-8 assay. Simply, a control experiment for WST-8 was performed by incubating different concentrations of nanomaterials with the assay reagent to check for possible formazan production ability without presence of cells. The result is presented in Figure 7.6. It is observed that the three materials (VS_2 , VSe_2 and VTe_2) can interfere with the WST-8 assay by reducing its active tetrazolium reagent to formazan crystal. Among them, VTe_2 shows the greatest reducing ability towards WST-8, while VS_2 and VSe_2 demonstrated similar reducing responses with each other.

Figure 7.6. WST-8 control experiments: Formazan produced upon incubation with varying concentrations of exfoliated VX₂ materials.

From these control experiments, we found that the exfoliated VX_2 materials can interfere moderately with both cell viability assay measurements when present at high concentrations (i.e. above 100 mg mL^{-1}) by reducing their active tetrazolium reagent to formazan dye. However, through the thorough washing steps implemented to minimise test materials interference prior to the introduction of assay reagents, we believe that the interference caused by the test materials would be minimised. Even though inevitably there would be some remnants of the materials remaining in the wells after the washing steps, this amount should be significantly reduced to at least half of the original concentration. The slight discrepancies observed between MTT and WST-8 cell viability data could be due to differences in reducing abilities of the materials towards assay reagents. On top of that, different sensitivities arising from different enzymes from viable cells involved in the reaction could also contribute to some variations in the result.⁷⁰

Nevertheless, both assays agree in the general trend obtained, whereby VS_2 showed the least toxicity among the vanadium dichalcogenides tested, while VSe_2 and VTe_2 displayed similar toxicity behaviours.

Comparison with other layered materials

Following the cytotoxicity assessment, the toxicological responses obtained can be compared with other exfoliated transition metal dichalcogenides for better understanding of their relative toxicities. By comparing our data with those reported for a Group 6 transition-metal dichalcogenides (namely MoS_2 , WS_2 and WSe_2) under similar conditions (i.e. 24 h treatment to A549 cells), we found that the Group 5 vanadium dichalcogenides are significantly more toxic in general (Figure 7.7).

Figure 7.7. Comparison of cytotoxicity data between various exfoliated Group 5 and 6 transition-metal dichalcogenides (i.e. MoS₂, WS₂, WSe₂, VS₂, VSe₂, VTe₂) for (A) MTT and (B) WST-8 assay. Data for exfoliated Group 6 dichalcogenides are obtained from reference 54.

Additionally, we also compared the effects of transition metal element as well as the chalcogen type in affecting the toxicological behaviour of TMD materials. For example, between MoS_2 , WS_2 and VS_2 , which share the same chalcogen element, the toxicity profile for VS_2 consistently show higher toxicity for both assays for almost all the concentrations tested. Similarly, for the selenides (i.e. WSe_2 and VSe_2), this is also observed. Since the structures of these layered transition-metal dichalcogenides resemble one another, our findings suggest that vanadium is inherently more toxic compared to molybdenum and tungsten.

Likewise, to compare the effects of chalcogen type towards the toxicity of TMDs, we can look at WS_2 , WSe_2 , and VS_2 , VSe_2 which share the same metal element. From here, we noticed that the metal sulfides show a higher toxicity relative to their corresponding metal selenides. This could imply that sulfides are generally less toxic compared to their selenide counterparts for TMDs. Tellurides, on the other hand, seem to have similar toxicity with selenides. However, this has to be further studied for verification.

Apart from the role of metal and chalcogen elements on toxicity of TMD materials, other factors may also influence their toxicity behaviour. This includes physical properties such as particle size and number of layers.⁷³ Previously, from material characterisation, we found that VS_2 has the highest degree of exfoliation followed by VSe_2 and VTe_2 . Even so, VS_2 presented a lower toxicity compared to VSe_2 . This could be due to varying extent of different factors contributing to the toxicity of a nanomaterial.

In comparison with the parent layered materials (i.e. graphene and its analogues), our group had previously reported that exfoliated TMDs exhibit a lower toxicity profile.³⁵ This comparison was done under similar conditions between exfoliated Group 6 transition-metal dichalcogenides (MoS_2 , WSe_2 and WS_2), graphene oxide prepared by different oxidation methods (Hummers and Hoffman) as well as halogen-doped graphene (Cl and I). However, through new insights from this study, we find that this observation

does not extend to Group 5 vanadium dichalcogenides. By changing the transition metal element from Group 6 to Group 5, the toxicity behaviour of exTMDs can drastically change to show a higher toxicity relative to graphene and its derivatives instead.

7.3. Conclusion

In view of their rising research and possible commercialisation in many applications, we have investigated the cytotoxicity of exfoliated Group 5 vanadium dichalcogenides (VS_2 , VSe_2 and VTe_2). This was conducted by incubating them with A549 cells over a period of 24 h followed by two cell viability measurements (MTT, WST-8). Control experiments performed in the absence of cells showed that all the test materials can moderately interfere with both assay reagents at high concentrations ($>100 \text{ mg mL}^{-1}$). However, thorough washing steps performed prior to cell viability assay measurements should be able to minimise these particle-induced interference effects. The toxicological profile obtained from both assay measurements agree that VS_2 is the least toxic among the three test materials, whereas VSe_2 and VTe_2 present similarly high toxicities with less than 20% cell viability remaining upon 24 h of exposure. In comparison with Group 6 TMDs (namely MoS_2 , WS_2 and WSe_2), we found that Group 5 vanadium dichalcogenides have higher toxicological response towards A549 cells. With this awareness, steps to reduce the toxicity of these TMD materials and/or necessary precautions can be taken to aid their actualisation for real life applications.

7.4. Experimental section

Synthesis of exfoliated vanadium dichalcogenides

The synthesis of vanadium dichalcogenides was performed from elements using quartz glass ampoules. Stoichiometric amounts of vanadium (99.5%, Strem) and chalcogen (sulfur, 99.999% from Strem; selenium, 99.999% from Chempur; tellurium 99.999% from Chempur) for synthesis of 10 g of dichalcogenide were placed in quartz glass ampoule. A 2 wt% excess of chalcogen was added and the ampoule was melt-sealed under vacuum (2×10^{-3} Pa) using an oxygen–hydrogen torch. The ampoule was heated to 400 °C for 24 h, then to 600 °C for 24 h and finally to 800 °C for 48 h. The excess of chalcogen was removed by heating an ampoule in thermal gradient (400 °C and 20 °C) for 1 h. More details on the synthesis were reported in reference 66. The composition of obtained chalcogenides was V_5S_8 , VSe_2 , and VTe_2 .

Exfoliation of TMDs was performed with butyllithium under argon atmosphere. The dichalcogenides (10 mmol) was dispersed in hexane (10 mL) and subsequently butyllithium (10 mL; 2.5 M in hexane) was added. The reaction mixture was stirred for 24 h at room temperature and separated by suction filtration. All operations were performed in a glovebox under an argon atmosphere. The exfoliation was performed by addition of water (5 mL) to the TMDs intercalated by lithium under an argon atmosphere. The suspension of exfoliated TMDs was diluted in water (100 mL) and purified by dialysis. Finally the exfoliated TMDs were separated by suction filtration and dried in vacuum oven at 50 °C for 48 h. The details of exfoliation procedure were reported in reference 66.

Apparatus

SEM, EDS and STEM images were obtained using JEOL 7600F. SEM was performed under SEI mode, 2.00 kV accelerating voltage (AV) and 4.7 mm working distance (WD). Under the same mode, EDS was carried out at 15.00 kV AV and 14.8 mm WD. STEM was conducted under TED mode, 30.00 kV AV and 8.2 mm WD.

A549 cell culture

Human lung carcinoma epithelial cell line (A549, PAA Laboratories) was cultured in Dulbecco's modified eagle medium (DMEM, Life Technologies) with supplementary 10% fetal bovine serum (FBS, Life Technologies) and 1% penicillin/streptomycin (Capricorn). Incubation conditions are 37 °C under 5 % CO₂. Similar incubation conditions were used for all incubation mentioned here, unless otherwise stated. The cells were seeded with a constant cell density (8.8×10^4 cells mL⁻¹) in 24-well plates (570 mL well⁻¹) for good reproducibility.

Cell exposure to vanadium dichalcogenides

After 24h of cell seeding, wells were washed with pH 7.2 phosphate buffer solution (PBS, Life Technologies) and replaced with different concentrations of test materials (570 mL). Cells were not incubated with any nanomaterial for the negative control, whereas for the positive control, cells were exposed to cell culture media with 10 % dimethyl sulfoxide (DMSO, Tedia) without test materials. The wells were then incubated for another 24 h.

Cell viability measurement using MTT and WST-8 assays

After treatment of seeded cells with nanomaterial, the cells were rinsed with PBS twice. Diluted MTT stock solution (1 mg mL^{-1} , Sigma Aldrich) or 10 % WST-8 stock solution (Dojindo Molecular Technologies) was then introduced into each well (300 μL per well) for the respective assays. Subsequently, the wells were incubated for 3 h for MTT and 1 h for WST-8 cell viability measurements. After the 3 h incubation period for MTT assay reagents, the solutions were replaced with dimethyl sulfoxide (DMSO from Tedia, 300 μL well⁻¹) to solubilise any formazan dyes generated. For better dissolution, the plates were shaken (500 rpm, 5 min). Thereafter, the solutions were centrifuged (8000 rpm, 10 min) to separate any VX_2 materials, which may interfere with subsequent absorbance measurement. After the centrifugation step, the supernatant (100 μL) was pipetted into 96-well plate for measurement of absorbance at 570 nm. For WST-8, after the 1 h incubation period, the solution was simply centrifuged and pipetted into 96-well plate for absorbance measurement at 450 nm.

Test for particle interference of test materials with cell viability assays

These control experiments were conducted in the absence of cells to check for possible interference effects. To do this, different test concentrations of the test materials were incubated with the assay reagents for 3 h (MTT) and 1 h (WST-8). After the incubation period, absorbance readings of the assay solutions were measured spectroscopically in a similar manner as the cell viability experiments mentioned above.

For MTT, there is an additional test performed due to possible binding of test material with insoluble formazan crystals produced. To test for this binding effect, 4 mM ascorbic (160 μL per well) was added to the MTT-test material solution (200 μL) after a 3 h incubation to reduce MTT to formazan crystals. The wells were then incubated for another 1 h. Following that, DMSO (720 μL) was introduced to each well. The wells were then left for 10 min at room temperature. Finally, the solution was centrifuged and their supernatant was pipetted for absorbance measurement at 570 nm.

References:

1. Ambrosi, A.; Chua, C. K.; Bonanni, A.; Pumera, M. *Chem. Rev.* **2014**, *114*, 7150–7188.
2. Ambrosi, A.; Chua, C. K.; Latiff, N. M.; Loo, A. H.; Wong, C. H. A.; Eng, A. Y. S.; Bonanni, A.; Pumera, M. *Chem. Soc. Rev.* **2016**, *45*, 2458–2493.
3. Novoselov, K. S.; Fal’ko, V. I.; Colombo, L.; Gellert, P. R.; Schwab, M. G.; Kim, K. *Nature* **2012**, *490*, 192–200.
4. a) Chia, X.; Eng, A. Y. S.; Ambrosi, A.; Tan, S. M.; Pumera, M. *Chem. Rev.* **2015**, *115*, 11941–11966; b) He, Z.; Que, W. *Appl. Mater. Today* **2016**, *3*, 23.
5. a) Ling, X.; Wang, H.; Huang, S.; Xia, F.; Dresselhaus, M. S. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 4523–4530; b) Zhou, J. *Appl. Mater. Today* **2016**, *2*, 24.
6. a) Bhimanapati, G. R.; Lin, Z.; Meunier, V.; Jung, Y.; Cha, J.; Das, S.; Xiao, D.; Son, Y.; Strano, M. S.; Cooper, V. R.; Liang, L.; Louie, S. G.; Ringe, E.; Zhou, W.; Kim, S. S.; Naik, R. R.; Sumpter, B. G.; Terrones, H.; Xia, F.; Wang, Y.; Zhu, J.; Akinwande, D.; Alem, N.; Schuller, J. A.; Schaak, R. E.; Terrones, M.; Robinson, J. A. *ACS Nano* **2015**, *9*, 11509–11539; b) Li, S.; Wang, S.; Tang, D.-M.; Zhao, W.; Xu, H.; Chu, L.; Bando, Y.; Golberg, D.; Eda, G. *Appl. Mater. Today* **2015**, *1*, 60.
7. Pumera, M.; Sofer, Z.; Ambrosi, A. *J. Mater. Chem. A* **2014**, *2*, 8981–8987.
8. Benck, J. D.; Hellstern, T. R.; Kibsgaard, J.; Chakthranont, P.; Jaramillo, T. F. *ACS Catal.* **2014**, *4*, 3957–3971.
9. Zou, X.; Zhang, Y. *Chem. Soc. Rev.* **2015**, *44*, 5148–5180.
10. Vesborg, P. C. K.; Seger, B.; Chorkendorff, I. *J. Phys. Chem. Lett.* **2015**, *6*, 951–957.
11. Voiry, D.; Yamaguchi, H.; Li, J.; Silva, R.; Alves, D. C. B.; Fujita, T.; Chen, M.; Asefa, T.; Shenoy, V. B.; Eda, G.; Chhowalla, M. *Nat. Mater.* **2013**, *12*, 850–855.
12. Yan, Y.; Xia, B.; Li, N.; Xu, Z.; Fisher, A.; Wang, X. *J. Mater. Chem. A* **2015**, *3*, 131–135.
13. Eng, A. Y. S.; Ambrosi, A.; Sofer, Z.; Simek, P.; Pumera, M. *ACS Nano* **2014**, *8*, 12185–12198.

14. Jiang, L.; Lin, B.; Li, X.; Song, X.; Xia, H.; Li, L.; Zeng, H. *ACS Appl. Mater. Interfaces* **2016**, *8*, 2680–2687.
15. Asadi, M.; Kumar, B.; Liu, C.; Phillips, P.; Yasaei, P.; Behranginia, A.; Zapol, P.; Klie, R. F.; Curtiss, L. A.; Salehi-Khojin, A. *ACS Nano* **2016**, *10*, 2167–2175.
16. Liu, Y.; Zhang, N.; Kang, H.; Shang, M.; Jiao, L.; Chen, J. *Chem.-Eur. J.* **2015**, *21*, 11878–11884.
17. Wang, H.; Wang, L.; Wang, X.; Quan, J.; Mi, L.; Yuan, L.; Li, G.; Zhang, B.; Zhong, H.; Jiang, Y. *J. Electrochem. Soc.* **2016**, *163*, A1627–A1632.
18. Balasingam, S. K.; Lee, J. S.; Jun, Y. *Dalton Trans.* **2015**, *44*, 15491–15498.
19. Karade, S. S.; Dubal, D. P.; Sankapal, B. R. *RSC Adv.* **2016**, *6*, 39159–39165.
20. Khalil, A.; Liu, Q.; He, Q.; Xiang, T.; Liu, D.; Wang, C.; Fang, Q.; Song, L. *RSC Adv.* **2016**, *6*, 48788–48791.
21. Wang, Q. H.; Kalantar-Zadeh, K.; Kis, A.; Coleman, J. N.; Strano, M. S. *Nat. Nanotechnol.* **2012**, *7*, 699–712.
22. Schmidt, H.; Giustiniano, F.; Eda, G. *Chem. Soc. Rev.* **2015**, *44*, 7715–7736.
23. Duan, X.; Wang, C.; Pan, A.; Yu, R.; Duan, X. *Chem. Soc. Rev.* **2015**, *44*, 8859–8876.
24. Mak, K. F.; Shan, J. *Nat. Photonics* **2016**, *10*, 216–226.
25. Choi, J.; Zhang, H.; Choi, J. H. *ACS Nano* **2016**, *10*, 1671–1680.
26. Tian, H.; Chin, M. L.; Najmaei, S.; Guo, Q.; Xia, F.; Wang, H.; Dubey, M. *Nano Res.* **2016**, *9*, 1543–1560.
27. Zibouche, N.; Kuc, A.; Musfeldt, J.; Heine, T. *Ann. Phys.* **2014**, *526*, 395–401.
28. Xu, X.; Yao, W.; Xiao, D.; Heinz, T. F. *Nat. Phys.* **2014**, *10*, 343–350.
29. Balis, N.; Stratakis, E.; Kymakis, E. *Mater. Today* **2016**, *19*, 580–594.
30. Yu, X.; Sivula, K. *ACS Energy Lett.* **2016**, *1*, 315–322.

31. Li, B. L.; Wang, J.; Zou, H. L.; Garaj, S.; Lim, C. T.; Xie, J.; Li, N. B.; Leong, D. T. *Adv. Funct. Mater.* **2016**, *26*, 7034–7056.
32. Mayorga-Martinez, C. C.; Ambrosi, A.; Eng, A. Y. S.; Sofer, Z.; Pumera, M. *Adv. Funct. Mater.* **2015**, *25*, 5611–5616.
33. Kalantar-zadeh, K.; Ou, J. Z.; Daeneke, T.; Strano, M. S.; Pumera, M.; Gras, S. L. *Adv. Funct. Mater.* **2015**, *25*, 5086–5099.
34. Nguyen, E. P.; Daeneke, T.; Zhuiykov, S.; Kalantar-zadeh, K. *Curr. Protoc. Chem. Biol.* **2016**, *8*, 97–108.
35. Li, Z.; Wong, S. L. *Mater. Sci. Eng. C* **2017**, *70*, 1095–1106.
36. Chen, Y.; Tan, C.; Zhang, H.; Wang, L. *Chem. Soc. Rev.* **2015**, *44*, 2681–2701.
37. Polcar, T.; Cavaleiro, A. *Surf. Coat. Technol.* **2011**, *206*, 686–695.
38. Yang, J.-F.; Parakash, B.; Hardell, J.; Fang, Q.-F. *Front. Mater. Sci.* **2012**, *6*, 116–127.
39. Yuan, J.; Wu, J.; Hardy, W. J.; Loya, P.; Lou, M.; Yang, Y.; Najmaei, S.; Jiang, M.; Qin, F.; Keyshar, K.; Ji, H.; Gao, W.; Bao, J.; Kono, J.; Natelson, D.; Ajayan, P. M.; Lou, J. *Adv. Mater.* **2015**, *27*, 5605–5609.
40. Qu, Y.; Pan, H.; Kwok, C. T.; Wang, Z. *Nanoscale Res. Lett.* **2015**, *10*, 480–487.
41. Liang, H.; Shi, H.; Zhang, D.; Ming, F.; Wang, R.; Zhuo, J.; Wang, Z. *Chem. Mater.* **2016**, *28*, 5587–5591.
42. Pan, H. *Sci. Rep.* **2014**, *4*, 5348.
43. Qu, Y.; Pan, H.; Kwok, C. T.; Wang, Z. *Phys. Chem. Chem. Phys.* **2015**, *17*, 24820–24825.
44. Chia, X.; Ambrosi, A.; Lazar, P.; Sofer, Z.; Pumera, M. *J. Mater. Chem. A* **2016**, *4*, 14241–14253.
45. Jing, Y.; Zhou, Z.; Cabrera, C. R.; Chen, Z. *J. Phys. Chem. C* **2013**, *117*, 25409–25431.
46. Wang, W.; Sun, Z.; Zhang, W.; Fan, Q.; Sun, Q.; Cui, X.; Xiang, B. *RSC Adv.* **2016**, *6*, 54874–54879.

47. Murphy, D. W.; Carides, J. N.; Salvo, F. J. D.; Cros, C.; Waszczak, J. V. *Mater. Res. Bull.* **1977**, *12*, 825–830.
48. Putungan, D. B.; Lin, S.-H.; Kuo, J.-L. *ACS Appl. Mater. Interfaces* **2016**, *8*, 18754–18762.
49. Sun, R.; Wei, Q.; Li, Q.; Luo, W.; An, Q.; Sheng, J.; Wang, D.; Chen, W.; Mai, L. *ACS Appl. Mater. Interfaces* **2015**, *7*, 20902–20908.
50. Fang, W.; Zhao, H.; Xie, Y.; Fang, J.; Xu, J.; Chen, Z. *ACS Appl. Mater. Interfaces* **2015**, *7*, 13044–13052.
51. Feng, J.; Sun, X.; Wu, C.; Peng, L.; Lin, C.; Hu, S.; Yang, J.; Xie, Y. *J. Am. Chem. Soc.* **2011**, *133*, 17832–17838.
52. Masikhwa, T. M.; Barzegar, F.; Dangbegnon, J. K.; Bello, A.; Madito, M. J.; Momodu, D.; Manyala, N. *RSC Adv.* **2016**, *6*, 38990–39000.
53. a) Wang, X.; Mansukhani, N. D.; Guiney, L. M.; Ji, Z.; Chang, C. H.; Wang, M.; Liao, Y.-P.; Song, T.-B.; Sun, B.; Li, R.; Xia, T.; Hersam, M. C.; Nel, A. E. *Small* **2015**, *11*, 5079–5087;
b) E. L. K. Chng, M. Pumera, *RSC Adv.* **2015**, *5*, 3074.
54. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Chem.-Eur. J.* **2014**, *20*, 9627–9632.
55. Wang, X.; Mansukhani, N. D.; Guiney, L. M.; Ji, Z.; Chang, C. H.; Wang, M.; Liao, Y.-P.; Song, T.-B.; Sun, B.; Li, R.; Xia, T.; Hersam, M. C.; Nel, A. E. *Small* **2015**, *11*, 5079–5087.
56. Love, S. A.; Maurer-Jones, M. A.; Thompson, J. W.; Lin, Y.-S.; Haynes, C. L. *Annu. Rev. Anal. Chem.* **2012**, *5*, 181–205.
57. Monteiro-Riviere, N. A.; Inman, A. O.; Zhang, L. W. *Toxicol. Appl. Pharmacol.* **2009**, *234*, 222–235.
58. Belyanskaya, L.; Manser, P.; Spohn, P.; Bruinink, A.; Wick, P. *Carbon* **2007**, *45*, 2643–2648.
59. Kong, B.; Seog, J. H.; Graham, L. M.; Lee, S. B. *Nanomedicine* **2011**, *6*, 929–941.
60. Ghosh, S. K.; Saha, R.; Saha, B. *Res. Chem. Intermed.* **2015**, *41*, 4873–4897.

61. Costigan, M.; R. Cary, S. Dobson in *Vanadium Pentoxide and Other Inorganic Vanadium Compounds*, World Health Organisation, Geneva, **2001**, pp. 16–17. Available from: <http://www.who.int/ipcs/publications/cicad/en/cicad29.pdf> [Accessed on 19 September 2016].
62. Cooper, R. G. *Indian J. Occup. Environ. Med.* **2007**, *11*, 97–102.
63. International Agency for Research on Cancer (IARC) Monographs, Vanadium Pentoxide, Vol. 86, World Health Organisation, France, 2006, pp. 259–262. Available from: <https://monographs.iarc.fr/ENG/Monographs/vol86/mono86-10.pdf> [Accessed on 19 September 2016].
64. Nemery, B. *Eur. Respir. J.* **1990**, *3*, 202–219.
65. Sohaebuddin, S. K.; Thevenot, P. T.; Baker, D.; Eaton, J. W.; Tang, L. *Part.Fibre Toxicol.* **2010**, *7*, 22–38.
66. Wang, Y.; Sofer, Z.; Luxa, J.; Pumera, M. *Adv. Mater. Interfaces* **2016**, *3*, 1600433.
67. Chng, E. L. K.; Sofer, Z.; Pumera, M. *Nanoscale* **2014**, *6*, 14412–14418.
68. Moravec, R. A.; Niles, A. L.; Duellman, S.; Benink, H. A.; Worzella, T. J.; Minor L. in *Cell Viability Assays* (Eds.: G. S. Sittampalam, N. P. Coussens, H. Nelson et al.), Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda, 2004. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK144065/> [Accessed on 19 September 2016].
69. Richards, D.; Ivanisevic, A. *Chem. Soc. Rev.* **2012**, *41*, 2052–2060.
70. Berridge, M. V.; Tan, A. S.; McCoy, K. D.; Wang, R. *Biochemica* **1996**, *4*, 4–19.
71. Ginouves, M.; Carme, B.; Couppie, P.; Prevot, G. *J. Clin. Microbiol.* **2014**, *52*, 2131–2138.
72. Węrlé-Knirsch, J. M.; Pulskamp, K.; Krug, H. F. *Nano Lett.* **2006**, *6*, 1261–1268.
73. Krug, H. F. *Angew. Chem. Int. Ed.* **2014**, *53*, 12304–12319

74. Alkilany, A. M.; Mahmoud, N. N.; Hashemi, F.; Hajipour, M. J.; Farvadi, F.; Mahmoudi, M. *Chem. Res. Toxicol.* **2016**, *29*, 943–948.
75. Holder, A. L.; Goth-Goldstein, R.; Lucas, D.; Koshland, C. P. *Chem. Res. Toxicol.* **2012**, *25*, 1885–1892.
76. Ong, K. J.; MacCormack, T. J.; Clark, R. J.; Ede, J. D.; Ortega, V. A.; Felix, L. C.; Dang, M. K. M.; Ma, G.; Fenniri, H.; Veinot, J. G. C.; Goss, G. G. *PLoS One* **2014**, *9*, e90650.

Chapter 8 –

Cytotoxicity of Metal Phosphorus Chalcogenides

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Cytotoxicity of layered metal phosphorus chalcogenides (MPX_Y) nanoflakes; FePS₃, CoPS₃, NiPS₃

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8.1. Introduction

Metal phosphorus chalcogenides have a general formula MPX_Y where M is a metal (e.g. Mn, Fe, Co, Ni, Cd), X is a chalcogen (i.e. S or Se) and Y is the number of chalcogen atoms (typically either 3 or 4). Many studies have reported interesting semiconducting, electronic, anisotropic, magnetic, dielectric, structural, and optical properties of these materials when they are exfoliated into few layers.¹⁻¹⁰ Similar to other layered materials such as graphite, transition metal dichalcogenides and black phosphorus, the layers in MPX_Y are held together by weak van der Waals forces and can easily be exfoliated into fewer sheets.¹¹⁻¹⁵ Their structures are also similar whereby within each layer, the metal and phosphorus atoms are sandwiched in between the chalcogen atoms.¹¹ Previously, these MPX_Y materials have been well researched for lithium battery applications dating back to the 1980s.¹⁶⁻²¹ More recently, this class of materials has witnessed a renewed interest as new discoveries unveil a wider scope of their possibilities. Over the past three years, research on $NiPS_3$, $FePS_3$, $CoPS_3$ and their derivatives in particular have been highlighted. For example, our group had reported that $CoPS_3$ and $NiPS_3$ exhibit promising electrocatalytic activity for water splitting (i.e. hydrogen evolution reaction, and oxygen evolution reaction).²² This finding was further supported by studies from Sampath, Xu, Schuhmann and co-workers for $NiPS_3$ and $FePS_3$ with good stabilities of the materials reported.²³⁻²⁶ Additionally, $NiPS_3$ also demonstrated good potential for hydrogen energy storage²⁷ while $FePS_3$ displayed robust intrinsic magnetism for numerous applications.^{22,28}

With increasing research on these materials for technological advances, it is highly possible that they may be commercialized in the future; especially for promising candidates $NiPS_3$, $FePS_3$ and $CoPS_3$. However, with limited knowledge pertaining to their toxicological effects thus far, it is important to fill in this research gap for us to be aware of the hazards that they may pose. Herein, we attempt to address this concern by investigating the cytotoxicity of MPX_Y ; particularly $FePS_3$, $CoPS_3$ and $NiPS_3$ on human lung carcinoma epithelial cells (A549) and normal human bronchial cells (BEAS-2B). To the best of our knowledge, this is the first cytotoxicity study reported for this class of materials.

These cell lines were chosen as the primary route of entry for nanomaterials into our body is likely to be the respiratory tract. Upon exposure of the cells to different concentrations of MPX_Y materials for 24 h, the viability of remaining cells was analysed using water soluble tetrazolium salt (WST-8) assay. The measurements were normalized with respect to a negative control where cells were not treated to any test materials.

8.2. Results and Discussion

Prior to cytotoxicity investigation, it is important to properly characterize the nanomaterials under study to verify the material synthesized and understand the physicochemical properties of the materials due to a wide range of toxicological behaviours reported for different variations of the same type of nanomaterial studied.²⁹ This can be achieved by scanning electron microscopy (SEM), scanning transmission electron microscopy (STEM), energy dispersive X-ray spectroscopy (EDS), high resolution transmission electron microscopy (HR-TEM), dynamic light scattering (DLS), Raman spectroscopy, X-ray powder diffraction (XRD) and X-ray photoelectron spectroscopy (XPS).

SEM images in Figure 8.1 confirm the layered structures of solid FePS₃, CoPS₃ and NiPS₃ samples. NiPS₃ showed a lower degree of exfoliation between layers in comparison with the other two samples. To produce well dispersed solutions of the solids, they were suspended in ultrapure water and sonicated for 3 h. Thereafter, the solutions were characterized by STEM to probe into the morphology and size of the prepared dispersions. This is important as the cytotoxicity assessment would be performed for the materials prepared in solution. From Figure 8.1, we can observe that NiPS₃ generally show thicker structures amongst the three MPX_Y materials studied. This could possibly be due to the smaller interlayer distance in its solid form as seen from the SEM images earlier. In the case of FePS₃ and CoPS₃, few layered sheets can be seen from the STEM images. Besides comparing the degree of exfoliation, we are also able to observe a large spectrum of lateral dimensions of the materials ranging from 1 to 5 μm .

Figure 8.1. Characterization of solid FePS₃, CoPS₃, NiPS₃ by SEM (left) and their liquid dispersions by STEM (right). The liquid suspensions were prepared by dispersing the solid samples in ultrapure water followed by ultrasonication for 3 hours.

Another characterization tool, EDS, was conducted to ascertain the composition and formula of the materials (refer to Figure 8.2). EDS results showed the ratios of P/metal and S/metal are close to the expected ratio of 1:1 and 3:1 (see Table 8.1). Additionally, we have also performed TEM-EDS which show similar results with regards to size and elements present (see Figure 8.S1).

Figure 8.2. Characterization of metal phosphorus chalcogenides under study (FePS_3 , CoPS_3 , NiPS_3) by EDS.

Table 8.1. Results from EDS analysis of the metal phosphorus chalcogenides MPX_Y (FePS_3 , CoPS_3 , NiPS_3) samples. Data below are reported in terms of atomic percentage. P to metal (P/M) and S to metal (S/M) ratios are also calculated.

	Metal (M)	P	S	O	C	P/M	S/M
FePS_3	13.64	13.27	34.83	6.34	31.92	0.97	2.55
CoPS_3	10.86	14.06	34.75	14.11	26.23	1.29	3.20
NiPS_3	10.35	11.93	33.44	2.56	41.72	1.15	3.23

Besides SEM, STEM and EDS, we also characterized the materials using zeta-potential measurement for their surface charges. This is important as surface charge has been reported as a parameter that can influence the toxicity of nanomaterials.³⁰ The surface charges of the MPX_Y nanomaterials suspended in cell culture media are as shown in Table 8.2 and revealed near zero values.

Table 8.2. Results from surface charge analysis of the metal phosphorus chalcogenides MPX_3 (FePS_3 , CoPS_3 , NiPS_3) samples.

	Surface charge (mV)
FePS_3	0.04 ± 0.08
CoPS_3	0.00 ± 0.03
NiPS_3	0.03 ± 0.04

Furthermore, we also conducted HR-TEM on the MPX_3 (FePS_3 , CoPS_3 , NiPS_3) samples (Figure 8.3). The samples consist of individual sheets with sizes up to few microns and typically have few layer thickness. The selected area electron diffraction (SAED) images in inserts shows the hexagonal symmetry of MPS_3 compounds lattice.²² The HR-TEM images in the right column shows the hexagonal arrangement of atoms within MPS_3 structure.

Figure 3. HR-TEM characterization of metal phosphorus chalcogenides under study (FePS_3 , CoPS_3 , NiPS_3).

Apart from the above characterizations, Raman spectroscopy was also performed (Figure 8.4). The Raman spectra corresponds to the typical spectra of MPS_3 compounds with C_2 symmetry consisting from P_2S_6 units.²² In the spectra, A_{1g} modes (590, 387, 255 cm^{-1}) as well as E_g modes (560, 283, 235, 180 and 150 cm^{-1}) were observed. The cation vibration frequencies can be found at low wavenumbers below 150 cm^{-1} .

Figure 8.4. Raman spectroscopy for metal phosphorus chalcogenides under study (FePS₃, CoPS₃, NiPS₃).

Following that, the MPX_Y materials were further characterized using XRD (Figure 8.5). The X-ray diffraction shows presence of single phase corresponding to FePS₃, CoPS₃ and NiPS₃ phases.²² All samples show high preferential orientation towards (00l) reflections due to its layered character.

Figure 8.5. XRD characterization of metal phosphorus chalcogenides under study (FePS_3 , CoPS_3 , NiPS_3).

The bonding state and the chemical composition of MPS_x ($M = \text{Fe, Co, Ni}$) were studied by analysis of XPS spectra. The survey spectrums are available in Figure 8.S2. The high-resolution XPS spectra of metals (Fe 2p, Co 2p, Ni 2p), P 2p, and S 2p of these materials are evaluated (see Figure 8.6). The positions of the respective signals are summarized in Table 8.S1. The high-resolution XPS spectra of metals for FePS_3 , CoPS_3 , and NiPS_3 show both metallic phase of Fe $2p_{(3/2;1/2)}$, Co $2p_{(3/2;1/2)}$, Ni $2p_{(3/2;1/2)}$ and oxidized phase of FeO (Fe $2p_{3/2}$), CoO (Co $2p_{3/2}$), and NiO (Ni $2p_{3/2}$, Ni $2p_{1/2}$).²² The peak fitting of the P 2p high-resolution XPS spectrum could be divided into three major peak components: 131.2 ± 0.2 eV and 131.9 ± 0.1 eV for P $2p_{3/2}$ and P $2p_{1/2}$ attributed to P-S bonding^{31,32} and 134.1 ± 0.5 eV for P $2p_{3/2}$ attributed to P_4O_{10} .³³ Two deconvoluted peaks appear in the S 2p region of all MPX_y . They can be attributed to S $2p_{3/2}$ and S $2p_{1/2}$ with average positions of 161.7 ± 0.2 and 162.9 ± 0.1 eV, respectively, it can be assumed that they confirm the presence of polysulfide.³¹ However, the third peak at position of 164.1 eV can be found in NiPS_3 , this peak corresponds to sulphur.³¹

Figure 8.6. High resolution XPS deconvolution of MPX_Y materials for metal (A), phosphorus (B) and sulphur (C).

For the cytotoxicity assessment of MPX_Y nanomaterials ($FePS_3$, $CoPS_3$ and $NiPS_3$), we considered using two commonly used cell viability assays; methylthiazolyldiphenyl tetrazoliumbromide (MTT) and water soluble tetrazolium salt (WST-8) assays.^{34–36} These assays are made of active coloured tetrazolium reagents that can receive electrons in the presence of viable cells thereby producing a different coloured formazan dye which can be easily measured by absorbance spectroscopy.^{34–36} However, due to reports of possible interferences between nanomaterials with these cell viability assay markers, it is important for us to perform control experiments to ensure reliability of cytotoxicity results.^{37–40} These control experiments were conducted by incubating different concentrations of MPX_Y with the MTT or WST-8 assay reagents in the absence of cells.

Data was normalized to the absorbance reading of a blank control which contains no nanomaterials.

In the case of MTT (see Figure 8.S3), more than 800% of normalized formazan was produced at the highest concentration studied (i.e. 100 $\mu\text{g/ml}$) for FePS_3 and CoPS_3 , whereas for WST-8 (see Figure 8.7), the normalized formazan produced did not exceed 200% throughout the range of concentration tested (0–100 mg/ml). This indicates that between the two assays, WST-8 assay proves to produce less interference with the MPX_γ materials for the range of concentration studied. As such, we proceeded to perform the cytotoxicity assessment using WST-8 assay for this study.

Figure 8.7. Results from control experiments from exposure of MPX_Y materials with WST-8 assay. The results shown are the averaged values with $\pm$ standard deviations obtained from at least three repeat experiments.

Previously, our group had performed two times washing steps with phosphate buffer solution (PBS, pH 7.2) to remove nanomaterials prior to cell viability measurements.^{38,41–47} However, for these samples, we observed that the washing steps were not able to remove traces of nanomaterials efficiently, particularly at the highest concentration of MPX_Y materials tested (i.e. 100 µg/ml). Trace metal analysis conducted by inductively coupled plasma mass spectrometry (ICP-MS) of the solutions removed from wells exposed to 100 µg/ml MPX_Y suspensions revealed that less than 30% of the MPX_Y samples were removed through the washing steps at the highest concentration tested (refer to Table 8.S2). This could be due to adsorption of the MPX_Y materials to A549 cells hindering their complete removal by washing with PBS.

Due to presence of strong nanomaterial-induced interference effects, we decided to explore a slightly different approach in conducting the cytotoxicity assessment in search for a more accurate analysis. For this study, the absorbance readings from control experiments (in the absence of cells) were used as background subtraction from the values obtained with cells and nanomaterials. In this way, any interference caused by the nanomaterials which affects the absorbance readings can be subtracted away from the measurement.

Using this new approach, we found the cell viabilities determined using WST-8 assay upon exposure to different concentrations of MPX_Y samples with A549 cells for 24 h to be as shown in Figure 8.8. All three MPX_Y materials studied (FePS_3 , CoPS_3 , NiPS_3) showed high cell viabilities ($> 75\%$) at low concentrations tested ($0\text{--}25\text{ }\mu\text{g/ml}$). This suggests that these MPX_Y samples are safe to use at such low concentration range. However, at higher concentrations ($\geq 50\text{ }\mu\text{g/ml}$), we start to see the toxic effects of the materials. The toxicological profile revealed CoPS_3 to be the most toxic among the three, followed by FePS_3 while NiPS_3 was found to be the least toxic. This can be clearly seen after incubation of A549 cells with $100\text{ }\mu\text{g/ml}$ of the MPX_Y samples, where cell viabilities reduce to 43%, 26% and 70% for FePS_3 , CoPS_3 and NiPS_3 respectively.

Figure 8.8. Cytotoxicity assessment of MPX_Y (FePS₃, CoPS₃, NiPS₃) samples with A549 cells analyzed by WST-8 cell viability assay. The results shown are the averaged values with $\pm$ standard deviations obtained from at least three repeat experiments.

To confirm the cellular uptake of these nanomaterials, MPX_Y materials penetrating inside A549 cells after 24 h exposure with the materials through microscope imaging (see Figure 8.9) was evaluated. In the cells indicated with red arrow and circles, nanomaterials can be identified to be present inside these cells. Previous reports have shown that cells can engulf nanomaterials through different pathways such as clathrin-mediated endocytosis, caveolae-dependent endocytosis, pinocytosis/macropinocytosis and phagocytosis.⁴⁸ Further studies are required to provide a deeper understanding for this observation.

Figure 8.9. Microscope images of A549 cells after 24 hours incubation with the MPX_Y materials showing penetration of the materials into the cells. The scale bar represents 10 μm .

Even though A549 is a commonly used cell line for nanotoxicology studies, it is a cancerous cell line and may not provide a good representation for the toxicity towards normal cells. To have a better representation of the cytotoxicity of these MPX_Y materials, we also conducted a cytotoxicity assessment of the materials on BEAS-2B human bronchial epithelial cells. The result from this experiment can be seen in Figure 8.10.

Figure 8.10. Cytotoxicity assessment of MPX_Y (FePS₃, CoPS₃, NiPS₃) samples with BEAS-2B cells analyzed by WST-8 cell viability assay. The results shown are the averaged values with $\pm$ standard deviations obtained from at least three repeat experiments.

Here, we also see a similar trend in toxicity; CoPS₃ (most toxic) > FePS₃ > NiPS₃ (least toxic). In comparison between the two cell lines investigated, we observe that BEAS-2B cells are generally more sensitive towards FePS₃ and CoPS₃ as the cell viability values decrease at a much faster rate compared to that seen for A549 cells (Figure 8.8). Interestingly, for NiPS₃, we found that the cell viability values remain above 75% throughout the range of concentration tested (0–100 µg/ml). This implies that NiPS₃ can be a potentially safe and biocompatible material.

Production of reactive oxygen species (ROS) is a common mechanism of cell death induced by nanomaterials by causing oxidative stress and damaging cell components such as proteins, lipids, and nucleic acids.⁴⁹ A nanomaterial's inherently high oxidizing property may result in a higher generation of ROS in the exposed cells, thereby increasing its oxidative stress to lead to higher toxicity. In a previous study, the inherent electrochemistry of these materials have been investigated.²² It revealed that there were strong innate oxidation signals by CoPS₃ while mild oxidation peaks was exhibited by FePS₃ in their first scans. On the other hand, NiPS₃ showed no inherent oxidation peak in its first scan. This suggest that inherently CoPS₃ is more easily oxidized compared to the other two MPX_Y samples. This is followed by FePS₃ while NiPS₃ is the least oxidizing. We find that the inherent oxidizing behavior of these MPX_Y material matches well with our toxicity trend and ROS production is likely to be a mechanism for inducing cell death.

Previously, there have been reports of thicker layers of 2D nanomaterials exhibiting lower toxicity.^{43,50} In this work, we found that NiPS₃, which show a lower degree of exfoliation compared to CoPS₃ and FePS₃ (as seen from STEM characterization) to be the least toxic among the three MPX_Y materials tested. This difference could contribute to NiPS₃ exhibiting a lower toxicity relative to the other two MPX_Y materials studied. Another possible factor that can contribute to the differences in toxicity of nanomaterials is the level of endotoxin contamination in the samples.⁵¹ We had not considered this factor in this study. Future work has to be done to investigate this effect on MPX_Y materials.

When we compare these results with our previous work on other 2D materials tested under similar conditions (see Table 8.3), we observe that the MPX_Y samples (i.e. FePS₃, CoPS₃, NiPS₃) display similar toxicities with transition metal dichalcogenide (TMD, e.g. MoS₂) and black phosphorus (BP) while showing lower toxicity compared to graphene oxide. With many recent reports of TMD and BP in the field of biomedicine, this preliminary result suggests good potential of MPX_Y expanding into such areas too.^{52,53} For future work, it would be interesting to study the toxicity effects of more inorganic metal chalcogenides such as GeSb₂Te₄, Rb₂Mn₃Se₄, and Cs₂Mn₃Se₄ which have yet been investigated.^{54–56}

Table 8.3. Comparison of normalized percentages as determined by WST-8 assay upon treatment of A549 cells for 24 h with 25 µg/ml of FePS₃, CoPS₃, NiPS₃, MoS₂, black phosphorus (BP) and 20 µg/ml of graphene oxide prepared by Hummers oxidation method (GO).

	Cell viability (%)	Reference
FePS₃	92	Our work
CoPS₃	81	Our work
NiPS₃	79	Our work
MoS₂	78	43
BP	81	44
GO	60	41

8.3. Conclusion

As more and more nanomaterials progress from research laboratories into our daily lives, it becomes increasingly important for us to keep up with their toxicity studies to safeguard the public and scientific community. Here, we investigated the cytotoxicity of metal phosphorus chalcogenides MPX_Y (NiPS₃, FePS₃, CoPS₃) nanoflakes on human lung carcinoma A549 cells and normal human bronchial BEAS-2B cells. For both cell lines, we found that CoPS₃ shows toxic behaviour followed by FePS₃ with mild toxicity while NiPS₃ showed the lowest toxicity amongst them. In comparison with other layered materials, MPX_Y samples showed similar toxicities with transition metal dichalcogenides and black phosphorus at low concentrations. Further toxicity assessments need to be conducted to further analyze the impacts of MPX_Y materials to humans and the environment, especially in the areas of understanding their toxicity mechanism and cellular uptake as well as the role of endotoxin contamination in the samples. Nevertheless, this preliminary study is an important first step in shedding light on the toxicity of this class of 2D materials.

8.4. Experimental section

Synthesis of metal phosphorus chalcogenides MPX_Y

The reagents and synthesis procedures for metal phosphorus chalcogenides MPX_Y (M= Fe, Co, Ni) are as reported in reference 22.

Characterizations

The MPX_Y samples were characterized by SEM-EDS and STEM using JOEL 7600F. SEM photographs were collected under gentle-beam (GB-H) setting at 2kV accelerating voltage and working distance of 4.7 mm whereas EDS images were collected using 15kV accelerating voltage and working distance of 14.8 mm. For STEM, the samples were prepared by drop casting 1 mg/mL MPX_Y suspensions in water on STEM Cu grids followed by drying under lamp overnight. ICP-MS was performed using an Agilent 7700x system from Japan. The samples were digested by concentrated nitric acid in a microwave digestion system (Milestone Ethos, Italy) before analysis. X-ray powder diffraction data were collected at room temperature on Bruker D8 Discoverer (Bruker, Germany) powder diffractometer with parafocusing Bragg–Brentano geometry using CuK α radiation (λ = 0.15418 nm, U = 40 kV, I = 40 mA). Data were scanned over the angular range 10–80° (2 θ) with a step size of 0.015° (2 θ). Data evaluation was performed in the software package EVA. inVia Raman microscope (Renishaw, England) in backscattering geometry with CCD detector was used for Raman spectroscopy. DPSS laser (532 nm, 50 mW) with applied power of 5 mW and 50 $\times$ magnification objective was used for the measurement. Instrument calibration was achieved with a silicon reference which gives a peak position at 520 cm⁻¹ and a resolution of less than 1 cm⁻¹. The samples were suspended in deionized water (1 mg/ml) and ultrasonicated for 10 min. The suspension was deposited on a small piece of silicon wafer and dried. High resolution transmission electron microscopy (HR-TEM) was performed using an EFTEM Jeol 2200 FS microscope (Jeol, Japan). A 200 keV acceleration voltage was used for measurement. Elemental maps and EDS spectra were acquired with SDD detector X-MaxN 80 TS from Oxford Instruments

(England). Sample preparation was attained by drop casting the suspension (1 mg mL^{-1} in water) on a TEM grid (Cu; 200 mesh; Formvar/carbon) and dried at 60°C for 12 h. High resolution X-ray photoelectron spectroscopy (XPS) was performed with ESCAProbeP spectrometer (Omicron Nanotechnology Ltd, Germany) with a monochromatic aluminum X-ray radiation source (1486.7 eV). Wide scan surveys of all elements were performed with subsequent high-resolution scans of the Fe 2p, Co 2p, Ni 2p, P 2p, and S 2p. The samples were placed in a conductive carrier made from a high purity silver bar.

MTT control experiment

This was conducted in a similar manner as that for WST-8. However, in this case, 1 mg/ml MTT assay was used, the reaction mixtures were incubation for 3 h and the absorbance readings were measured at 570 nm.

WST-8 control experiment

For cell-free WST-8 control experiments, different concentrations of MPX_y suspensions were incubated with 10 % WST-8 assay in cell culture media for 1 h. Thereafter, the absorbance reading was measured at 450 nm. The results were normalized with a blank control which was not subjected to any test materials.

Cell culture

A549 human lung carcinoma epithelial cells (PAA laboratories) and BEAS-2B human bronchial epithelial cells (ATCC) were cultured in DMEM (Dulbecco's modified eagle medium, Life Technologies) supplemented with 10 % FBS (foetal bovine serum, Life Technologies) and 1% penicillin/streptomycin (Capricorn). The cells were incubated at 37°C under 5 % CO_2 atmosphere.

Cell exposure to MPX_Y

Cells were prepared on ninety-six well plates (five thousand cells/well) overnight. Following that, the cell culture media was removed and replaced with different concentrations of MPX_Y suspensions in cell culture media (0, 3.125, 6.25, 12.5, 50, 100 µg/mL; 100 µl/well). The MPX_Y suspensions in cell culture media were prepared by dilution from 1 mg/mL MPX_Y suspensions in ultrapure water upon sonication for 3 h. A set of positive control containing 10 % DMSO (dimethyl sulfoxide, Tedia) was prepared for every set of experiment to check on the vitality of the cells used.

WST-8 Cell viability assay

Following 24 h exposure of the A549 cells to varying concentrations of MPX_Y suspensions, WST-8 cell viability assay were conducted. 10 µl of WST-8 assay was added directly to each well after the exposure with test materials. Following addition of WST-8 assay, the plates were wrapped in Aluminium foil and incubated at 37 °C and 5 % CO₂ for 1h before absorbance measurement at 450 nm. Absorbance readings obtained from a corresponding set of control experiments were subtracted away from the absorbance readings obtained from wells with cells. Results from these readings were then normalization with the negative control which was not subjected to any test materials.

8.5. Supporting Information

Figure 8.S1. TEM-EDS images of MPX₃ materials confirming presence of metal, sulfur and phosphorus and their identical homogeneous distribution.

Figure 8.S2. XPS survey spectra of MPX_3 materials.

Table S1. Summary of peak positions for XPS deconvolution of high-resolution scans of M 2p, P 2p and S 2p.

MPS_x	Position of M 2p peaks (eV)	Positions of P 2p peaks (eV)	Positions of S 2p peaks (eV)
$FePS_3$	Fe 2p _{3/2} $\approx$ 708.2 Fe 2p _{3/2} $\approx$ 712.4 (FeO) Fe 2p _{1/2} $\approx$ 721.4	P 2p _{3/2} $\approx$ 131.0 P 2p _{3/2} $\approx$ 133.6 (P_4O_{10}) P 2p _{1/2} $\approx$ 131.8	S 2p _{3/2} $\approx$ 161.5 S 2p _{1/2} $\approx$ 162.7
$CoPS_3$	Co 2p _{3/2} $\approx$ 779.3 Co 2p _{3/2} $\approx$ 783.3 (CoO) Co 2p _{1/2} $\approx$ 794.7	P 2p _{3/2} $\approx$ 131.1 P 2p _{3/2} $\approx$ 133.6 (P_4O_{10}) P 2p _{1/2} $\approx$ 131.8	S 2p _{3/2} $\approx$ 161.6 S 2p _{1/2} $\approx$ 162.8
$NiPS_3$	Ni 2p _{3/2} $\approx$ 854.4 Ni 2p _{3/2} $\approx$ 858.4 (NiO) Ni 2p _{3/2} $\approx$ 864.5 Ni 2p _{1/2} $\approx$ 871.9 Ni 2p _{1/2} $\approx$ 876.1 (NiO) Ni 2p _{1/2} $\approx$ 881.8	P 2p _{3/2} $\approx$ 131.4 P 2p _{3/2} $\approx$ 134.5 (P_4O_{10}) P 2p _{1/2} $\approx$ 132.0	S 2p _{3/2} $\approx$ 161.9 S 2p _{3/2} $\approx$ 164.1 (sulphur) S 2p _{1/2} $\approx$ 162.9

Figure 8.S3. Control experiments: Normalized formazan produced upon incubation with varying concentrations of MPX_y materials for MTT assay. The results shown are the averaged values with $\pm$ standard deviations obtained from at least three repeat experiments.

Table 8.S2. Amount of Fe, Ni and Co removed through removal of media and two times washing steps with phosphate buffer solution analysed by ICP-MS from the wells exposed to 100 µg/ml of MPX_y suspensions.

		Fe (ppm)	Co (ppm)	Ni (ppm)
Amount of metal removed by	First removal of media	12.77	24.68	6.42
	First washing step	5.17	2.27	0.41
	Second washing step	2.33	2.03	0.21
Total amount of metal removed through two times washing steps		20.27	28.98	7.04

References:

1. Kuch, W.; Schäfer, R.; Fischer, P.; U. F. Hillebrecht in *Springer Series in Surface Sciences* [Online], Vol. 57, Springer, Heidelberg, **2015**, pp 1–9.
<http://www.springer.com/gp/book/9783662445310> (assessed Sep 18, 2017).
2. Lagadic, I.; Lacroix, P. G.; Clément, R. *Chem. Mater.* **1997**, 9, 2004–2012.
3. Ouvrard, G.; Brec, R.; Rouxel, J. *Mat. Res. Bull.* **1985**, 20, 1181–1189.
4. Brec, R.; Ouvrard, R. G.; Rouxel, J. *Mat. Res. Bull.* **1985**, 20, 1257–1263.
5. Taylor, B. E.; Steger, J.; Wold, A. *J. Solid State Chem.* **1973**, 7, 461–467.
6. Joy, P. A.; Vasudevan, S. *Phys. Rev. B* **1992**, 46, 5425–5433.
7. Zhang, X. et. al. *Adv. Sci.* **2016**, 3, 1600062.
8. Li, X. et. al. *J. Am. Chem. Soc.* **2014**, 136, 11065–11069.
9. Chittari, B. L.; Park, Y.; Lee, D.; Han, M.; MacDonald, A. H.; Hwang, E.; Jung, J. *Phys. Rev. B* **2016**, 94, 184428.
10. Clement, R.; Lomas, L.; Leaustic, A.; Codjovi, E.; Audiere, J. P.; Read, J. A.; Francis A. H. in *Inorganic and Organometallic Polymers with Special Properties* [Online], (Ed.: R. M. Laine), Springer, Netherlands, **1992**, pp. 115-129.
<http://www.springer.com/us/book/9789401051545> (assessed Sep 18, 2017).
11. Du, K. et. al. *ACS Nano* **2016**, 10, 1738–1743.
12. Jariwala, D. et. al. *ACS Nano* **2014**, 8, 1102–1120.
13. Chia, X.; Eng, A. Y. S.; Ambrosi, A.; Tan, S. M.; Pumera, M. *Chem. Rev.* **2015**, 115, 11941–11966.
14. Ambrosi, A.; Chua, C. K.; Latiff, N. M.; Loo, A. H.; Wong, C. H. A.; Eng, A. Y. S.; Bonanni, A.; Pumera, M. *Chem. Soc. Rev.* **2016**, 45, 2458–2493.
15. Gusmão, R. et. al. *Angew. Chem. Int. Ed.* **2017**, 56, 8052–8072.
16. Foot, P. J. S.; Suradi, J.; Lee, P. A. *Mat. Res. Bull.* **1980**, 15, 189–193.
17. Saikh, I.; Julien, C.; Balkanski M. in *Solid State Microbatteries* [Online], (Eds.: J. R. Akridge, M. Balkanski), NATO ASI Series (Series B: Physics), Springer, United States, **1990**, pp. 297–300. <http://www.springer.com/gp/book/9780306435058> (assessed Sep 18, 2017).
18. Foot, P. J. S.; Katz, T.; Patel, S. N.; Nevett, B. A.; Piecy, A. R.; Balchin, A. A. *Phys. Stat. Sol. A* **1987**, 100, 11–29.

19. Thompson, A. H.; Whittingham, M. S. *Mater. Res. Bull.* **1977**, *12*, 741–744.
20. Foot, P. J. S.; Suradi, J.; Lee, P. A. *Mat. Res. Bull.* **1980**, *15*, 189–193.
21. Raymond, B. R. E. C. *Solid State Ionics* **1986**, *22*, 3–30.
22. Mayorga-Martinez, C. C. et. al. *ACS Appl. Mater. Interfaces* **2017**, *9*, 12563–12573.
23. Jenjeti, R. N.; Austeria, M. P.; Sampath, S. *ChemElectroChem* **2016**, *3*, 1392–1399.
24. Li, K.; Rakov, D.; Zhang, W.; Xu, P. *Chem. Commun.* **2017**, *53*, 8199–8202.
25. Konkena, B.; Masa, J.; Botz, A. J. R.; Sinev, I.; Xia, W.; Koßmann, J.; Drautz, R.; Muhler, M.; Schuhmann, W. *ACS Catal.* **2017**, *7*, 229–237.
26. Mukherjee, D.; Austeria, P. M.; Sampath, S. *ACS Energy Lett.* **2016**, *1*, 367–372.
27. Ismail, N.; Madian, M.; El-Meligi, A. A. *J. Alloys Compd.* **2014**, *588*, 573–577.
28. Lee, J.; Lee, S.; Ryoo, J. H.; Kang, S.; Kim, T. Y.; Kim, P.; Park, C.-H.; Park, J.-G.; Cheong, H. *Nano Lett.* **2016**, *16*, 7433–7438.
29. Fojtů, M. et. al. *Environ. Sci.: Nano* **2017**, DOI: 10.1039/c7en00401j.
30. Sharifi, S.; Behzadi, S.; Laurent, S.; Forrest, M. L.; Stroeve, P.; Mahmoudi, M. *Chem. Soc. Rev.* **2012**, *41*, 2323–2343.
31. Lau, W. M.; Jin, S.; Wu, X.-W. *J. Vac. Sci. Technol. B* **1990**, *8*, 848–855.
32. Zhang, J.; et al. *J. Mater. Chem. A* **2017**, *5*, 23536–23542.
33. Qi, Z.; Lee, W. *Tribol. Int.* **2010**, *43*, 810–814.
34. Riss, T. L.; Moravec, R. A.; Niles, A. L.; Duellman, S.; Benink, H. A.; Worzella, T. J.; Minor L. in *Assay Guidance Manual* [Online], (Eds.: G. S. Sittampalam, N. P. Coussens, K. Brimacombe, et al.), Bethesda (MD), Eli Lilly & Company and the National Center for Advancing Translational Sciences, **2004**.
<https://www.ncbi.nlm.nih.gov/books/NBK92019/> (assessed Sep 18, 2017).
35. Präbst, K.; Engelhardt, H.; Ringgeler, S.; Hübner H. in *Cell Viability Assays* [Online], (Eds.: D. F. Gilbert, O. Friedrich), Springer Nature, New York, **2017**, pp. 1–17.
https://link.springer.com/protocol/10.1007%2F978-1-4939-6960-9_1 (assessed Sep 18, 2017).
36. Tominaga, H.; Ishiyama, M.; Ohseto, F.; Sasamoto, K.; Hamamoto, T.; Suzuki, K.; Watanabe, M. A. *Anal. Commun.* **1999**, *36*, 47–50.
37. Wörle-Knirsch, J. M.; Pulschke, K.; Krug, H. F. *Nano Lett.* **2006**, *6*, 1261–1268.
38. Latiff, N.; Teo, W. Z.; Sofer, Z.; Huber, Š.; Fisher, A. C.; Pumera, M. *RSC Adv.* **2015**, *5*,

67485–67492.

39. Dhawan, A.; Sharma, V. *Anal. Bioanal. Chem.* **2010**, *398*, 589–605.
40. Liang, L.; Cui, M.; Zhang, M.; Zheng, P.; Deng, Z.; Gao, S.; Wang, X.; Zhang, X.; Wang, C.; Liu, Y.; Xie, L. *RSC Adv.* **2015**, *5*, 67327–67334.
41. Chng, E. L. K.; Pumera, M. *Chem. –Eur. J.* **2013**, *19*, 8227–8235.
42. Latiff, N. M.; Sofer, Z.; Fisher, A. C.; Pumera, M. *Chem. –Eur. J.* **2017**, *23*, 684–690.
43. Chng, E. L. K.; Sofer, Z.; Pumera, M. *Nanoscale* **2014**, *6*, 14412–14418.
44. Latiff, N. M.; Teo, W. Z.; Sofer, Z.; Fisher, A. C.; Pumera, M. *Chem. –Eur. J.* **2015**, *21*, 13991–13995.
45. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Chem. –Eur. J.* **2014**, *20*, 9627–9632.
46. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Nanoscale* **2014**, *6*, 1173–1180.
47. Chng, E. L. K.; Zhao, G.; Pumera, M. *Nanoscale* **2014**, *6*, 2119–2124.
48. Zhao, F.; Zhao, Y.; Liu, Y.; Chang, X.; Chen, C.; Zhao, Y. *Small* **2011**, *7*, 1322–1337.
49. Fu, P. P.; Xia, Q.; Hwang, H. -M.; Ray, P. C.; Yu, H. *J. Food Drug Anal.* **2014**, *22*, 64–75.
50. Rasulev, B.; Gajewicz, A.; Puzyn, T.; Leszczynska, D.; Leszczynski J. in *Towards Efficient Designing of Safe Nanomaterials: Innovative Merge of Computational Approaches and Experimental Techniques* [Online], (Eds.: J. Leszczynski, T. Puzyn, T.), RSC Publishing, United Kingdom, **2012**, pp. 220–256.
<http://pubs.rsc.org/en/content/chapter/bk9781849734530-00220/978-1-84973-453-0>
 (assessed Sep 18, 2017).
51. Li, Y.; Boraschi, D. *Nanomedicine (Lond.)* **2016**, *11*, 269–287.
52. Kalantar-zadeh, K.; Ou, J. Z.; Daeneke, T.; Strano, M. S.; Pumera, M.; Gras, S. L. *Adv. Funct. Mater.* **2015**, *25*, 5086–5099.
53. Fojtů, M.; Chia, X.; Sofer, Z.; Masařík, M.; Pumera, M. *Adv. Funct. Mater.* **2017**, *27*, 1701955.
54. Hardtdegen, H.; Rieß, S.; Schuck, M.; Keller, K.; Jost, P.; Du, H.; Bornhöfft, M.; Schwedt, A.; Mussler, G.; Ahe, M. V.; Mayer, J.; Roth, G.; Grützmacher, D.; Mikulics, M. *J. Alloys Compd.* **2016**, *679*, 285–292.
55. Hardtdegen, H.; Mikulics, M.; Rieß, S.; Schuck, M.; Saltzmann, T.; Simon, U.; Longo, M. *Prog. Cryst. Growth Charac. Mater.* **2015**, *61*, 27–45.
56. Bronger, W.; Hardtdegen, H.; Kanert, M.; Müller, P.; Schmitz, D. Z. *Anorg. Allg. Chem.* **1996**, *622*, 313–318.

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Chapter 9 –

Cytotoxicity of Black Phosphorus and Its Allotropes

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Part of the writing presented in this chapter was published in the following journal article:

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9.1. Introduction

Research on 2D layered materials continues to fascinate the scientific community with one of the latest trends showing a recent wave of publications on black phosphorus (BP). Similar to graphite, black phosphorus consists of a single element and exhibits many exciting properties as the bulk states are exfoliated into single/few-layers.^{1,2} BP hold several advantages over graphene and transition metal dichalcogenides (e.g. MoS₂, MoSe₂, WS₂) in terms of bandgap and hole mobility. With many distinguished properties, it is not surprising that there are numerous reports on BP and its exfoliated forms displaying great potential in diverse applications ranging from ultrafast electronics, high frequency optoelectronics, telecommunications, thermal imaging, field effect transistors, photodetectors, sensing, photothermal therapy, theranostic nanomedicine, energy storage/conversion and more.³⁻¹⁴ In view of their promising prospects for application in real life, it is important for us to be aware of the possible hazards they can pose to humans and the environment.¹⁵ This is especially true for new materials such as black phosphorus where literature pertaining to their toxicological behaviour is still in its infancy.

Previously, our group had reported the toxicity of bulk BP synthesized through vapour phase growth method from red phosphorus and found it be relatively toxic.¹⁶ The vapour phase growth method of black phosphorus is a kinetically controlled and low-pressure route from red phosphorus, with Sn/SnI₄ as a mineralization additive.^{2,12} Hittorf's phosphorus, also known as violet phosphorus, has been reported as the intermediate in the vapour phase growth method.¹⁷ To the best of our knowledge, the effect of different synthesis methods on the toxicity effects of BP has not yet been explored. With many aspects that still remain unclear pertaining to potential health hazards of these new materials, more toxicity assessments are needed. In this report, we attempt to address this concern by studying another synthesis method of BP via high pressure conversion from red phosphorus (BP HPC) which is an older method and requires more energy consumption relative to the vapour phase growth method (BP VPG). In order to better understand the effect of synthesis method on the toxicity of

phosphorus materials, we have also included red phosphorus (RP) and violet phosphorus (VP) in our investigation. This would enable us to track the changes in toxicity that occurs as the precursor material transforms into BP HPC and BP VPG. These materials are all allotropes of phosphorus with structures as depicted in Scheme 9.1.

Scheme 9.1. Crystal structures of different P allotropes.

Human lung carcinoma epithelial (A549) cells are chosen as the respiratory tract is likely to be the first point of entry for nanomaterials through inhalation.^{18–24} Upon 24 h treatment of the cells with different concentrations of the test materials, the remaining cell viability was determined using methylthiazolyldiphenyltetrazolium bromide (MTT) and watersoluble tetrazolium salt (WST-8) assays. Two assays were used to ensure

reliable data. Additionally, control experiments in the absence of cells were performed to monitor for possible interferences between the phosphorus materials and the assays used.

9.2. Results and Discussion

Before cytotoxicity assessment, it is important to characterize the materials first to understand their properties. The solid P allotrope materials synthesized were characterized using scanning electron microscopy (SEM) to study their morphology (Figure 9.1). Plate-like and distinct layered structures were observed in BP VPG while this was less clearly seen in the case of BP HPC. On the other hand, VP showed layered and ribbon-like structures whereas RP appeared like stones without observation of layered structures. Interestingly, we see different morphologies for the different phosphorus allotropes and BP materials.

Figure 9.1. SEM images of black phosphorus synthesized by vapour phase growth (BP VPG), black phosphorus synthesized by high pressure conversion (BP HPC), violet phosphorus (VP) and red phosphorus (RP) used in this study.

To prepare homogenous liquid suspensions, the solid materials were dispersed in ultrapure water and sonicated for 6 h. The obtained materials were further characterized for their structure, sizes, surface charge, bonding arrangement and composition by scanning transmission electron microscopy (STEM), transmission electron microscopy (TEM), dynamic light scattering (DLS), ζ -potential measurements, Raman spectroscopy and X-ray photoelectron spectroscopy (XPS).

As seen from STEM images in Figure 9.2, the sonication process results in exfoliation of BP VPG into few layer sheets of lateral sizes in the nano and micrometre range. However, for the other phosphorus materials, thin sheets were not observed. Instead, the materials appeared to be broken down into smaller pieces of lateral sizes in the nanometer and micrometre range.

Figure 9.2. STEM images of P materials under study in low (left) and high (right) magnifications. The scale bars represent 1 μm .

Subsequently, DLS was conducted to determine the size distribution and the ζ -potential of the P materials. These parameters have been reported to influence the cytotoxicity of nanomaterials.²⁵ The size distribution for the P materials are as shown in Supporting Information Figure 9.S1. The sizes for the materials generally range between 200 nm and 1 μ m. We find that the ζ -potential for all the P materials under study are close to zero (Table 9.S1).

Following that, Raman spectroscopy was performed for the four materials evaluated in this study (Figure 9.3). The Raman spectra of BP VPG and BP HPC show characteristic peaks of A_{1g} , B_{2g} and A_{2g} at approximately 360 cm^{-1} , 440 cm^{-1} and 465 cm^{-1} respectively.^{2,3,26,27} The higher intensity ratio of A_{2g} to A_{1g} modes in BP VPG (2.46 ± 0.24) relative to BP HPC (2.09 ± 0.09) suggests a decrease in number of layers as suggested from other studies.^{2,28} Also, broader A_{1g} , B_{2g} , and A_{2g} peaks of BP HPC indicate larger number of layers relative to BP VPG as associated in another study²⁹, in agreement with the STEM results. A complex Raman spectrum of VP and RP were observed similar to previous studies.^{4,30,31} The complexity arises from presence of multiple vibration modes due to low symmetry and numerous different crystallographic atoms present in the unit cell.²⁹

Figure 9.3. Raman spectra of black phosphorus synthesized by vapour phase growth (BP VPG), black phosphorus synthesized by high pressure conversion (BP HPC), violet phosphorus (VP) and red phosphorus (RP).

X-ray photoelectron spectroscopy (XPS) was later performed to analyze the composition of the phosphorus materials. Figure 9.4 shows the high resolution XPS spectra of the samples. Two peaks, P 2p_{3/2} and P 2p_{1/2} doublet, were seen at 129.7 ± 0.6 eV and 130.6 ± 0.6 eV respectively which are characteristic peaks of crystalline BP.³² An oxidized phosphorus (PO_x) peak is apparent at 134 ± 0.6 eV which could be formed through oxidation in water and ambient conditions.³³ The different samples show varying amounts of oxidations in comparison with the P 2p peaks.

Figure 9.4. High resolution P 2p X-ray photoelectron spectroscopy (XPS) spectra of the P materials used in this study.

To observe this difference better, we calculated the ratio of areas under the PO_x peak to the sum of P 2p peaks (i.e. PO_x/P ratio) and presented them in Table 9.1. Among the P allotropes studied here, the two BP materials show higher contents of PO_x compared to VP and RP. Between BP VPG and BP HPC, we found BP VPG to have higher amounts of oxidized PO_x species. In addition, the EDX mapping from TEM images (Figure 9.S2 in SI) were performed showing the high oxidation degree of BP VPG with regards to the other P allotropes under study.

Table 9.1. Comparison for ratio of oxidized phosphorus (PO_x) to phosphorus determined from XPS deconvolution of high-resolution spectrum of P 2p core level for black phosphorus synthesized by vapour phase growth (BP VPG), black phosphorus synthesized by high pressure conversion (BP HPC), violet phosphorus (VP) and red phosphorus (RP).

P material	PO_x/P ratio
BP VPG	1.09
BP HPC	0.70
VP	0.31
RP	0.21

To further support the trends seen for oxidation of the different phosphorus allotropes, we studied the intrinsic redox properties of the materials using cyclic voltammetry (CV) in N_2 purged 50 mM phosphate buffer solution (PBS, pH 7.2). The recorded CVs in anodic and cathodic direction for phosphorus allotropes were shown in Figure 9.5. The BP VPG and BP HPC show an oxidation peak around 0.5V that corresponds to the oxidation of P^0 to P^{5+} .^{34,35}

Figure 9.5. Inherent electrochemistry of black phosphorus synthesized by vapour phase growth (BP VPG), black phosphorus synthesized by high pressure conversion (BP HPC), violet phosphorus (VP) and red phosphorus (RP) performed in the anodic direction (left) and cathodic direction (right). Conditions used: 50 mM phosphate buffer solution (PBS) pH 7.2 at a scan rate of 100 mV/s. The arrows indicate the scan direction.

Interestingly in the case of BP VPG, the intensity of the oxidation peak enhances if the CV was recorded in cathodic direction. This was not seen in BP HPC, and this is in good agreement with the XPS data which showed BP VPG having higher surface oxidation compared to BP HPC. In this way, the high pressure conversion method used for synthesizing BP enhance the stability and decrease the passivation observed in BP synthesized by vapour phase growth after the sonication process.

After material characterization, we proceeded to toxicity investigation. In our previous study, BP was found to interfere with the WST-8 and MTT assay by reducing the active tetrazolium salts reagents to the formazan products which are generated by metabolically active cells, thereby giving false negative results.¹⁶ Also, MTT assay can bind with the formazan crystal produced to cause an overestimation in cell viability readings. To minimize these interferences, washing steps with PBS were implemented in that study to remove the phosphorus materials prior to addition of the cell viability assay reagents.

Here, we modified the previous method to minimize these nanomaterial-induced effects by studying the lower concentration range (3.125–25 $\mu\text{g/ml}$) where the interference effects are comparably smaller (see Figure 9.6). These control experiments were carried out by incubating the phosphorus materials with the MTT and WST-8 assay in the absence of cells. Data was normalized to a blank control which does not contain any phosphorus materials. At these low concentration range, the percentage of control formazan produced was reduced significantly to below 200% as compared to 800% as seen from our previous study for BP VPG (tested between 3.125 and 400 $\mu\text{g/ml}$).

Figure 9.6. Control experiments (in the absence of cells) to check for interferences between (A) MTT and (B) WST-8 cell viability assays with black phosphorus synthesized by vapour phase growth (BP VPG), black phosphorus synthesized by high pressure conversion (BP HPC), violet phosphorus (VP) and red phosphorus (RP).

Moreover, for a more accurate analysis, we used the data obtained from control experiments as a blank subtraction for analyzing the results collected from experiments with cells. This would enable us to reduce any nanomaterial-induced interference including possible binding interaction between nanomaterials with MTT formazan crystals for the cytotoxicity evaluation.

Using these modifications to the previous method, results from the cytotoxicity assessment (Figure 9.7) reveal that BP VPG has higher toxicity compared to BP HPC. To illustrate, BP VPG shows ca. 20–30% lower viable cells than BP HPC upon 25 µg/ml BP tested according to both MTT and WST-8 assays, despite its greater ease while sharing the same precursor (RP). Interestingly starting precursor material (RP) show relatively low toxicities with cell viability values remaining above 75% upon 24 h treatment of 25 µg/ml RP. These trends are in agreement for both the MTT and WST-8 assay. These finding suggest that the toxicity of BP is contributed by synthesis processes involved during the conversion from RP to BP HPC and BP VPG. To confirm this assertion, another allotrope VP that is an intermediate material for BP synthesized by vapour phase growth synthesis was studied as well. This P allotrope shows low toxicities with cell viability values remaining above 70% upon 24 h treatment of 25 µg/ml VP, demonstrating that the higher toxicity of BP VPG is not related to the toxicity of VP. In view of the different reagents involved during the synthesis process of BP VPG and BP HPC, one may think that SnI_4 introduced through the vapour growth phase synthesis method could possibly contribute to the higher toxicity of BP VPG. However, the synthesis method of VP also involves SnI_4 and yet we found VP to be a relatively safe material. This hints that there are other factors apart from metal content which play a greater role in influencing the toxicity of these phosphorus materials.

Figure 9.7. Cytotoxicity assessment of black phosphorus synthesized by vapour phase growth (BP VPG), black phosphorus synthesized by high pressure conversion (BP HPC), violet phosphorus (VP) and red phosphorus (RP) using (A) MTT assay and (B) WST-8 assay.

To further verify our results, we also performed staining of the live and dead cells using calcein and ethidium homodimer respectively, after treatment with the P materials (Figure 9.8). We found a similar trend where BP VPG shows the highest ratio of dead to live cells, followed by BP HPC and VP, while RP shows the lowest ratio of dead/live cells.

Figure 9.8. Live/Dead staining of A549 cells after 24 h exposure to 25 µg/ml of the various P materials under study. Live cells were stained green using calcein while dead cells were stained red using ethidium homodimer. The scale bars represent 50 µm.

Comparing the trend of toxicological behaviour towards A549 cells with the material characterization results, there appears to be a correlation between a higher degree of exfoliation and higher PO_x/P content to higher toxicity of phosphorus materials. The differences in degree of exfoliation is likely to arise from differences in morphology of the materials. BP VPG shows distinct layered structures from SEM imaging (see Figure 9.1) unlike the other phosphorus materials studied. Here, it is important to highlight that only BP VPG shows exfoliated nanosheets structures after the sonication process (see Figure 9.2 and Figure 9.S1). It is well known that liquid phase exfoliation method oxidizes and degrades BP.³⁴ However, thinner sheets of layered materials has been reported to have increased toxicological effects.³⁵

The correlation between toxicity and oxidation degree is similar to a previous report on cytotoxicity of graphene oxide (GO) prepared by different oxidation methods used, i.e. Staudenmaier, Hummers, Hoffman and Tours methods. In that study, Chng et al. found that cytotoxicity of the GO materials was influenced by their C/O ratios and C=O contents.³⁶ The Hummers and Tours oxidation methods, which involved permanganate treatment, result in a greater extent of oxidation and produced GO materials with a stronger toxic effect on the A549 cells. On the other hand, the Staudenmaier and Hoffman oxidation methods, which involved chlorate treatment, produced GO materials with lower oxygen contents and lower toxicities. This similarity is interesting as both graphite and black phosphorus are layered materials that consist of only one element, unlike other layered materials such as transition metal dichalcogenides.

Moreover, a higher oxidative activity seen from inherent electrochemistry analysis could indicate higher potential for the material to produce oxygen radicals. This has been associated in a previous study which found ZnO nanoparticles to have higher chemical activity relative to silicon oxide nanoparticles and resulted in higher levels of oxidative stress produced.³⁷ With many in vitro studies of BP reporting the production of reactive oxygen species (ROS) as a mechanism to induce apoptosis, the higher chemical

activity of BP VPG particles is likely to be the reason for its higher cytotoxicity behaviour observed.^{38,39}

To ascertain whether the mechanism for cell death is by ROS production, we measured the ratio of the reduced to oxidized forms of glutathione (GSH/GSSG). A decrease in GSH/GSSG ratio has been widely reported to be a useful indicator for oxidative stress.⁴⁰ We found this value to decrease from 65 for the negative control (without test materials) to 11, 13, 19 and 26 after exposure to 25 $\mu\text{g}/\text{ml}$ of BP VPG, BP HPC, VP and RP respectively (Figure 9.9). This trend correlates well with the toxicity results obtained in Figure 9.7, thereby suggesting that the generation of ROS is a cause for cell death induced by the P materials.

Figure 9.9. GSH/GSSG ratio determined for A549 cells after 24 h treatment with 25 $\mu\text{g}/\text{ml}$ of different P materials.

Apart from finding a mechanism for cell death, we also investigated the cell apoptosis process using a real-time assay that measures the presence of phosphatidylserine on the outer layer of cell membranes during apoptosis, through binding of annexin V which produces a luminescence signal. Necrosis process was also detected concurrently using a cell-impermeant fluorescent DNA dye for simultaneous real-time monitoring of apoptotic progression. The results as shown in Figure 9.10 revealed signs of early apoptosis when A549 cells were treated with 25 $\mu\text{g}/\text{ml}$ of the different P allotrope materials, as evident from the increase in luminescence signal while the fluorescence signal remains low.

Figure 9.10. Real-time monitoring of net luminescent (A) and fluorescent (B) signals measured for A549 cells treated with 25 g/ml of BP VPG, BP HPC, VP and RP over 24 h, reported in relative luminescence units (RLU) and relative fluorescence units (RFU), respectively.

Besides A549 cells, we have also tested the P materials with other cell lines such as human embryonic kidney cells (HEK293), human cervical carcinoma cells (HeLa), human breast cancer cells (MCF7) and human liver carcinoma cells (HepG2) with WST-8 assay to extend the scope of this study (see Supporting Information Figure 9.S3). Interestingly, we did not find a similar trend as seen for A549. This may be due to different sensitivities of the different cell lines.

We compared our data here with our previous work of other layered materials tested under similar conditions (Table 9.2). However, it should be noted that the degree of exfoliation varies for the different samples depending on the exfoliation method used and the interlayer distance between layers of the material. It has been reported by several studies that size, thickness, shape, oxygen content, surface charges and elements of nanomaterials can greatly influence the toxicological property of nanomaterials.^{35,36,39,41–44} The large number of possible factors that can vary makes good comparison between different reports challenging. Further and systematic studies have to be conducted to further elucidate this evaluation. Generally, our finding shows that BP can have large differences in toxicity depending on the synthesis method used.

Table 9.1. Comparison of cytotoxicity of nanomaterials using WST-8 assay for cell viability measurement upon treatment of 25 µg/ml of material for 24 h. GO-HU represents graphene oxide synthesized by Hummers oxidation method. Hummers oxidation method is chosen over the other methods as it is the more commonly used oxidation method seen in literature.^{45,46}

Material	Cell viability (%)	Reference
BP VPG	51	This work
BP HPC	83	This work
VP	92	This work
RP	92	This work
GO-HU	60	36
MoS ₂	78	35

9.3. Conclusion

With rising interest in research on allotropes of phosphorus, especially for black phosphorus, there is great possibility of these materials entering the commercial market in the future. However, as new materials, more studies are required to better understand the toxicological effects of black phosphorus. Here, we investigated the cytotoxicity of black phosphorus synthesized by vapour phase growth and high pressure conversion, as well as their precursor red phosphorus. The intermediate for vapour phase growth synthesis method, violet phosphorus, was also included for a more complete study. We found black phosphorus synthesized by vapour growth transport have higher toxicity effects compared to black phosphorus produced via high pressure conversion. In comparison with black phosphorus, red and violet phosphorus show relatively low toxicities. Even though both black and violet phosphorus were synthesized from red phosphorus, they have significantly different toxicities. These findings demonstrate how synthesis methods can greatly influence the properties of the BP materials produced as well as its toxicological effects. We find that the toxicity of phosphorus materials is likely to be influenced by their degree of exfoliation and extent of oxidation. BP VPG with thinner sheets was observed to be more toxic compared to BP HPC with thicker structures. Furthermore, our results showed that phosphorus materials with higher content of oxidation resulted in higher toxicity profiles. This preliminary study advances our understanding pertaining to toxicity of phosphorus materials to prepare for their actual commercialization in real life.

9.4. Experimental section

Synthesis of different P allotropes

The materials were synthesized following reference 33:

Black phosphorus materials were prepared by vapour phase growth (BP VPG) and high pressure conversion (BP HPC). For the former method (BP VPG), 500 mg of Au/Sn alloy, 15 mg SnI_4 and 720 mg of red phosphorus were placed in a quartz ampoule and sealed using an oxygen/hydrogen torch. The Au/Sn alloy was prepared by melting stoichiometric amounts of tin (Sigma–Aldrich, Czech Republic) and gold (Safina, Czech Republic) under high vacuum while SnI_4 was prepared by refluxing iodine (Penta, Czech Republic) and tin in chloroform (Penta, Czech Republic), and purification by recrystallization from chloroform. Following that, the ampoule was heated using a muffle furnace to 400 °C for 1 h and then left at these conditions for another 2 h. Subsequently, the temperature was raised to 600 °C for one day (24 h) and cooled overnight to room temperature. The BP plates formed were washed with CS_2 (Penta, Czech Republic) to remove traces of white phosphorus produced during synthesis. For the latter method (BP HPC), 10 g of red phosphorus (Sigma–Aldrich, Czech Republic) was wrapped in graphite foil and pressurized to 6 GPa and heated to 600 °C. Later, the product was cooled slowly to room temperature and removed from the graphite foil mechanically.

For violet phosphorus, it was synthesized by vapour transport of 1 g of red phosphorus (Sigma–Aldrich, Czech Republic) with 40 mg of Sn and 20 mg of SnI_4 in a quartz glass ampoule. The ampoule was then placed in a muffle furnace for heating to 600 °C, at a heating rate of 5 °C/min. After 24 h, it was then cooled to room temperature at a cooling rate of 1 °C/min. Violet phosphorus was produced in the middle part of the ampoule while mixture of violet and black phosphorus was formed at the end of the ampoule. The product was washed with CS_2 to remove traces of white phosphorus produced during synthesis.

To prepare their liquid dispersions, the solid materials were suspended in ultrapure water to achieve a concentration of 1 mg/ml followed by a 6 h sonication process.

Characterization

Size and morphology characterization of phosphorus allotropes were conducted by SEM using JOEL 7600F. Chemical composition was characterized using XPS (Phoibos 100 spectrometer, SPECS, Germany) with a monochromatic Mg X-ray radiation source. Raman analysis was performed by LabRam HR instrument (Horiba Scientific). DLS measurements were made using Zetasizer Nano ZSP (Malvern).

Inherent electrochemistry studies were conducted by cyclic voltammetry measurements using compact and modular potentiostat/galvanostat (Autolab PGSTAT204/FRA32M, Eco Chemie, Utrecht, Netherlands) at a scan rate of 100 mV/s. A three electrode configuration was used with Pt, glassy carbon (GC) and Ag/AgCl. GC was modified with 0.0015 mg of test material using the drop-cast method.

MTT control experiment

This was conducted in a similar manner as that for WST-8. However, in this case, 1 mg/ml MTT assay was used, the reaction mixtures were incubation for 3 h and the absorbance readings were measured at 570 nm.

WST-8 control experiment

For cell-free WST-8 control experiments, different concentrations of MPX_y suspensions were incubated with 10 % WST-8 assay in cell culture media for one hour. Thereafter, the absorbance was recorded at 450 nm. The results were normalized with a blank control which was not subjected to any test materials.

Cell culture

A549 human lung carcinoma epithelial cells (PAA laboratories) and BEAS-2B human bronchial epithelial cells (ATCC) were cultured in DMEM (Dulbecco's modified eagle medium, Life Technologies) supplemented with 10 % FBS (foetal bovine serum, Life Technologies) and 1 % penicillin/streptomycin (Capricorn). The cells were incubated at 37 °C under 5 % CO₂ atmosphere.

Exposure of cells to test materials

Cells were prepared on ninety-six well plates (five thousand cells/well) overnight. Following that, the cell culture media was removed and replaced with different concentrations of material suspensions in cell culture media (0, 3.125, 6.25, 12.5, 50, 100 µg/mL; 100 µl/well). A set of positive control containing 10 % DMSO (dimethyl sulfoxide, Tedia) was prepared for every set of experiment to check on the vitality of the cells used.

Cytotoxicity assessment

Following overnight treatment of A549 cells with the material suspensions, MTT (Sigma Aldrich) and WST-8 (Dojindo) cell viability assays were conducted. In the case of MTT assay, the solutions were removed from the wells and 100 µl of 1 mg/ml MTT stock solution were added to each well. For WST-8 assay, 10 µl of WST-8 assay was added directly to each well after the exposure with test materials. Following addition of WST-8 assay, the plates were wrapped in Aluminium foil and incubated at 37 °C and 5 % CO₂ for 1h before absorbance measurement at 450 nm. In the case of MTT, the incubation period was 3 h and the absorbance measurement was performed at 570 nm. Absorbance readings obtained from a corresponding set of control experiments were subtracted away from the absorbance readings obtained from wells with cells. Results from these readings were then normalization with the negative control which was not subjected to any test materials.

Live and dead cells were stained using 1 M calcein and 2 M ethidium homodimer from Life Technologies, after treatment of the A549 cells with the P materials. Upon 45 min incubation of the cells with the dye, the live and dead cells were viewed under Nikon fluorescent microscope at 485/530 nm and 530/645 nm respectively.

Mechanistic studies

GSH/GSSG and apoptosis/necrosis real-time monitoring were determined using GSH/GSSG-Glo Assay and RealTime-Glo™ Annexin V from Promega following the instructions provided.

9.5. Supporting Information

Figure 9.S1. Dynamic light scattering (DLS) results obtained for black phosphorus synthesized by vapour phase growth (BP VPG), black phosphorus synthesized by high pressure conversion (BP HPC), violet phosphorus (VP) and red phosphorus (RP) suspended in ultrapure water.

Table 9.S1. Surface charge of various P materials suspended in cell culture media determined by ζ -potential measurement.

P material	Surface charge (mV)
BP VPG	-0.01
BP HPC	0.01
VP	-0.04
RP	-0.04

Figure 9.S2. Energy-dispersive X-ray (EDX) mapping from TEM images of BP VPG, BP HP and VP and RP.

Figure 9.S3. Cytotoxicity assessment using WST-8 assay after 24 hours incubation of black phosphorus synthesized by vapour phase growth (BP VPG), black phosphorus synthesized by high pressure conversion (BP HPC), violet phosphorus (VP) and red phosphorus (RP) with human embryonic kidney cells (HEK293), human cervical carcinoma cells (HeLa), human breast cancer cells (MCF7) and human liver carcinoma cells (HepG2).

References:

1. Gusmão, R. et. al. *Angew. Chem. Int. Ed.* **2017**, *56*, 8052–8072.
2. Eswaraiah, V.; Zeng, Q.; Long, Y.; Liu, Z. *Small* **2016**, *12*, 3480–3502.
3. Bagheri, S.; Mansouri, N.; Aghaie, E. *Int. J. Hydrogen Energy* **2016**, *41*, 4085–4095.
4. Ma, Y.; Shen, C.; Zhang, A.; Chen, L.; Liu, Y.; Chen, J.; Liu, Q.; Li, Z.; Amer, M. R.; Nilges, T.; Abbas, A. N.; Zhou, C. *ACS Nano* **2017**, *11*, 7126–7133.
5. Shao, J.; Xie, H.; Huang, H.; Li, Z.; Sun, Z.; Xu, Y.; Xiao, Q.; Yu, X.-F.; Zhao, Y.; Zhang, H.; Wang, H.; Chu, P. K. *Nat. Commun.* **2016**, *7*, 12967.
6. Qian, X.; Gu, Z.; Chen, Y. *Mater. Horiz.* **2017**, *4*, 800–816.
7. Ling, X.; Wang, H.; Huang, S.; Xia, F.; Dresselhaus, M. S. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 4523–4530.
8. Mayorga-Martinez, C. C. et. al. *Anal. Chem.* **2016**, *88*, 10074–10079.
9. Li, P. et. al. *ACS Appl. Mater. Interfaces* **2015**, *7*, 24396–24402.
10. Abbas, A. N.; Liu, B.; Chen, L.; Ma, Y.; Cong, S.; Aroonyadet, N.; Köpf, M.; Nilges, T.; Zhou, C. *ACS Nano* **2015**, *9*, 5618–5624.
11. Mayorga-Martinez, C. C. et. al. *Angew. Chem. Int. Ed.* **2015**, *54*, 14317–14320.
12. Sajedi-Moghaddam, A.; Mayorga-Martinez, C. C.; Sofer, Z.; Bouša, D.; Saievar-Iranizad, E.; Pumera, M. *J. Phys. Chem. C* **2017**, *121*, 20532–20538.
13. Yew, Y. T.; Sofer, Z.; Mayorga-Martinez, C. C.; Pumera, M. *Mater. Chem. Front.* **2017**, *1*, 1130–1136.
14. Wang, L. et. al. *ChemElectroChem* **2015**, *2*, 324–327.
15. Fojtů, M. et. al. *Environ. Sci.: Nano* **2017**, *4*, 1617–1633.

16. Latiff, N. M.; Teo, W. Z.; Sofer, Z.; Fisher, A. C.; Pumera, M. *Chem. –Eur. J.* **2015**, *21*, 13991–13995.
17. Zhang, Z.; Xing, D.-H.; Lib, J.; Yan, Q. *CrystEngComm* **2017**, *19*, 905–909.
18. Krug, H. F.; Wick, P. *Angew. Chem. Int. Ed.* **2011**, *50*, 1260–1278.
19. Teo, W. Z. et. al. *RSC Adv.* **2015**, *5*, 107158–107165.
20. Teo, W. Z. et. al. *Chem. –Eur. J.* **2015**, *21*, 13020–13026.
21. Chng, E. L. K.; Chua, C. K.; Pumera, M. *Nanoscale* **2014**, *6*, 10792–10797.
22. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Chem. –Eur. J.* **2014**, *20*, 9627–9632.
23. Chng, E. L. K.; Sofer, Z.; Pumera, M. *Chem. –Eur. J.* **2014**, *20*, 6366–6373.
24. Teo, W. Z.; Chng, E. L. K.; Sofer, Z.; Pumera, M. *Nanoscale* **2014**, *6*, 1173–1180.
25. Sharifi, S.; Behzadi, S.; Laurent, S.; Forrest, M. L.; Stroeve, P.; Mahmoudi, M. *Chem. Soc. Rev.* **2012**, *41*, 2323–2343.
26. Lin, S.; Liu, S.; Yang, Z.; Li, Y.; Ng, T. W.; Xu, Z.; Bao, Q.; Hao, J.; Lee, C.-S.; Surya, C.; Yan, F.; Lau, S. P. *Adv. Funct. Mater.* **2016**, *26*, 864–871.
27. Olego, D. J.; Baumann, J. A.; Kuck, M. A.; Schachter, R.; Michel, C. G.; Raccach, P. M. *Solid State Commun.* **1984**, *52*, 311–314.
28. Lu, W. L.; Nan, H. Y.; Hong, J. H.; Chen, Y. M.; Zhu, C.; Liang, Z.; Ma, X. Y.; Ni, Z. H.; Jin, C. H.; Zhang, Z. *Nano Res.* **2014**, *7*, 853–859.
29. Sofer, Z. et. al. *Chem. Commun.* **2016**, *52*, 1563–1566.
30. Zaug, J. M.; Soper, A. K.; Clark, S. M. *Nat. Mater.* **2008**, *7*, 890–899.
31. Baumer, F.; Ma, Y.; Shen, C.; Zhang, A.; Chen, L.; Liu, Y.; Pfister, D.; Nilges, T.; Zhou, C. *ACS Nano* **2017**, *11*, 4105–4113.

32. Huang, H.; Xiao, Q.; Wang, J.; Yu, X.-F.; Wang, H.; Zhang, H.; Chu, P. K. *npj 2D Materials and Applications* **2017**, *1*, 20, DOI:10.1038/s41699-017-0022-6.
33. Mayorga-Martinez, C. C.; Sofer, Z.; Sedmidubsky, D.; Luxa, J.; Pumera, M. Metallic impurities in black phosphorus nanoflakes prepared by different synthetic routes. *Nanoscale* **2018**, *10*, 1540–1546.
34. Plutnar, J.; Sofer, Z.; Pumera, M. *ACS Nano* **2018**, *12*, 8390–8396.
35. Chng, E. L. K.; Sofer, Z.; Pumera, M. *Nanoscale* **2014**, *6*, 14412–14418.
36. Chng, E. L. K.; Pumera, M. *Chem. –Eur. J.* **2013**, *19*, 8227–8235.
37. Dayem, A. A.; Hossain, M. K.; Lee, S. B.; Kim, K.; Saha, S. K.; Yang, G.-M.; Choi, H. Y.; Cho, S.-G. *Int. J. Mol. Sci.* **2017**, *18*, 120.
38. Mu, X. et. al. *ACS Appl. Mater. Interfaces* **2017**, *9*, 20399–20409.
39. Zhang, X. et. al. *Small* **2017**, *13*, 1701210.
40. Zitka, O.; Skalickova, S.; Gumulec, J.; Masarik, M.; Adam, V.; Hubalek, J.; Trnkova, L.; Kruseova, J.; Eckschlager, T.; Kizek, R. *Oncol. Lett.* **2012**, *4*, 1247–1253.
41. Latiff, N. M.; Sofer, Z.; Fisher, A. C.; Pumera, M. *Chem. –Eur. J.* **2017**, *23*, 684–690.
42. Andersson, P. O. et. al. *Small* **2011**, *7*, 514–523.
43. Badawy, A. M. et. al. *Environ. Sci. Technol.* **2011**, *45*, 283–287.
44. Krug, H. F. *Angew. Chem. Int. Ed.* **2014**, *53*, 12304–12319.
45. Marcano, D. C.; Kosynkin, D. V.; Berlin, J. M.; Sinitskii, A.; Sun, Z.; Slesarev, A.; Alemany, L. B.; Lu, W.; Tour, J. M. *ACS Nano* **2010**, *4*, 4806–4814.
46. Kang, J. H.; Kim, T.; Choi, J.; Park, J.; Kim, Y. S.; Chang, M. S.; Jung, H.; Park, K. T.; Yang, S. J.; Park, C. R. *Chem. Mater.* **2016**, *28*, 756–764.

Chapter 10 –

Conclusion and Future Outlook

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In summary, we have looked at two aspects of layered materials for energy applications. One concerns advancing our knowledge in the development of their electrochemical properties for energy storage and generation systems, particularly for applications in supercapacitors and as electrocatalysts for hydrogen evolution reaction (HER). In this aspect, we have studied the effects of B, Cl and N dopants on the capacitive performance of microwave exfoliated graphene. Our findings show that type of graphene oxide (GO) precursor used (i.e. Hummers, Hoffman, Staudenmaier) can produce varying quantity of dopants, and that the Hummers and Hoffman oxidation methods are better routes for doping graphene via microwave exfoliation relative to the Staudenmaier method. Apart from this project, we had also investigated the influence of valence and oxide impurities in transition metal dichalcogenides (TMDs) MoS_2 and WS_2 on their HER activity. Our results demonstrated that small amounts of catalytic impurities of MoS_3 or WS_3 can completely dominate the electrochemistry of the corresponding TMD. These findings can serve as platforms for us to further improve on the HER performance and capacitance of these materials.

Besides electrochemical studies, we are also concerned with the potential health hazards posed by these layered materials. This has led us to conduct preliminary cytotoxicity studies of several emerging layered materials namely semiconductor chalcogenides (GaSe , GeS), vanadium dichalcogenides (VS_2 , VSe_2 , VTe_2), metal phosphorus chalcogenides (CoPS_3 , NiPS_3 , MnPS_3 , FePS_3) as well as black phosphorus and its allotropes (red and violet). These materials have been selected as their toxicological effects are not yet well understood and they have shown promising potential for energy-related applications such as batteries, capacitors and HER electrocatalysts. Our investigations revealed the importance of conducting control experiments to be aware of possible interferences between the layered materials and the cell viability assay used. As seen in the case of GaSe , GeS and black phosphorus, these interferences can be significant and lead to unreliable results. We found that vanadium ditelluride to be more toxic as compared to its sulfides and selenides counterparts. We also observed that the toxicity of metal phosphorus chalcogenides (MPX_n) are influenced by the type of metal element

present with NiPS_3 showing the least toxicity amongst the MPX_n materials studied. Besides this, we noticed the changes in toxicities as red phosphorus transformed into violet phosphorus and later to black phosphorus. We found a correlation between toxicity and oxygen content. These results can facilitate our understanding on factors that can affect the toxicity of layered materials.

However, we recognize that these results are still in the preliminary phase and more research can be done to deepen our understanding in these two aspects (i.e. electrochemistry and toxicity studies of layered materials). In the area of electrochemistry, further work can be performed to tap on our previous findings to enhance their present performances. Examples include designing hybrid of WS_3 decorated on WS_2 and introducing other types of dopants to find the best combination and quantity to improve the capacitance of graphene. In the case of toxicity studies, it would be insightful to go into the mechanisms behind the toxicity behaviour and design controlled systematic experiments to assess how different parameters (i.e. number of layers, elements present, oxygen content, synthesis method) can affect the toxicity of each family of layered materials.

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