Voltammetric Detection, Transformation and Toxicity of Engineered Nanomaterials in Aqueous Environment and Application of Micro-/Nanomotors for Environmental Remediation

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Voltammetric Detection, Transformation and Toxicity of Engineered Nanomaterials in Aqueous Environment and Application of Micro-/Nanomotors for Environmental Remediation

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Acknowledgement

“Every accomplishment starts with the decision to try.” Though I did not accomplish much in my PhD journey, this quote gave me the courage to pursue the research pathway four years ago when I had my first research experience during my undergraduate studies. This journey has not been smooth-sailing, but I am glad that I made the decision to try, for the bonds and friendships that were forged and the valuable life lessons that I learnt along the way made it all worthwhile.

I would like to express my deepest gratitude to my advisor, Associate Professor Martin Pumera, for his guidance and support throughout my PhD journey. I truly appreciate his efforts in ensuring that I was making good progress in my research, and being available to advise me whenever I encounter any obstacles while working on the project. His interest and passion in various research areas allowed me to branch into different aspects of nanotechnology, giving me a broader perspective of the field. It was an honour to work under Prof Pumera who is always so patient and understanding and I am glad to be part of his research group.

I am also grateful to Dr Toh Chee Seng who agreed to be my FYP supervisor even though I had no previous research experience back then. He brought me into the field of electrochemistry and provided me with ample advices on the approach towards scientific research. He was also very supportive when he came to know about my decision to pursue a PhD.

I would also like to thank Dr Adriano Ambrosi for his guidance in writing an academic paper when I started my PhD, Dr Poh Hwee Ling for teaching me how to operate the instruments in the lab, Dr Zhao Guanjia for his motivational talks, and Dr CK Chua for his constructive feedbacks on my work and occasional coffee treats throughout the past four years. Special thanks to Dr Elaine Chng who mentored me and ensured that I could pick up the knowledge on nanotoxicology research quickly.

I believe that my PhD journey will be less enjoyable without the strong camaraderie in Pumera’s group. This is especially so if the trio (Adeline, Alex, Colin) who begun the PhD journey with me in August 2012 were not in the group. I will definitely miss the fun and laughter we had during our overseas conference, the anxiety we shared during our QE period, and the spontaneous decisions to order food online for lunch. I am deeply indebted to Alex for his continuous assistance whenever I needed them. His vast knowledge in Chemistry and kind personality made him very approachable if one faces any difficulty in research. I would also like to acknowledge Wang Hong and James for their help in the micromotors research, Naziah for her trust in me as a mentor, and the rest of the group members for enriching my life and sharing my joy when I got married last year.

Most importantly, I am greatly thankful to my parents for ensuring that I could pursue my education without worrying about the financials and having faith in my capabilities since I was young. Last but not least, I am sincerely grateful to have my wife, Lingxin, by my side throughout the past 8 years. She would never fail to cheer me up and kept me sane at the end of the day. Thank you for walking my PhD journey with me and being my main pillar of support in life.
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**Chapter 1** Objectives of the thesis

*In this Chapter, the objectives of the thesis were outlined.*

**Chapter 2** Background and literature review

*In this Chapter, the general features of engineered nanomaterials and micro-/nanomotors were discussed. Analytical techniques for characterisation and quantification of engineered nanomaterials, their transformation in water, in vitro toxicity assessments methods, properties and toxicity profiles of halogenated nanocarbons and transition metal dichalcogenides, and environmental remediation application of micro-/nanomotors were reviewed.*

**Chapter 3** Voltammetric determination of engineered nanomaterials I

*In this Chapter, the possibility of quantifying copper (II) oxide nanoparticles in alkaline media using cyclic voltammetry was investigated.*

**Chapter 4** Voltammetric determination of engineered nanomaterials II

*In this Chapter, the electrochemistry of iron (II,III) oxide nanoparticles and probability of quantifying iron-based nanoparticulate impurities in carbon nanotubes with cyclic voltammetry were examined.*

**Chapter 5** Fate of engineered nanomaterials in natural waters I

*In this Chapter, the transformation of silver nanoparticles in different environmental waters were investigated and compared using various analytical techniques, including cyclic voltammetry.*
Chapter 6  Fate of engineered nanomaterials in natural waters II

In this Chapter, the transformation of three different graphene oxides in various natural waters over three months were investigated and compared using different analytical techniques, including cyclic voltammetry.

Chapter 7  Toxicity of engineered nanomaterials I

In this Chapter, the cytotoxicities of non-two-dimensional fluorinated nanocarbons were examined to determine the effect of size and morphology on its toxicity.

Chapter 8  Toxicity of engineered nanomaterials II

In this Chapter, the cytotoxicities of fluorinated graphenes were investigated and compared.

Chapter 9  Toxicity of engineered nanomaterials II

In this Chapter, the cytotoxicities of halogenated graphenes (excluding fluorinated graphene) were investigated and compared.

Chapter 10  Toxicity of engineered nanomaterials IV

In this Chapter, the cytotoxicities of exfoliated MoS\textsubscript{2}, WS\textsubscript{2}, and WSe\textsubscript{2} nanosheets were investigated and compared with graphene derivatives.

Chapter 11  Towards real-world application of self-propelled micro-/nanomotors for environmental remediation I

In this Chapter, the locomotion of silver-catalyst based bubble-propelled tubular micromotors was examined.

Chapter 12  Towards real-world application of self-propelled micro-/nanomotors for environmental remediation II

In this Chapter, the locomotion of Fe(0) nanomotors that could be prepared in tons quantities and their utilisation inazo-dye pollutant degradation were investigated.

Chapter 13  Towards real-world application of self-propelled micro-/nanomotors for environmental remediation III

In this Chapter, the effect of bubble generation and motion of bubble-propelled micro-/nanomotors on mechanical mixing at macroscale level was investigated.

Chapter 14  Conclusion and Epilogue

In this Chapter, a summary of the findings in this PhD thesis and a future perspective for the relevant research areas were described.
Abstract

Nanotechnology has made remarkable progress in the 21st century owing to advances in instrumentations and techniques. Many consumer products are now incorporated with engineered nanomaterials that possess unique properties to enhance the product features. Limited regulations on the use of these potential pollutants led to rising concerns about the effect of engineered nanomaterials presence in the environment on the ecosystems and our health. Hence, this project examined the possibility of utilising voltammetric methods for accurate and efficient determination of engineered nanomaterials. Subsequently, the transformation of nanomaterials in different environmental waters were investigated and compared to find out how its interactions with the components in the water could affect its physiochemical properties, which in turn would influence its toxicity. Following that, cytotoxicity assessments of emerging engineered nanomaterials were performed to better understand their toxicity profiles they are introduced commercially. Besides engineered nanomaterials, micro- and nanometre scale devices which could undergo self-propulsion were successfully fabricated due to nanotechnology advances and they possess remarkable potential for various applications. Despite showing the capability to improve the rate of pollutant removal and degradation in proof-of-concept studies, there are challenges that impede its progression towards real-world application. Some of these challenges were addressed in this project through the replacement of platinum catalyst, large-scale and low cost fabrication of nanomotors, and studying the effect micromotors motion at macroscale level.
List of Publications


Thesis Organisation

This thesis is organised into fourteen (14) Chapters. The reader is presented with the motivations and ideas towards the collation of this thesis in Chapter 1. The following Chapter 2 will provide the reader with an overview of engineered nanomaterials, self-propelled micro-/nanomaterials, and in vitro toxicology assessment techniques. In depth review on earlier published works in relation to the analytical techniques applied in the characterisation and quantification of engineered nanomaterials, their physiochemical changes in water, the toxicity of halogenated nanocarbons and transition metal dichalcogenides, and environmental remediation application of self-propelled micro-/nanomotors is also included in the same Chapter. With appropriate information relating to the scientific background of this thesis, the reader will be directed to Chapters 3 – 13, which is made up of a compilation of research towards the objectives established in Chapter 1.

In detail, Chapter 3 and 4 will expand on existing works involving direct voltammetric quantification of engineered nanoparticles. Chapters 5 and 6 will cover the physiochemical transformation of engineered nanomaterials after dispersion in natural waters for prolonged periods. Chapters 7, 8, 9, and 10 will touch on the cytotoxicity of emerging nanomaterials. Chapters 11, 12, and 13 will emphasize on issues that need to be addressed before employing self-propelled micro-/nanomotors in real-world environment for remediation applications.

A summary and perspective of the work presented in this thesis are described in Chapter 14.
**List of Abbreviations**

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<th>Definition</th>
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<tr>
<td>A549</td>
<td>Human Lung Carcinoma Epithelial Cell Line</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<tr>
<td>at.%</td>
<td>Atomic Percentage</td>
</tr>
<tr>
<td>BTB</td>
<td>Bromothymol Blue</td>
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<tr>
<td>CNT</td>
<td>Carbon Nanotubes</td>
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<tr>
<td>CPI</td>
<td>Nanotechnology Consumer Product Inventory</td>
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<tr>
<td>CV</td>
<td>Cyclic Voltammetry</td>
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<tr>
<td>DCFDA</td>
<td>2',7' dichlorodihydrofluorescein Diacetate</td>
</tr>
<tr>
<td>DMF</td>
<td>$N,N'$-dimethylformamide</td>
</tr>
<tr>
<td>DPV</td>
<td>Differential Pulse Voltammetry</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy-dispersive X-ray Spectroscopy</td>
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<tr>
<td>ENMs</td>
<td>Engineered Nanomaterials</td>
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<tr>
<td>FNC</td>
<td>Fluorinated Nanocarbons</td>
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<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
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<tr>
<td>GC</td>
<td>Glassy Carbon</td>
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<tr>
<td>GO</td>
<td>Graphene Oxide</td>
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<tr>
<td>ICP-MS</td>
<td>Inductively Coupled Plasma Mass Spectrometry</td>
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<tr>
<td>IS</td>
<td>Ionic Strength</td>
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<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide</td>
</tr>
<tr>
<td>MB</td>
<td>Methylene Blue</td>
</tr>
<tr>
<td>MO</td>
<td>Methyl Orange</td>
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<tr>
<td>NOM</td>
<td>Natural Organic Matter</td>
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<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Solution</td>
</tr>
<tr>
<td>PEDOT</td>
<td>Poly(3,4-ethylenedioxythiphene)</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<tr>
<td>TOC</td>
<td>Total Organic Carbon</td>
</tr>
<tr>
<td>TMDs</td>
<td>Transition Metal Dichalcogenides</td>
</tr>
<tr>
<td>WST-8</td>
<td>Water-soluble Tetrazolium Salt</td>
</tr>
<tr>
<td>wt.%</td>
<td>Weight Percentage</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray Diffraction</td>
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<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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<td>-G</td>
<td>Graphenes</td>
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Chapter 1

Objectives of the thesis
Advances in instrumentations and techniques have allowed researchers to make remarkable progress in the field of nanotechnology. Many engineered nanomaterials with unique properties have been synthesised and are found in various consumer products today. In addition, micro- and nanometre scale devices which could undergo self-propulsion were successfully fabricated and they possess remarkable potential for various applications.

The exponential increase in the number of commercial products containing engineered nanomaterials available in the market will inevitably lead to the release of these nanomaterials into the environment, posing potential threat to our health and the ecosystem. As such, it is important to devise sensitive and rapid analytical methods to detect these nanomaterials, as well as to study changes in their physiochemical properties after prolonged exposure in environmental waters. Electrochemistry represents a facile and suitable method that could be employed for the direct determination of metallic and metallic oxide nanoparticles owing to the electroactivity of most of these nanoparticles.

Halogenated carbon-based nanomaterials have been placed under the limelight in recent years as they were found to exhibit interesting physiochemical properties that were not found in the original nanomaterial. The discovery has widened the potential applications of such nanomaterials. Inorganic derivatives of graphene such as transition metal dichalcogenides are also emerging nanomaterials with various envision applications. However, their toxicological profiles ought to be completely understood before introducing the halogenated carbon-based nanomaterials and two-dimensional transition metal dichalcogenides nanomaterials into consumer products.
Self-propelling micro-/nanomotors were reported to be able to increase the rate of pollutants removal and degradation, making these small scale devices suitable as environment remediation agents. In order to apply these micro-/nanomotors for remediation in real-world environment, however, issues pertaining to the catalyst and fuel used for the propulsion, as well as the fabrication methods of these micro-/nanomotors need to be addressed.

Therefore, as depicted in Figure 1.1, a total of three objectives were set for this thesis to address and improve the research areas mentioned above. A comprehensive background information on engineered nanomaterials and micro-/nanomotors, directed at rationalising the objectives, will be discussed in Chapter 2. The engineered nanomaterials studied in this thesis to address Objective 1 were chosen because they represent some of the common nanomaterials which are incorporated into consumer products and exhibit toxicity, or they show potential for bioapplication.

![Figure 1.1 Summary of the challenges and objectives of this project.](image-url)
Objective 1: Development of voltammetric techniques to detect engineered nanomaterials and study its transformation in natural waters

Metallic and metallic oxide nanoparticles are already being incorporated in various consumer products such as food packaging, sunscreens, textiles, and personal care products. As these products are used, washed, or discarded, the nanoparticles embedded within will ultimately be released into environmental waters where they accumulate and undergo interactions with the media.

Studies have revealed that some of these nanoparticles are likely to cause adverse health effects and thus there is a need to develop simple and efficient analytical techniques to detect and quantify these toxic nanomaterials. Due to the electroactivity of most metallic and metallic oxide nanoparticles, voltammetry is seen as a suitable method for this purpose and voltammetric determination of engineered nanomaterials is demonstrated in Chapters 3 and 4.

Reports have also suggested that toxicological effects induced by nanomaterials could be altered by changes in their physiochemical properties after exposure to different aqueous media. In lieu of this, it is important to investigate the transformation of the nanomaterials in different types of environmental waters in order to understand the level of environmental damage that these nanomaterials will cause as a result of their accumulation in these natural waters. Investigations on the fate of silver nanoparticles and graphene oxide nanomaterials in natural waters with the integrative use of conventional analytical techniques and cyclic voltammetry as a novel method are presented in Chapters 5 and 6.
Chapter 1: Objectives of the thesis

Objective 2: Investigation on the cytotoxicity of halogenated nanocarbons and emerging 2D nanomaterials

Carbon-based nanomaterials functionalised with heteroatoms such as halogens have been found to possess enhanced physicochemical properties which are otherwise missing in the parent nanomaterial, making them attractive for possible commercial applications including lubricants, optical, and energy storage systems. Large-scale production and subsequent leaching of these nanomaterials into the environment will be inevitable should they be incorporated into everyday products, hence it is necessary to explore the toxicological aspect of doing so. As the physicochemical properties of engineered nanomaterials could influence its toxicity, thorough characterisation of the halogenated nanocarbons were carried out before investigating their cytotoxicity.

Following the successful progress in the study of graphene, other two-dimensional nanomaterials derived from another class of materials known as transition metal dichalcogenides are currently under immense research. These nanomaterials exhibit superior electronic and structural properties and would probably be commercialised in the near future. Therefore, it will be of interest to elucidate their toxicities as well.

The cytotoxicity of various halogenated nanocarbons and exfoliated transition metal dichalcogenides nanosheets are discussed in Chapters 7, 8, 9, and 10.
Objective 3: Development of self-propelled micro-/nanomotors towards real-world environmental remediation

Self-propelled micro-/nanomotors have been demonstrated to enhance the efficiency of pollutants removal and degradation, suggesting its possible utilisation for environmental remediation. However, it remained a challenge for real-world application of these small scale motors as most of them required scarce and costly platinum as catalyst to decompose hazardous hydrogen peroxide fuel for propulsion. In addition, current fabrication techniques are unable to synthesise the micro-/nanomotors in a truly large-scale quantity at low cost. As such, development of micro-/nanomotors that tackle these issues is crucial and this is presented in Chapters 11 and 12.

It has been shown that the acceleration in the remediation process through the incorporation of micro-/nanomotors in the polluted solution was the result of mechanical mixing arising from the motors’ continuous motion and bubbling. However, these studies typically involved small volumes of solution, failing to examine the impact of the bubbles formation and micromotors propulsion at the macroscale level which is important as we advance towards real-world environmental remediation using these motors. Therefore, we investigated this impact and the findings are elaborated in Chapter 13.
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Background and Literature Review

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<td>2.3.2</td>
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2.1 Engineered nanomaterials (ENMs)

Inspired by the lecture “There’s Plenty of Room at the Bottom” from physicist and Nobel Prize laureate Richard Feynman in 1959, the development of scanning tunnelling microscope in 1981, and the discovery of buckminsterfullerene \( \text{C}_{60} \) in 1985 and carbon nanotubes in 1991, the past two decades saw an exponential increase in the research of nanometre scale materials and the amount of funding towards nanotechnology-related research and development.\(^1\)\(^3\) Today, engineered nanomaterial, broadly referred to any anthropogenic substance with lateral sizes between 1 and 100 nm, can be found in many consumer products like food packaging, sunscreens, cosmetics, washing machines, detergents, fabric and paints.\(^4\)\(^6\) According to the Nanotechnology Consumer Products Inventory (CPI) created by the Woodrow Wilson International Center for Scholars and the Project on Emerging Nanotechnology in 2005 to document products containing nanomaterials that are marketed and distributed into the commercial marketplace, there are a total of 1814 nanomaterial-containing consumer products listed in the inventory as of March 2015.\(^7\) This number represents a 500% increase in the amount of such consumer products listed in the online CPI when it was launched a decade ago, in 2006.\(^8\)

Consumer products listed in the CPI are grouped into eight consumer goods categories and Figure 2.1 shows the number of products listed in each major category between 2007 and 2014.\(^7\) The Health and Fitness category, which comprises of seven sub-categories like Personal Care, Clothing, Cosmetics, and Sunscreen products, has the largest listing of products in the CPI (42%). Among these sub-categories, Personal Care is the largest, constituting about one-third of the products listing in the Health and Fitness category. Sports equipment such as tennis rackets, baseball bats, and bicycle frames are
embedded with carbon nanotubes for improved strength and these products come under the Health and Fitness category as well.\(^9\)

![Figure 2.1](image-url)  
*Figure 2.1* Number of nanomaterial-containing consumer products listed in the CPI from 2007 in each major category and in the Health and Fitness subcategories. Reproduced with permission from reference 7.

With the increasing availability of consumer goods with ENMs, the release of these nanomaterials into the environment and their eventual accumulation in water systems will be inevitable as the products are being utilised, washed or discarded. Benn and co-workers showed in their work that leaching of silver nanoparticles and silver ions could be achieved in six types of silver nanoparticle-containing socks simply through immersion and shaking them in water.\(^10\) In two other separate studies, silver nanoparticles were also found to be discharged from an advertised silver nanoparticles-
producing washing machine and painted outdoor facades under normal usage at ambient conditions. ENMs have been listed as emerging pollutants and their presence in the environment might have adverse effects on the ecology and our health. Furthermore, several studies have already demonstrated that some ENMs could induce cytotoxicity and genotoxicity to mammalian cells, and their transformation upon release into the environment may ultimately affect their toxicity and environmental fate. Therefore, it is important to devise simple, sensitive, and efficient analytical techniques to detect and monitor the level of ENMs in the environment, as well as to investigate their transformation after interacting with the environment, so as to determine if their usage in consumer products should be restricted to prevent irreversible environmental damage.

In the following sub-chapters, the general features of ENMs and some of the methodologies used for the characterisation for these ENMs will be discussed. We will also review available studies which examined the fate and transformation of ENMs in water over time.
2.1.1 General features of engineered nanomaterials

ENMs can be broadly classified into carbon-based (organic) and inorganic nanomaterials. Fullerenes, carbon nanotubes, and graphene are some examples of carbon-based nanomaterials while metallic and metallic oxide nanoparticles fall under inorganic nanomaterials. They can be found in various sizes and shapes, including but not limited to spheres, tubes, rods, platelets, and dendritic structures. The size and morphology of an ENM would, in turn, influence other properties that it displays. For instance, the optical properties between gold (or silver) nanoparticles with identical composition but differing sizes and morphologies would vary significantly, giving rise to monodispersed nanoparticles with different colours under visible light.\textsuperscript{22, 23}

The rapid emergence of ENMs and the phenomena of increasing introduction of these anthropogenic materials into consumer products can be explained by the interesting and unique physiochemical properties they exhibit, which seemed to exist only when the materials are in nanoscale dimensions. It has been suggested that such distinctive properties were observed mainly with nanomaterials because their size lie in a region where quantum mechanics has predominance over classical physics in determining the physiochemical properties of the nanomaterials.\textsuperscript{24} An example of a remarkable property displayed by ENMs is the UV blocking capability of zinc oxide and titanium oxide nanoparticles. Both nanoparticles are transparent and pleasant to touch, on top of having the ability to reflect and scatter UVA and UVB radiations, thus resulting in their utilisation in sunscreen and cosmetic products.\textsuperscript{25} Antimicrobial activity is another noteworthy property exhibited by ENMs which led to their increased application in consumer products, especially in areas such as food packaging and storage, textiles, and medical devices. Silver nanomaterials, being well-known for its antimicrobial properties,
are the most frequently added component in nanomaterial-containing consumer products which are advertised to possess antimicrobial capabilities.\textsuperscript{7,26,27} The superior mechanical strength and stiffness discovered in some carbon-based nanomaterials are also properties that attracted researchers’ interest in these nanomaterials and the subsequent incorporation of these nanomaterials commercially.\textsuperscript{28} For instance, carbon nanotubes have been reported to be able to withstand extreme strains of up to 25 GPa without breaking, and possess high weight-to-strength ratio as compared to materials like steel.\textsuperscript{29,30} Fishing rods, car body panels, and golf clubs manufactured using materials reinforced with carbon nanotubes have enhanced strength and lightness. Lastly, the high surface area-to-volume ratio of nanomaterials has made them ideal candidates for catalysis, like in the case of catalytic converters in cars, where platinum, palladium, or rhodium nanoparticles served as catalyst to convert nitrogen oxides, carbon monoxide, and unburnt hydrocarbons to nitrogen, oxygen, carbon dioxide and water.\textsuperscript{31}

When ENMs enter into aqueous systems, agglomeration and settling of the nanomaterials may occur over time, affecting their stability in the water.\textsuperscript{32} There are many factors affecting the tendency and rate of nanomaterials aggregation in water, including its size, concentration, composition, surface area, and zeta potential, as well as the pH, salinity, and ionic strength of the water.\textsuperscript{33,34} Modifying the surface of the ENM with a layer of coating could aid in suppressing agglomeration.\textsuperscript{35} The physiochemical properties of ENMs can be altered by their transformation in environmental waters, thus it will be interesting to study their behaviour in varying natural waters with differing water conditions.
2.1.2 Characterisation and quantification methods – current and emerging

As mentioned earlier, ENMs can be fabricated in various shapes and sizes, which in turn could affect other physiochemical properties that these nanomaterials exhibit, including its toxicity. Moreover, ENMs have the tendency to undergo transformation in aqueous systems over time. Therefore, it is crucial to characterise the ENMs, as well as determine their concentration in the consumer products or aqueous systems. Some of the current and emerging techniques adopted to characterise and quantify ENMs are discussed below.

2.1.2.1 Current characterisation and quantification techniques

Microscopic techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) are currently some of the methods employed to find out the size, shape and structure of the nanomaterials. They are utilised because high-resolution visual images of the nanomaterials can be obtained from the measurements, thus allowing further analysis of the nanomaterials’ size, morphology, and aggregation state to be possible. Both SEM and TEM are operated under vacuum conditions, thus the nanomaterial sample must be dried before measurement. This is a major drawback of using these techniques for characterising ENMs dispersed in liquids, as artifacts and alteration of the nanomaterials might arise during sample preparation and drying. Another disadvantage of using SEM or TEM is the possibility of electron charging effects in less conducting nanomaterials during measurement. This problem is caused by the inability of these nanomaterials to conduct away electrons irradiated on them efficiently, resulting in accumulation of negative charges on the samples and making imaging impossible. However, this issue can be overcome by sputtering the sample with a
conducting material (e.g. platinum) prior to the analysis. As opposed to SEM and TEM, AFM can be carried out under atmospheric conditions, allowing the nanomaterials to be characterised in their original environment. Dynamic light scattering (DLS) technique can also be used for determining the size and aggregation states of ENMs in aqueous systems. However, the data obtained are susceptible to error caused by artifacts like dust particles in the solution.

SEM or TEM, when operated in tandem with energy-dispersive X-ray spectroscopy (EDX), can provide a rough estimation of the nanomaterial’s elemental composition. A more accurate quantification on the elemental composition of the ENM can be achieved through inductively coupled plasma mass spectrometry (ICP–MS) measurement. This technique is especially useful for quantifying minute amounts of metallic nanomaterials in a sample as the limit of detection are generally very low, in the sub-parts-per-trillion (ppt) range. However, ICP–MS is a destructive method, implying that the sample cannot be further analysed with other techniques after ICP–MS measurement. ICP–MS is also unable to detect lighter elements, thus making it unsuitable for the elemental composition analysis of carbon-based nanomaterials. Combustible elemental analysis method, which gives the carbon, hydrogen, nitrogen, and oxygen content of the sample, can be utilised in place of ICP–MS to examine the amount of these elements in the ENMs. Elemental composition of ENMs can also be measured with X-ray diffraction (XRD), which is a non-destructive technique. In addition, the data collected can facilitate the interpretation of the nanomaterial’s crystallographic structure. X-ray photoelectron spectroscopy (XPS) is a sensitive semi-quantitative analysis method that enables the study of elemental composition of nanomaterials, up to a depth of 10 nm from their surface. Distinctive signals corresponding to individual elements will appear at specific binding energies during a
XPS wide survey scan, thus allowing them to be identified. On top of that, XPS can also reveal the element’s chemical state or chemical bonding present (e.g. C–H; C–O bonds) on the surface of the nanomaterial.\(^{44}\) This is made possible by running a high-resolution XPS-core level scan of the relevant peak corresponding to the element of interest, for example the C1s peak, and analysing it through careful fitting of the spectra with the use of relatively sensitive factors.\(^{45}\)

Raman spectroscopy can be employed to obtain the density of defects of graphene and related carbon nanomaterials.\(^{46,47}\) In these nanomaterials, presence of defects or sp\(^3\) hybridised carbon atoms in the network will be indicated by a D band at approximately 1350 cm\(^{-1}\) in Raman spectroscopic measurements while a G band at around 1560 cm\(^{-1}\) signifies pristine sp\(^2\) lattice carbon atoms in the network.\(^{47}\) The ratio of the intensities \(I_D/I_G\) calculated will give the density of defects present in the nanomaterial. Fourier transform infrared (FTIR) spectroscopy is also useful in the characterisation of carbon-based nanomaterials, as functional groups present on the surface of the nanomaterials, including carbonyl, hydroxyl, and nitrile groups, can be qualitatively identified with this technique.\(^{48,49}\)

### 2.1.2.2 Emerging characterisation and quantification techniques

Environmental scanning electron microscopy (ESEM) is introduced as an alternative to SEM for the imaging of ENMs. In contrast to SEM, the sample chamber of an ESEM is operated at around 10–50 Torr, enabling measurements to be conducted under more natural conditions.\(^{37}\) This allows nanomaterials to be characterised closer to their natural state, thereby reducing alterations and artifacts resulting from sample preparation and drying. In addition, electron charging effects are eliminated in ESEM,
therefore non-conducting nanomaterials could be imaged without surface modification. However, in exchange, ESEM suffer from a lower resolution as compared to SEM.

Most of the metallic and metallic oxide nanoparticles are electroactive and some of these nanoparticles exhibit distinct voltammetric responses when subjected to voltammetry measurements such as cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The unique voltammogram acquired from CV or DPV provides quantitative and qualitative information about the electroactive nanoparticle and its redox reactions, thus voltammetry could be utilised for the purpose of detecting and quantifying these metallic and metallic oxide nanoparticles.

In fact, DPV has been employed for the determination of colloidal gold as early as 1995 by Costa-García and co-workers with the use of a pretreated carbon paste electrode (CPE). The electrode was immersed in colloidal gold in open circuit for 10 minutes to accumulate the gold nanoparticles before proceeding with the DPV measurements. Later, another group of researchers demonstrated the direct determination of gold nanoparticles through DPV as well, using a renewable graphite-epoxy composite electrode. Subsequently, the direct determination of other metallic and metallic oxide nanoparticles with voltammetry techniques were reported and the detection was generally carried out by first immobilising the nanoparticles on the surface of a working electrode before measuring the redox signal generated electrochemically. Through the analysis of values derived from the voltammograms (for example, peak potential or current of the redox signals), one will be able to deduce the identity of the nanoparticle and its concentration in the analyte. Besides CV and DPV, another electrochemical method, known as particle coulometry, could be used to detect and quantify the metallic and metallic oxide nanoparticles too. This technique
works on the principle that nanoparticles in the analyte solution will undergo either oxidation or reduction when they strike an electrode surface with a sufficiently positive or negative potential. By analysing the oxidation or reduction signals generated, the size, aggregation and amount of the nanoparticles present can be calculated, and this has been shown to be successful in the determination of gold, molybdenum, nickel, silver, as well as magnetite (Fe$_3$O$_4$) nanoparticles.$^{59-65}$

As ENMs are increasingly being incorporated into consumer products and some of these nanomaterials have the potential to damage our health, it is of utmost importance to introduce simple, sensitive and efficient analytical methods to detect and quantify the amount of hazardous ENMs present in a sample. Voltammetry represents such facile and suitable technique for the direct determination of ENMs which exhibit inherent electroactivity. We are interested to find out which metallic oxide nanoparticles, besides those already reported in the literature, can be quantified using this technique.

In this project, the electrochemistry of copper(II) oxide and iron(II,III) oxide nanoparticles will be investigated using CV and their voltammetric responses will be analysed methodically to examine the possibility of quantifying them through CV measurements. The findings are presented in Chapters 3 and 4.
2.1.3 Physiochemical transformation after releasing in water

It is estimated that the amount of ENMs that are released directly into surface water globally can be as much as 66,000 metric tons/yr.\textsuperscript{66,67} Upon release into water, these ENMs would likely undergo transformation in the form of aggregation, dissolution, oxidation, sulphidation, or changes in chemical state.\textsuperscript{66,68} The rate and extent of these transformations depend mainly on the chemical properties of the water and the duration that the ENMs spent in the water. Other factors such as size, morphology, and surface properties of the nanomaterials will also affect the degree of transformations. The transformation will cause alteration in the nanomaterials physiochemical properties, affecting their interactions with the environment and probably their toxicity.\textsuperscript{20,69,70} We will review some of the studies that were conducted to examine the transformation of selected ENMs in different types of water (eg. freshwater; seawater) below.

In the first study, the stability of three commercial metallic oxide nanoparticles, TiO\textsubscript{2}, SiO\textsubscript{2}, and ZnO, in pure water and real water samples was examined.\textsuperscript{71} It was found that the dry nanoparticles aggregated rapidly upon addition to pure water, forming flocs of nanoparticles with particle sizes much higher than the size observed in their dry form. ZnO experienced the greatest aggregation out of the three nanoparticles, forming aggregates of about 10 µm. The aggregated nanoparticles could be dispersed by ultrasonicing the suspension for 10 mins, resulting in particle sizes of 146 nm, 225 nm, and 244 nm for SiO\textsubscript{2}, TiO\textsubscript{2}, and ZnO respectively. According to results obtained from previous reports, the existence of cations in a water sample would cause nanoparticles to aggregate rapidly, especially if the valence and concentration of the cation was high. However, contrary to expectations, the three nanoparticles remained relatively stable after being released into the real water samples (lake water and wastewater), and their
particle sizes were between 200 – 600 nm after 10 h in lakewater and 4 hr in wastewater respectively. The authors attributed this observation to the presence of humic substances in the lakewater and surfactants in the wastewater respectively, which would adsorb to the nanoparticles in the water samples and prevented them from agglomerating. The second study also examined the stability of three metallic oxide nanoparticles, namely TiO$_2$, ZnO and CeO$_2$ in real water samples obtained from eight different sources. The authors in this study observed that the stability of the three nanoparticles is highly dependent on the total organic carbon (TOC) level and the ionic strength (IS) of the water sample. Under low TOC and high IS conditions, as in the case of seawater, the nanoparticles would aggregate quickly and create a sediment. Aggregation occurred within tens of minutes when TiO$_2$ and CeO$_2$ were placed in seawater, forming agglomerates of micrometre sizes. On the other hand, high TOC and low IS conditions, as in the case of freshwater, would slow down the rate of aggregation, allowing the size of the three nanoparticles aggregates to remain stable at approximately 300 nm for the whole duration of the experiment (400 min). It was suggested that the presence of high concentrations of organic molecules in freshwater samples acted as a barrier to agglomeration. Out of the three nanoparticles, TiO$_2$ showed the most distinct difference in aggregation and sedimentation rate between water samples with high IS and low TOC conditions and those with low IS and high TOC conditions.

Zhou and co-workers compared the rate of aggregation of two ZnO nanoparticles with different morphology under various conditions; one of the ZnO nanoparticles was nearly spherical in shape (diameter ≈ 20 nm) while the other was rod-like/slab-like nanoparticle (200 nm by 20 nm). It was discovered that, in the range of IS examined, the nearly spherical ZnO nanoparticles displayed a positive correlation between its aggregation and the IS of the solution. On the other hand, IS had minimal effect on the
aggregation of the rod-like/slab-like ZnO nanoparticles. It was also found that natural organic matter (NOM) could impede the aggregation of both types of ZnO nanoparticles. However, both ZnO nanoparticles exhibited similar aggregation behaviours when placed in different types of real water: retaining stability in freshwater with high NOM and low IS while agglomerating and settling in seawater with low NOM and high IS. A similar study was conducted recently on silver nanomaterials to determine the effect of morphology on their transformations in different types of water. The authors compared silver nanocubes with silver nanoparticles and discovered that in all the water samples tested (synthetic hard water, pond water, and seawater), silver nanocubes experienced minimal aggregation over 4 days of residence in the samples, in contrast to silver nanoparticles. This difference was particularly prevalent in the case of seawater. It was also observed that the silver nanocubes lost their cubic structure through preferential dissolution at its edge in water, resulting in the rounding of the nanomaterials.

The effect of the type of natural waters on the dissolution of ZnO nanoparticles was investigated in another study. By measuring the amount of free Zn$^{2+}$ released from the ZnO nanoparticles in four water samples, namely tap water, Qiantang River water, West Lake water, and Xixi River water, it was concluded that the dissolution of the ZnO nanoparticles was affected by pH, IS, and concentrations of HCO$_3^-$ and HPO$_4^{2-}$ in the water. The order of free Zn$^{2+}$ concentration in the four water samples was as follows: tap water > Qiantang River water > West Lake water > Xixi River water. A lower pH and concentrations of HCO$_3^-$ and HPO$_4^{2-}$ led to the highest dissolution of ZnO nanoparticles in tap water while the same level of pH but higher concentrations of HCO$_3^-$ and HPO$_4^{2-}$ in Xixi River water resulted in ZnO nanoparticles dissolving the least in the water sample.
A higher total dissolved zinc concentration was found in Qiantang River water as compared to West Lake water due to the former having a higher IS. Li and co-workers carried out a comprehensive study to determine the effect of capping layer and light on the aggregation and dissolution of silver nanoparticles (AgNPs) in freshwater samples. Three types of silver nanoparticles: bare silver nanoparticles (bare-AgNPs), citrate-coated silver nanoparticles (Citrate-AgNPs), and Tween-coated silver nanoparticles (Tween-AgNPs) were placed in freshwater samples and studied over 15 days in the dark or light for any changes in their stability and dissolution. The authors found that sterically stabilised silver nanoparticles such as Tween-AgNPs were less susceptible to aggregation and existed as individual particles in the freshwater samples over the whole 15 days while bare-AgNPs and Citrate-AgNPs agglomerated as soon as they are placed in the water. The well-dispersed and nano-sized Tween-AgNPs with higher surface area to volume ratio inevitably resulted in a faster initial rate of dissolution as compared to the larger sized aggregates of bare-AgNPs and Citrate-AgNPs. The authors then concluded that while the dissolution of AgNPs might be affected by sun light and photoactive capping agents (eg. citrate), aggregation of the AgNPs would render this relationship invalid.

The roles of pH, IS and concentration of NOMs in real water samples (freshwater and wastewater) on the aggregation and stability of graphene oxide and reduced graphene oxide were also investigated. It was found that graphene oxide has significantly higher stability in freshwater systems as compared to reduced graphene oxide, where only about 8% of the reduced graphene oxide remained suspended after one month in the freshwater sample. The authors attributed the lower aggregation of graphene oxide to the low IS and the presence of NOM that could reduce agglomeration.
In contrast, the high IS in wastewater resulted in the sedimentation of almost all graphene oxide and reduced graphene oxide in just one day. This indicated the significant impact of IS on the stability of graphene oxide and reduced graphene oxide.

In this project, we will investigate and compare the transformations of silver nanopowder in different types of water using both current analytical techniques and voltammetry in Chapter 5. A similar study will be carried out with graphene oxides prepared from different synthetic procedures in Chapter 6 to compare their transformations in different types of water with respect to residence duration in the water.
2.2 *In vitro* toxicity assessment of engineered nanomaterials

Research in the toxicity of ENMs has been rising over the past decade as a result of concerns over escalating human and environmental exposure of these nanomaterials arising from their growing incorporation in consumer products.\(^{77}\) Besides investigating the toxicity of ENMs that are already introduced into consumer products, nanomaterials that exhibit potential for future applications, especially in the biomedical field, should be examined for possible toxicological effects prior to their utilisation. In this way, we could better determine their suitability to be employed in the respective fields.

Toxicity of an ENM is highly influenced by its interactions with the biological systems, which in turn can be affected by the nanomaterial’s physiochemical properties such as shape, size, and chemical compositions. For instance, graphene oxides can exist in sheets of various layers and sizes, with different functional groups such as carbonyl, epoxyl, hydroxyl, and carboxyl, thus leading to significant differences in their reported toxicity.\(^{78}\) Therefore, toxicity studies should be carried out systematically on well-characterised nanomaterials so that proper correlation between the materials’ physiochemical variations and their toxicological effects on biological systems can be established. As shown in Figure 2.2, a thorough characterisation of the ENM should be performed to elucidate their physiochemical properties prior to analysing any data obtained from toxicity assessment of the nanomaterial. Characterisation techniques that can be employed to determine these properties have been discussed in the previous sub-chapter.

Compared to *in vivo* assessments, *in vitro* testing methods are fast, low cost, and does not involve ethical issues of animal testing, making them the ideal model systems for examining the toxicity of ENMs as they require immediate attention.\(^{79}\) Precise and
quantifiable measurements with good reproducibility obtained from these *in vitro* assessments will serve as an initial step towards the evaluation of the nanomaterial’s biocompatibility, which is extremely crucial for biomedical applications. There are various known routes for ENMs induced toxicity, including disruption of metabolic activity, cell membrane disintegration, DNA damage, and oxidative stress.\textsuperscript{77} *In vitro* methods can be used to probe whether these processes have taken place and these techniques can generally be grouped into two categories: viability assays and functional assays.

\textbf{Figure 2.2} *In vitro* and *in vivo* assessments to determine toxicity of nanomaterials. Reproduced with permission from reference 79.
Viability assays examine the extent of cell damage/death caused by exposing the ENMs to the cells. Cellular properties such as mitochondrial activity, membrane integrity, and membrane structure will be probed by specific assays to determine cell viability. For example, metabolic assays like MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and WST-8 (water-soluble tetrazolium salt) assays assess mitochondrial activity. The tetrazolium reagents in the assays will be reduced in the presence of metabolically active cells to generate coloured formazan dyes whose colour intensity will reflect the percentage of viable cells present. Necrosis assays measure cell viability through assessing membrane integrity, and LDH (lactate dehydrogenase) assay is an example of such assays. In the presence of lysed cells, LDH will be released into the cell media where it oxidises lactate to pyruvate, which in turn promotes the reduction of the active tetrazolium reagent in the LDH assay to coloured formazan. The percentage cell viability can then be derived through optical absorbance measurements.

Functional assays enable users to determine the toxicity mechanism through the assessment of particular cellular processes. For example, DNA damage to the cells can be investigated using the Comet assay, which is by far the most commonly employed technique. The ratio of fluorescence intensity emitted from the comet “tail” (containing damaged DNA) to that emitted from the “head” (containing intact DNA) is measured using the assay, and the value will be indicative of the degree of DNA damage caused by the nanomaterials. Besides damaging the DNA, nanomaterials can disrupt cellular processes by inducing oxidative stress, thereby increasing the formation of intracellular reactive oxygen species (ROS) like superoxide, hydroxyl radical, peroxyl radical and hydrogen peroxide to abnormal levels. The amount of ROS in the cells can be quantified by 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) assay, a method
that has been applied in several studies concerning toxicity of nanomaterials. In principle, the cell permeable non-fluorescent probe DCFDA reacts with ROS to produce a highly fluorescent 2',7'-dichlorofluorescein (DCF) as the former gets accumulated within the cell, and the intensity of the fluorescence will reflect the quantity of ROS in the cell.

In the following sub-chapters, we will discuss the properties of halogenated nanocarbons and transition metal dichalcogenides, as well as review their toxicity profiles and other related materials. These two groups of nanomaterials represent potential candidates to be introduced commercially in the future.

2.2.1 Halogenated nanocarbons – properties and toxicity profile

Carbon-based nanomaterials have been under the limelight for their remarkable physiochemical properties since the successful preparation of fullerene and carbon nanotubes. Interest in nanocarbons soared even further following the successful isolation of graphene in 2004. Currently, they are ranked the third most commonly incorporated nanomaterial in consumer products. When modified with elements such as oxygen, sulphur, nitrogen, or halogens, some of the original physiochemical properties exhibited by the nanocarbons will be altered. For example, the zero band gap in graphene will be opened once they are functionalised with fluorine. In addition, unlike the original graphene, fully fluorinated graphene acts like an insulator. Research in halogenated nanocarbons, in particular halogenated graphenes, experienced rapid growth over the past few years owing to interest in the enhanced optical, electronic, thermal, magnetic, mechanical, biological, and chemical properties possessed by these nanomaterials in comparison to their nanocarbon counterparts.

The structure of halogenated graphenes consists of a mixture of sp² hybridised carbon atoms interlinked to each other, and sp³ hybridised carbon atoms that are
connected to both halogens and other carbon atoms. In the extreme case of fully halogenated graphene, all the carbon atoms will become sp\(^3\) hybridised while maintaining the two-dimensional hexagonal symmetry.\(^{90}\) Physiochemical properties exhibited by halogenated graphenes may be influenced by the degree of their halogenation. For example, it was reported that pristine, partially fluorinated, and fully fluorinated graphene display different optical properties; highest transparency was observed for fully fluorinated graphene, followed by partially fluorinated graphene and the least transparent was pristine graphene.\(^{95}\) Since the studies conducted on halogenated graphenes thus far in the scientific community have focused largely on fluorinated graphenes, we will review the properties of fluorinated graphene first.

The band gap of fully fluorinated graphene has a theoretical value of 3.1 eV and value of the band gap will decrease with lower F/C ratio. This property thus allows for tuning of band gap to suit specific requirements for applications. Fluorine functionalisation could also change the photoluminescence property of graphene. For instance, fluorinated graphene quantum dots (F-GQDs) were demonstrated to exhibit bright blue photoluminescence and a strong upconversion photoluminescence which allow the F-GQDs to be excited by visible light, showing a great potential in the future applications of environmental and energy technology.\(^{91}\) Improved thermal and chemical stability were also observed in fully fluorinated graphene as compared to its graphene counterpart, probably the result of strong C–F bonding present in the nanomaterial.\(^{95}\) The presence of the C–F bonds also resulted in paramagnetism in the nanomaterial, and the effect was found to be dependent on the extent of fluorination as well. Romero-Aburto and co-workers demonstrated that fluorinated graphene oxide with paramagnetism could be utilised as a magnetic resonance imaging (MRI) contrast agent or drug carrier that is responsive to magnetic control.\(^{96}\) In that study, the fluorinated
graphene oxide was determined to be biocompatible as the nanomaterial did not induce cytotoxicity towards human breast cancer cells, even after 3 days of incubation with the cells at concentrations of up to 576 µg mL\(^{-1}\).

As compared to fluorinated graphenes, the rest of the halogenated graphenes are less stable and no graphene that is fully chlorinated, brominated, or iodinated has been successfully fabricated so far. In addition, there are limited studies involving partially chlorinated, brominated, or iodinated graphenes. Similar to fluorinated graphene, chlorinated graphene displays a non-zero band gap and the value of the band gap can be tuned by controlling the extent of chlorine functionalisation.\(^\text{90, 97}\) As for iodinated graphene, a recent study showed that it has excellent oxygen reduction reaction (ORR) electrocatalytic activity, long-term stability, as well as an exceptional tolerance to crossover effects for ORR, thus making it a suitable candidate to replace current commercial Pt/C catalysts.\(^\text{98}\) The formation of I\(^3^-\) within its structure was proposed to be the reason for the excellent ORR electrocatalytic activity.

Although there are numerous cytotoxicity data on pristine graphene, graphene oxide, and reduced graphene oxide, studies on the toxic effect of halogenated graphene are few. Therefore, we will discuss some of the cytotoxicity studies performed on these nanomaterials and the toxicity of organohalides in general. Generally, \textit{in vitro} assessments on graphenes, graphene oxides, and reduced graphene oxides have indicated that the toxicological effects induced by these materials are mainly dose-dependent. However, other factors such as shape and size of the nanomaterials, fabrication techniques (particularly for graphene oxide), duration of nanomaterials exposure to the cells, types of cells tested and the cell culture conditions also affect the toxicity induced by the nanomaterials.\(^\text{78, 99}\) Zhang and co-workers demonstrated that
Graphene could cause severe cytotoxic effects and mitochondrial damage in human neuronal cells.\textsuperscript{88} In the study, the authors compared the cytotoxicity of graphene and single-walled carbon nanotubes with MTT and LDH assays. They observed that the toxic response was concentration- and shape-dependent, and graphene was more toxic than single-walled carbon nanotubes at lower concentrations but less toxic at higher concentrations. In addition, results from DCFDA assay suggested that toxicity of graphene was induced through an oxidative stress mechanism. In another study, oxidised graphene nanoribbons (O-GNR) were screened for cytotoxicity in four different types of cell lines for up to 48 h of incubation.\textsuperscript{100} Besides demonstrating dose- and time-dependent toxic effects, O-GNR induced different levels of toxicity towards the four cell lines, with significantly higher cell death observed in human cervical cancer cells (HeLa) than in human breast cancer cells (SKBR3 or MCF7). Recently, Chng and co-workers investigated the cytotoxicity of different graphene oxides using human lung carcinoma epithelial cells.\textsuperscript{101} The cell viability results obtained from the study indicated that the cytotoxicity of graphene oxide was influenced by concentration of the nanomaterials and the oxidative methods adopted to fabricate them, as the latter would affect the oxygen content/functional groups present in the nanomaterial.

Group VII elements are known to be hazardous and therefore their presence in nanocarbon could affect its cytotoxicity.\textsuperscript{102} Information on the toxicity of fluorocarbon-based organic compounds are readily available as these compounds have been around since the early 19\textsuperscript{th} century.\textsuperscript{103, 104} Fluoroacetate, a pesticide component that was introduced in 1940s, was reported to be highly toxic to mammals and insects.\textsuperscript{105, 106} Conversely, fluoro-substituted alkanes and alkenes only induce little to no acute inhalation toxicity.\textsuperscript{107} Toxicity of fluorinated organic compounds was later speculated to be linked to their molecular characteristics, instead of the displacement of fluoride ions
during metabolic reactions.\textsuperscript{108} Few studies have discussed about the toxicity of fluorinated graphene. In one such study, it was demonstrated that fluorinated graphene could be utilised to enhance cell adhesion and proliferation of bone marrow derived mesenchymal stem cells, indirectly proving that their fluorinated graphene films were non-toxic.\textsuperscript{109} On the other hand, another group showed that fluorinated graphene sheets were toxic to human nerve cells (SH-SY5Y).\textsuperscript{110}

In this project, we will perform \textit{in vitro} toxicity assessments on various types of halogenated nanocarbons to bridge the gap in the lack of cytotoxicity data for this class of graphene derivatives, and to further our understanding on the health implications regarding their commercialisation in the future. Specifically, we will assess the cytotoxicity of different non-two-dimensional fluorinated nanocarbons in Chapter 7. Then, we will evaluate the toxicity profiles of fluorinated graphenes with different fluorine content in Chapter 8, and compare the cytotoxicity of graphenes functionalised with different halogens in Chapter 9.

\subsection*{2.2.2 Transition metal dichalcogenides – properties and toxicity profile}

Transition metal dichalcogenides (TMDs) are a family of around 60 materials, some of which exist as naturally occurring minerals.\textsuperscript{111,112} They can be represented with a general formula of MX\textsubscript{2}, where M represents any transition metal element from group 4, 5, or 6 (eg. Ti, Zr, V, Nb, Mo and W) in the periodic table and X represents a chalcogen (S, Se or Te).\textsuperscript{113} Research in TMDs has been ongoing ever since 1960s and several interesting properties exhibited by the materials were observed.\textsuperscript{112,114,115} For instance, TMDs can be insulating, semiconducting, metallic, or even superconducting, depending on the identity of the transition metal and chalcogen present in the material.\textsuperscript{116-118} In the bulk form, two-thirds of the TMDs exist as layered structures. Each of these layers
consists of covalently bonded chalcogen and transition metal atoms, arranged as three planes of atoms (X-M-X), as seen in Figure 2.3. The adjacent layers of the bulk TMDs are held together by weak van der Waals forces of attraction, like in the case of graphite, except that the TMDs exhibit a wide array of polytypes. Because of their amazing properties, bulk TMDs can be utilised in many applications, including batteries, electrocatalysts, lubricants, and solar cells.

Figure 2.3 Three-dimensional schematic representation of a typical layered MX$_2$ structure. The figure shows three layers of MX$_2$, each layer consisted of covalently bonded M-X arranged as three planes of atoms, with M forming the centre plane and X forming the top and bottom planes. Reproduced with permission from reference 113.

In the recent decade, two-dimensional TMDs such as MoX$_2$ and WX$_2$, which are often regarded as inorganic analogues of graphene, have been studied intensively due to their superior electronic and structural properties. They can be synthesised through top-down exfoliation or bottom-up synthesis approaches, some of which are analogous to those employed for graphene preparation. The thickness (from multilayers to monolayer) of the semiconducting MoX$_2$ and WX$_2$ were reported to influence their band gap behaviour, transiting from indirect band gap in multilayer TMDs to direct band gap in monolayer TMDs. With direct and sizable band gaps between...
1 to 2 eV, monolayer TMDs can be applied as field-effect transistors (FETs) that possess high on/off current ratios and decent carrier mobility.\textsuperscript{113, 134, 135} A monolayer TMD can exist in two coordination phases, namely trigonal prismatic (2H) and octahedral metal (1T) coordination phase.\textsuperscript{136} Studies have indicated that in the case of Group 6-based TMDs (eg. MoX\textsubscript{2}; WX\textsubscript{2}), a phase transition from 2H to 1T will cause the TMD to change from semiconducting to metallic, and this phase transition can be made possible through alkali intercalation.\textsuperscript{137, 138} The highly conductive 1T-MoS\textsubscript{2} can be utilised as an electrocatalyst for hydrogen evolution reaction as it has been demonstrated to exhibit excellent catalytic activity towards this reaction.\textsuperscript{139} Monolayer MoS\textsubscript{2} is also found to display photoluminescence property whose luminescence intensity is material thickness-dependent, making them suitable to be used in devices such as light-emitting diodes and photosensors.\textsuperscript{133, 140} Another important feature of two-dimensional TMDs is that they are highly elastic, as established by studies conducted on MoS\textsubscript{2} nanosheets (single to few-layers).\textsuperscript{141, 142} These semiconducting materials will be required in next generation electronics that are flexible, and in composite films as reinforcing agents.\textsuperscript{143}

Besides monolayer TMDs, inorganic nanotubes (INT) and fullerene-like nanomaterials (IF) of TMDs, in particular WS\textsubscript{2} and MoS\textsubscript{2}, have attracted much interest from scientific communities and industries for their excellent tribological properties and potential applications.\textsuperscript{144-146} In fact, products containing IF-WS\textsubscript{2} such as solid lubricants has already been commercialised since 2008.\textsuperscript{147} Recently, this class of nanomaterials has also shown potential to be impregnated into metallic coatings for medical administration.\textsuperscript{148} For example, a study demonstrated that the use of orthodontic wires coated with metallic film containing IF-WS\textsubscript{2} in dentistry could significantly reduce the mechanical forces required for teeth realignment, thus preventing unnecessary excess
forces that would lead to unacceptable teeth movement, longer treatment, and adverse damage to the roots of the teeth.\textsuperscript{147, 149}

With the IF-WS\textsubscript{2} already commercially available in the market and the likelihood of introducing IF- and INT-TMDs into medical applications in the future, extensive investigations on the biocompatibility and toxicity of these nanomaterials ought to be performed in order to ensure that they are safe for usage. Preliminary results from \textit{in vivo} toxicology tests of IF-WS\textsubscript{2} showed no apparent toxic effects on mammals, suggesting its high biocompatibility.\textsuperscript{150} In addition, \textit{in vitro} cytotoxicity examination of IF-MoS\textsubscript{2} on three different human cell lines revealed that it is non-toxic to the cells, as the cell viability results derived from MTT measurements remained close to 100\% even after 48 h exposure to the nanomaterial.\textsuperscript{151}

As the research on the toxicity of TMDs nanomaterials is still in its infancy with only a handful of assessments performed on IF-MoS\textsubscript{2} and IF-WS\textsubscript{2}, it is not surprising that almost no parallel studies have been conducted to determine the toxicity of two-dimensional TMDs nanomaterials yet. In one of the two available studies, \textit{in vitro} toxicity assessment of chemically exfoliated two-dimensional MoS\textsubscript{2} was conducted and it was reported that the nanomaterial was non-toxic to HeLa cells.\textsuperscript{152} Percentage cell viability remained at more than 80\% after 24 h treatment with up to 40 ppm of the chemically exfoliated MoS\textsubscript{2} nanomaterial. In the other study, Chng and co-workers compared the toxicity of two-dimensional MoS\textsubscript{2} that were exfoliated with different lithium intercalating agents, which resulted in these nanomaterials having different thickness.\textsuperscript{153} It was determined through cell viability assays that the thickness of the exfoliated MoS\textsubscript{2}, and thus the extent of exfoliation has an effect on the cytotoxicity of these
nanomaterials; cytotoxicity of two-dimensional exfoliated MoS$_2$ increases with higher degree of exfoliation (lower thickness).

While research on the cytotoxicity of two-dimensional MoS$_2$ nanomaterials has already begun, no similar studies have been performed for other Group 6-based two-dimensional TMDs, thus we will examine and compare the cytotoxicity of two-dimensional exfoliated MoS$_2$, WS$_2$, and WSe$_2$ in Chapter 10.
2.3 Micro-/nanomotors and their application in environmental remediation

Since pioneering works started slightly more than a decade ago, self-propelled micro- and nanomotors have attracted huge interest from the scientific community and tremendous efforts have been placed on the development of these synthetic devices over the past ten years. This is because the self-propelled micro-/nanomotors have shown great promise to revolutionise many areas of research, especially in the biomedical field for drug delivery and biosensing, as well as in environmental field for remediation purposes.\textsuperscript{154-165} We will focus on the environmental remediation application of micro-/nanomotors in this project and contribute towards the real-world environmental application of these devices.

In the following sub-chapters, the general features of self-propelled micro-/nanomotors and current studies involving the use of micro-/nanomotors for environmental remediation will be discussed.

2.3.1 General features of self-propelled micro-/nanomotors

A micro-/nanomotor can be broadly defined as a micro- or nanoscale device that has the ability to convert energy into movement and force. As these micro-nanomotors exist at small length scales, the effects of Brownian motion and viscous forces at low Reynolds numbers on their motion become significant, thereby causing difficulty for them to propel in water.\textsuperscript{166} There are several methods to fabricate micro-/nanomotors, and the frequently adopted fabrication techniques include electrochemical deposition, physical vapor deposition, and rolled-up technology.\textsuperscript{167}
Figure 2.4 Schematic depicting the motion of a self-propelled micro-/nanomotor through (A) self-electrophoresis, (B) self-diffusiophoresis, or (C) bubble propulsion mechanism. The schematic in (A) is reproduced with permission from reference 168.

Motion of self-propelled micro-/nanomotors is typically driven by mechanical forces generated from the conversion of stored energy in fuels, achieved through self-electrophoresis, self-diffusiophoresis, or bubble propulsion mechanisms. The shape and symmetry of the micro-/nanomotor play a major role in influencing the propulsion mechanism. Figure 2.4 illustrates the principles that lead to the generation of micro-/nanomotors motion in each of the three mechanisms. Self-electrophoretic micro-/nanomotors are usually bimetallic nanowires or Janus particles that would undergo opposing electrochemical half reactions with the fuel on each end of the conducting motor. This generates an electroosmotic flow on the micro-/nanomotors’ surface, which consequently induces propulsion of the micro-/nanomotor in the opposite direction. Pt/Au nanowire, as shown in Figure 2.4A, is an example of a self-electrophoretic
Catalytic oxidation and reduction of the $\text{H}_2\text{O}_2$ fuel occur at the Pt and Au end of the nanowire respectively, inducing electrons flow from the Pt end to the Au end inside the nanowire, accompanied by protons migration in the same direction on the surface of the nanowire. An electroosmotic flow will ensue on the surface of the nanowire, propelling the nanomotor in the direction of the Pt end. Self-diffusiophoresis of micro-/nanomotors is achieved through the creation of a concentration gradient of solutes around the micro-/nanomotors’ surface and the subsequent fluid flow from low solute concentration to high solute concentration regions. The concentration gradient is generated because fuel is preferentially reacted at the catalytic end of the micro-/nanomotor. Therefore, many spherical Janus micro-/nanomotors with asymmetrically distributed catalyst, like the one shown in Figure 2.4B, propel via the self-diffusiophoretic mechanism, with the direction of motion opposite to that of the fluid flow. Micro-/nanomotors powered by bubble propulsion mechanism typically possess tubular structure, but some catalytic Janus motors can also undergo bubble propulsion. As depicted in Figure 2.4C, $\text{H}_2\text{O}_2$ fuel will be decomposed by the inner surface of the microtube to produce oxygen bubbles which, upon detachment from the tubular micromotor, will give rise to a recoiling force on the micromotor, propelling it away from the bubbles.

Among the three mechanisms, bubble-propelled micro-/nanomotors tend to exhibit the highest velocity and power, thus such micro-/nanomotors attracted the most attention, and were preferentially utilised in many literature involving micro-/nanomotors. $\text{H}_2\text{O}_2$ is usually used as the fuel for bubble generation to propel the micro-/nanomotors, and platinum metal commonly served as the catalyst for the decomposition of $\text{H}_2\text{O}_2$ into water ($\text{H}_2\text{O}$) and oxygen ($\text{O}_2$) bubbles. Recently, efforts were made to replace $\text{H}_2\text{O}_2$ fuel with more biocompatible alternatives or eliminate the
need for external fuel source altogether by developing micro-/nanomotors capable of harvesting energy from water.\textsuperscript{184-188} For instance, Gao and co-workers fabricated Al–Ga/Ti Janus micromotors which could generate hydrogen bubbles through the reaction between aluminium and water to propel the micromotor.\textsuperscript{184} The Al–Ga binary alloy ensured continuous generation of hydrogen bubbles by efficiently removing the reaction-hindering aluminium oxide layer. Magnesium-based Janus micromotors have also been reported to undergo self-propulsion in water through similar reaction with water.\textsuperscript{187, 188} For example, it was demonstrated that Mg-Ti-Ni-Au Janus micromotors were able to propel in chloride-rich seawater.\textsuperscript{187} The authors explained that the Au layer of the micromotor and the high chloride content of the seawater, which resulted in macrogalvanic corrosion and pitting corrosion of Mg respectively, facilitated the efficient Mg-water reaction and subsequent hydrogen bubble formation. Besides water, acids and bases can also be utilised to power micro-/nanomotors through metal-acid/base reaction.\textsuperscript{185, 186} In one particular study, Pd/Al Janus micromotors were shown to propel autonomously under both acidic and basic conditions.\textsuperscript{186} This is because the amphoteric aluminium metal on the Janus micromotor can react with both acid and base to produce hydrogen bubbles for motion.

The speed of the micro-/nanomotors can be affected by several parameters of the fluid that they are propelling in, namely viscosity, ionic strength, and the presence of entities that can poison the catalyst on the micro-/nanomotors.\textsuperscript{163} It has been reported that an increase in viscosity of the solution will result in a corresponding decrease in the velocity of the micro-/nanomotors.\textsuperscript{189} Ionic strength, which has a positive correlation to the amount of ions present, also has negative influence on the velocity of the micro-/nanomotors. Zhao and co-workers studied the locomotion of bubble-propelled Cu/Pt micromotors in different types of real water, such as tap water, lake water, rainwater,
and seawater, and found that the micromotors moved at lower speeds when the percentage of each type of real water in the solution became higher.\textsuperscript{190} The authors then concluded that the concentration of inorganic ions in the water sample was the major contributor to the retardation in the micromotors’ speed, after working out the amount of ions present in each type of real water. Presence of chemicals that can potentially poison the catalytic surface of the micro-/nanomotors will cause the velocity of the motors to decline too. One such example is sulphur-containing molecules like cysteine. The thiol group in cysteine can inhibit the catalytic activity of platinum by attaching itself onto the active sites of the platinum catalyst, thus affecting the speed of micro/nanomotors that rely on platinum catalyst for propulsion.\textsuperscript{191} Besides sulphur-containing molecules, heavy metal ions like Pb\textsuperscript{2+} can also poison platinum catalyst, causing the speed of platinum-based micromotors to decrease.\textsuperscript{183} Therefore, we need to continue to design and develop new micro-/nanomotors whose velocity would be less influenced by these factors in order to maximise their full potential for real-world environmental remediation.

2.3.1 Environmental remediation using micro-/nanomotors

The utilisation of micro-/nanomotors for environmental remediation is still in its infancy. Research in this area only started a few years ago and most of the studies conducted thus far were mainly proof-of-concept studies that demonstrated the micro-/nanomotors’ ability to remove/degrade pollutants.\textsuperscript{157-160, 173, 174, 192-194} For example, Guix and co-workers reported the use of catalytic micromotors functionalised with a hydrophobic layer for removal of oil.\textsuperscript{192} The tubular micromotor consisted of an outer gold layer for functionalisation with alkanethiol through thiol chemistry to create a hydrophobic layer, and an inner platinum surface to power the micromotor via bubble
propulsion. Upon contact with the self-propelling micromotor, oil droplets in the solution will attach strongly to the hydrophobic surface of the micromotor through hydrophobic interaction, and move along with it, thereby achieving oil removal. A similar study involving alkanethiol functionalised microcap-like Pt/Ni/Au micromotors also illustrated the oil removal capability of the micromotors.

Besides oil removal application, micro-/nanomotors can serve as remediation agents for the degradation of pollutants. For instance, Soler and co-workers fabricated tubular bimetallic bubble-propelled micromotors for removal of organic pollutants such as rhodamine 6G. The metallic iron outer layer of the micromotors served as remediation agents, to degrade rhodamine 6G through the Fenton oxidation mechanism; the inner platinum layer functioned as the catalytic surface for H₂O₂ decomposition to produce bubbles for the micromotors’ propulsion. The group observed that the rate of removal of rhodamine 6G was approximately 12 times faster in the presence of the moving bimetallic micromotors as compared to similar experiments consisting of stationary rolled-up tubes made of iron only. They attributed the accelerated decontamination process to the enhanced mixing of the moving micromotors with the pollutants. In a separate work conducted by another group of researchers, Orozco and co-workers discovered that the use of PEDOT/Pt (PEDOT = poly(3,4-ethylenedioxythiphene)) microtubular motors can speed up the oxidative detoxification of organophosphate contaminants. The authors placed PEDOT/Pt micromotors into solutions containing organophosphate contaminants and H₂O₂, and observed that the micromotors’ continuous movement and bubble ejection led to an accelerated oxidative detoxification process. H₂O₂ acted as both the decontaminating reagent and the fuel for the PEDOT/Pt micromotors in this study. Similar to the study by Soler and co-workers, it was deduced that the enhanced degradation process observed
was the consequence of mechanical mixing resulting from the micromotors’ continuous motion and bubbling. The same group conducted a fundamental study later which provided strong evidence to support their deduction.\textsuperscript{195} Another study discussed the employment of activated carbon/Pt Janus micromotor that can result in the efficient removal of a wide variety of pollutants, including heavy metals (eg. Pb\textsuperscript{2+}), nitroaromatic explosives (eg. 2,4-dinitrotoluene), organophosphates (eg. methyl-paraoxon), and azo-dye compounds (eg. Rhodamine 6G), through adsorption to the activated carbon surface of the micromotors.\textsuperscript{160} As expected, the continuous propulsion and bubble generation of the Janus micromotors were essential for the accelerated decontamination observed.

As the research in self-propelled micro-/nanomotors continue to progress in the field of environmental remediation, there are several challenges that need to be addressed before we could realise the real-world application these devices.\textsuperscript{163, 173, 174} First of all, many of the current bubble-propelled micro-/nanomotors rely on the scarce and expensive platinum catalyst for the decomposition of H\textsubscript{2}O\textsubscript{2} fuel to generate bubbles for propulsion. This could be a potential problem when huge quantities are required to decontaminate large amounts of polluted water. Therefore, investigations should be carried out to source for alternative catalysts to replace platinum in micro-/nanomotors. In fact, several studies have already demonstrated the use of metals such as silver (Ag) and manganese oxide (MnO\textsubscript{2}) in micro-/nanomotors to catalyse the decomposition of H\textsubscript{2}O\textsubscript{2} for bubble propulsion and they have shown promising results.\textsuperscript{196-200} Of particular interest is a very recent study that described the detoxification of chemical and biological warfare agent (CBWA) simulants with bubble-propelled cubic silver-exchanged zeolite (Ag-Z) micromotors.\textsuperscript{198} The Ag-Z micromotor consisted of metallic Ag asymmetrically deposited on its outer surface to catalyse the decomposition of H\textsubscript{2}O\textsubscript{2} fuel for propulsion, and Ag\textsuperscript{+} incorporated within the pores of the zeolite, which functioned as
catalyst for the degradation of diethyl chlorophosphate and as an antibacterial agent for the elimination of *E. coli* bacteria. New micromotors that rely on Mg-water reaction to produce H₂ bubbles for propulsion were also developed to eliminate the use of platinum metal. A recent paper by Li and co-workers illustrated the utilisation of water-driven TiO₂/Au/Mg micromotors for photocatalytic degradation of CBWA simulants.¹⁵⁹ The micromotors were synthesised by first coating a layer of gold nanoparticles on a magnesium particle, followed by a layer of TiO₂ on top of the gold nanoparticles surface, leaving only a small opening to expose the magnesium core for its reaction with water. In presence of UV light, the TiO₂ surface reacted with water to produce highly oxidative species required for the degradation process. While magnesium-based micromotors have an added benefit of being powered by biocompatible fuel, they suffer a major drawback of having limited lifespan, as the metal will be consumed during the Mg-water reaction, thus limiting their practical usage. The issue of limited lifespan of micro-/nanomotors is the second challenge that needs to be looked into as we progress towards their application in the real-world. Thirdly, current fabrication techniques for bubble-propelled micro-/nanomotors are tedious, expensive, and might require advanced equipment, making it difficult to fabricate them in a truly large-scale (kilograms/tons) quantity at low cost and with ease to meet the demands for real-world application. Mou and co-workers have looked into this issue recently and presented a novel approach, termed as “growing-bubble-templated nanoparticle assembly”, for the fabrication of bubble-propelled micromotors.¹⁹⁴ The fabrication method is simple, low-cost, and is able to synthesise the micromotors in large-scale quantity. The authors prepared single-layered MnFe₂O₄ pot-like hollow particulate micromotors through this fabrication approach and demonstrated their oil removal capability. This study also highlighted the use of manganese (instead of platinum) for the catalytic decomposition
of H$_2$O$_2$ to power the micromotors. Lastly, current research in this area typically involved small volumes of solutions to investigate the micro-/nanomotors’ ability to degrade/remove the pollutant, which is not a true representation of a real-world scenario. As such, the findings from these studies might differ if the experiments were scaled-up. Therefore, it is important to verify whether the results obtained from the proof-of-concept studies will remain the same when the micro-/nanomotors are examined at the macroscale level.

In this project, some of the aforementioned challenges will be addressed. We will investigate the locomotion of a newly developed silver-catalyst based micromotor in Chapter 11. The utilisation of large-scale fabricated Fe(0) nanomotors for the degradation of azo-dye will be examined in Chapter 12. We will also determine the effect of continuous motion and bubbling of micromotors on mechanical mixing at the macroscale level in Chapter 13.
Chapter 2: Background and Literature Review

References


Chapter 2: Background and Literature Review


Chapter 3

Voltammetric determination of engineered nanoparticles I

Detection and quantification of copper oxide nanoparticles using cyclic voltammetry
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**Contributions:**

The experiments were performed by W. Z. Teo. The project was conceptualised and directed by A. Ambrosi and M. Pumera. All authors contributed to discussions and wrote the manuscript.
The quantification of ENMs using electrochemical techniques have been demonstrated for numerous metallic and metallic oxide nanoparticles and the utilisation of voltammetric techniques to achieve fast and reliable determination of metallic and metallic oxide nanoparticles will be extended in Chapters 3 and 4 to include ENMs that have yet to be examined.

In Chapter 3, the possibility of detecting and quantifying copper (II) oxide in alkali medium using cyclic voltammetry was investigated. Then, in Chapter 4, iron (II,III) oxide nanoparticles were studied with cyclic voltammetry in phosphate buffer solution to establish its electrochemical behaviour. Subsequently, the technique was used to determine if it is suitable for quantifying the amount of iron (II,III) oxide nanoparticles impurities in carbon nanotubes.

Chapters 3 and 4 are part of a collective work in addressing Objective 1.
3.1 Introduction

The increasing use of metallic and metallic oxide nanoparticles in consumer products will lead to inevitable release of emerging pollutants into the environment. Therefore, there is a high demand for facile and sensitive analytical methods for the rapid detection of these ENMs. Due to the electroactivity of most metallic and metallic oxide nanoparticles, electrochemistry represents a suitable method that can be used for this purpose. The detection can be carried out after the immobilisation of the nanoparticles on the electrode surface, and has in fact been applied for direct determination of a wide range of metal and metal oxide nanoparticles. Copper (II) oxide nanoparticles (CuO NPs) have been identified to induce severe cytotoxicity, thereby suggesting the importance of developing sensitive and rapid analytical tool to quantify their amount in a sample.

The electrochemistry at copper oxide nanoparticles (CuO NPs) has been studied previously and used as an electrochemical sensor for $\text{H}_2\text{O}_2$ determination. It was found that when the CuO NPs was subjected to cyclic voltammetry in an alkaline media, it exhibited a distinctive voltammogram with 2 oxidation and 2 reduction peaks. In this study, we utilised this unique feature of CuO NPs to investigate the relationship of the peak currents with the mass of CuO NPs present, with the objective to introduce an efficient and cost effective method to detect small amounts of CuO NPs in the environment.
3.2 Results and Discussion

![Cyclic voltammograms of copper (II) oxide nanoparticles of mass 0.5–15 µg deposited on GC electrode. Conditions: 0.1 M NaOH, scan rate, 0.1 V s⁻¹. The inset shows an SEM image of the copper (II) oxide nanoparticles, scale bar of 100 nm.](image)

**Figure 3.1** Cyclic voltammograms of copper (II) oxide nanoparticles of mass 0.5–15 µg deposited on GC electrode. Conditions: 0.1 M NaOH, scan rate, 0.1 V s⁻¹. The inset shows an SEM image of the copper (II) oxide nanoparticles, scale bar of 100 nm.

Figure 3.1 shows the typical cyclic voltammograms of CuO NPs modified glassy carbon electrode in 0.1 M NaOH. The small oxidation wave generated at about −0.6 V has been attributed to the adsorption of oxygen,⁸ while the two main anodic signals at −0.4 V (A1) and −0.1 V (A2) correspond to the oxidation of Cu(0) to Cu(I) and Cu(I) to Cu(II), respectively, in agreement with the following reactions:

\[
2Cu + 2OH^- \rightarrow Cu_2O + H_2O + 2e^- \tag{1}
\]

\[
Cu_2O + 2OH^- \rightarrow 2CuO + H_2O + 2e^- \tag{2}
\]

The cathodic peak at −0.35 V (C2) represents the reduction of CuO NPs to Cu(I). The cathodic peak C1 at −0.8 V results from the combination of two processes: reduction.
of Cu(I) to Cu(0) and also the reduction of Cu(II) to Cu(0). This explains why the reduction peak C1 involves a larger peak current with respect to the C2, although the mechanism of this double reduction is still unclear. The inset of the figure shows an SEM image of CuO NPs deposited on the glassy carbon electrode.

Figure 3.2 Plots of peak current of (I) A1, (II) A2, (III) C2 and (IV) C1 against varying mass of copper (II) oxide nanoparticles deposited on GC electrode. Data points from 0.05 to 15 μg. Other conditions as in Figure 3.1.

In order to investigate the relationship between redox peak response and the mass of CuO NPs, different concentrations of CuO NPs colloidal suspensions were drop-cast on the GC electrode prior to the cyclic voltammetric measurements. Figure 3.2 shows the concentration dependence of peak current of the 4 redox peaks against varying mass of CuO NPs. It can be seen that a linear plot was obtained for all the redox peaks investigated with the amount of CuO NPs deposited on the electrode surface, with the highest $R^2$ values obtained using the peak A1 (0.997) and C1 (0.995). Clearly the highest sensitivity (58.6 μA/μg) was achieved using the cathodic peak C1 being the
largest. This plot can therefore be adopted to quantify the amount of CuO NPs present in a sample.

![Figure 3.3](image.png)

**Figure 3.3** Cyclic voltammograms of copper (II) oxide nanoparticles of mass 25–100 ng deposited on GC electrode. Conditions: 0.1 M NaOH, scan rate, 0.1 V s\(^{-1}\).

To determine the limit of quantification (LOQ), which is the lowest level at which two concentrations can be distinguished, the GC electrode was modified with decreasing mass of CuO NPs and used for the cyclic voltammetry experiments (Figure 3.3). It was found that when 0.025 μg of CuO NP-modified GC electrode was subjected to cyclic voltammetry in 0.1 M NaOH, the peak currents measured were indistinguishable from those obtained with 0.050 μg of CuO NPs. 0.050 μg is therefore the lowest limit of quantification of CuO NPs using this method.

In summary, we have demonstrated that it is possible to determine the amount of CuO NPs based on its voltammetric signal in alkaline media. The voltammetric response was linear, with limit of quantification of 50 ng.
3.3 Experimental

3.3.1 Materials

Copper (II) oxide nanoparticles (Product No.: 544868), N,N-dimethyl formamide (DMF), potassium phosphate dibasic, sodium phosphate monobasic, sodium chloride, and potassium chloride were purchased from Sigma-Aldrich.

3.3.2 Apparatus

All voltammetric measurements were carried out using Autolab PGSTAT101 (Eco Chemie, The Netherlands) connected to a personal computer. Electrochemical experiments were performed in a 10-mL voltammetric cell, at room temperature (23 °C), using a three electrode configuration. A platinum electrode served as an auxiliary electrode and an Ag/AgCl electrode was used as reference electrode. A glassy carbon electrode (GC, diameter 3 mm) was purchased from CH Chemicals, USA and used as a working electrode.

3.3.3 Procedures

Colloidal suspensions of CuO NPs (diameter < 50 nm) of varying concentration were prepared in DMF. The suspensions were subjected to 15 min of ultrasonication for dispersion of the nanoparticles. Subsequently, 1 μL of the colloidal suspension of CuO NPs was drop-cast on the GC electrode and left to dry. Cyclic voltammetry was carried out in 0.1 M NaOH using the CuO modified electrode. The potential was scanned from 0.2 to −1.4 V (versus Ag/AgCl) and then reversed to 0.2 V for 5 cycles at a scan rate of 0.1 V s⁻¹. SEM analysis was performed using a JEOL-7600F semi-in-lens FE-SEM, with a working distance of 5.3 mm, carried out in gentle-beam mode at 5 kV.
Chapter 3: Detection and quantification of copper oxide nanoparticles using cyclic voltammetry

References


Chapter 4

Voltammetric determination of engineered nanoparticles II

Determination of redox-active iron-based nanoparticles in carbon nanotubes

4.1 Introduction

4.2 Results and Discussion

4.2.1 Electrochemical behaviour of Fe₃O₄ nanoparticles

4.2.2 Quantification of iron-based nanoparticles in carbon nanotubes

4.3 Experimental
The results in this chapter were published in the following manuscripts:


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Contributions:

The experiments were performed by W. Z. Teo. The project was conceptualised and directed by M. Pumera. All authors contributed to discussions and wrote the manuscript.
4.1 Introduction

The commercialisation of ENMs has led to concerns about their adverse long-term effects on our health and the ecosystem in recent years.\textsuperscript{1,2} Over the past decade, carbon nanotubes (CNT) were incorporated in a wide array of commercial products, including batteries, sporting equipment, boat hulls, and electrochemical sensors\textsuperscript{3-7}, despite being demonstrated to be toxic in both \textit{in vitro} and \textit{in vivo} studies.\textsuperscript{8,9}

CNT are commonly synthesised through the chemical vapour deposition (CVD) method, where metallic nanoparticles (e.g. iron, nickel, and molybdenum) are deployed as catalysts for the growth of the tubular carbon nanomaterials from carbon containing gas.\textsuperscript{10-12} This synthetic route typically produces CNT with embedded metallic impurities which remained in the nanomaterial (at 0.5 – 1.0 wt.\%) even after thorough washings.\textsuperscript{7,13-15} These impurities have been demonstrated to be responsible for the electrocatalytic effects of CNT toward various compounds like hydrogen peroxide\textsuperscript{16}, amino acids\textsuperscript{17}, sulphides\textsuperscript{18}, glucose\textsuperscript{19,20} and hydrazine\textsuperscript{21}. Furthermore, studies have shown that toxicological effects induced by CNT are influenced mainly by bioavailable metallic impurities present on the surface of the CNT.\textsuperscript{22-25} For instance, it was reported that by selectively removing the bioavailable portion of residual nickel and iron from CNT, the toxicity of CNT could be reduced significantly.\textsuperscript{26,27} Therefore, it will be of interest to determine the amount of redox-active/bioavailable metallic impurities in CNT samples by using rapid and simple analytical techniques such as cyclic voltammetry (CV). The quantification can be performed by immobilising the nanoparticles on the surface of a working electrode and measuring their voltammetric responses in suitable electrolytes, as demonstrated with the quantification of CuO NPs in \textbf{Chapter 3}. 

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Given the conditions of post treatments employed on the as prepared CNT, it has been proposed that the iron impurity present in the CNT samples should be in the form of iron oxide.\textsuperscript{19, 28} Thus, in this study, we investigated the electrochemistry of Fe\textsubscript{3}O\textsubscript{4} NPs in phosphate buffer solution (PBS) and exploited its voltammetric features to determine the possibility of quantifying the redox available iron-based nanoparticulate impurities in CNT. Iron oxides, for example Fe\textsubscript{3}O\textsubscript{4} NPs, have been suggested to induce oxidative stress and toxicity through the direct production of ROS or by releasing Fe\textsuperscript{2+} ions which in turn generate ROS via the Fenton reaction.\textsuperscript{29, 30} It is hence necessary to quantify these iron-based nanoparticulate impurities in the CNT.
4.2 Results and Discussion

4.2.1 Electrochemical behaviour of Fe₃O₄ nanoparticles

The electrochemical behaviour of Fe₂O₃ NPs in PBS have been studied previously by Marken group using tin-doped indium oxide (ITO) electrodes. In that work, it was revealed that when the Fe₂O₃ NPs were subjected to cyclic voltammetry, they were reduced to Fe²⁺ ions in the first cycle and the Fe²⁺ ions underwent reversible redox reaction with HPO₄²⁻ in the subsequent cycles. We conducted similar CV measurements on Fe₃O₄-NPs-modified glassy carbon (GC) electrodes in N₂ purged PBS (pH 4.0) and the corresponding voltammograms obtained are shown in Figure 4.1.

![Figure 4.1 Cyclic voltammograms showing the first three voltammetric cycles of Fe₃O₄ NPs (mass = 1 µg) deposited onto a GC electrode. Conditions: 50 mM PBS buffer solution (N₂ purged; pH 4.0), scan rate, 0.1 V s⁻¹. Potential measured against Ag/AgCl reference electrode.](image)

Generally, it is observed that Fe₃O₄ NPs displayed similar electrochemical features as Fe₂O₃ NPs. As potential was scanned towards the negative direction in the first cycle of the CV measurement, a broad cathodic peak at around −0.3 V, which could correspond to the reduction of Fe₃O₄ to Fe²⁺ [Eq. (1)], was detected. Upon reversal of the scan direction towards the positive potential, oxidation of Fe²⁺ by HPO₄²⁻ [Eq. (2)]
produced an anodic peak at approximately 0.15 V and this signal forms a redox couple with the reduction signal that appeared at about –0.18 V in the subsequent cycle. As the cathodic signal in the second cycle was the most well-defined, it was used for the quantification of the iron content in this study.

\[
\text{Fe}_3\text{O}_4 + 2e^- + 8H^+ \rightleftharpoons 3\text{Fe}^{2+} + 4\text{H}_2\text{O} \tag{1}
\]

\[
\text{Fe}^{2+} + \text{HPO}_4^{2-} \rightleftharpoons \text{FePO}_4 + e^- + \text{H}^+ \tag{2}
\]

**Figure 4.2** Cyclic voltammograms of Fe₃O₄ nanoparticles (mass = 1 µg) deposited onto a GC electrode. The Fe₃O₄-NP-modified GC electrodes were subjected to CV measurements in 50 mM PBS buffer solutions (N₂ purged) of different pH (2.0, 4.0, 10.0 and 12.0). Scan rate: 0.1 V s⁻¹; reference electrode: Ag/AgCl.

Apart from investigating the changes in the electrochemistry of Fe₃O₄ NPs with the number of voltammetric scans, we studied the effect of pH on the voltammetric response pertaining to the redox reaction of Fe²⁺ and HPO₄²⁻. Figure 4.2 shows the cyclic voltammograms (second scan) of bare and Fe₃O₄-NPs-modified GC electrodes in both acidic (pH 2.0, 4.0) and basic (pH 10.0, 12.0) PBS electrolyte (N₂-purged). Based on the
data illustrated in the figure, it can be concluded that the occurrence of the cathodic peak corresponding to the reduction of FePO$_4$ to Fe$^{2+}$ at $-0.18$ V is pH dependent, as the signal was detected only when the pH of the PBS electrolyte was 4.0. According to the pKa values of phosphoric acid, the predominant species in the PBS electrolyte are H$_3$PO$_4$ and H$_2$PO$_4^-$ when the pH = 2.0.$^{32}$ The absence of HPO$_4^{2-}$ ions in the electrolyte thus prevented the formation of FePO$_4$ in the first voltammetric scan, which in turn gave no cathodic signal at $-0.18$ V in the second cycle. On the other hand, the lack of H$^+$ ions under basic conditions could possibly be the main reason for the missing FePO$_4$ reduction peak.

### 4.2.2 Quantification of iron-based nanoparticles in carbon nanotubes

With a better understanding of the electrochemistry of Fe$_3$O$_4$ NPs, we proceeded to examine whether it was possible to attain the FePO$_4$ reduction signal from CV measurements of CNT-modified GC electrodes in N$_2$-purged PBS (pH 4.0) electrolyte. Three different CNT samples, namely CNT-pure, CNT-A and CNT-B, were dispersed and ultrasonicated for 15 min in DMF prior to drop-casting the respective suspensions on GC electrodes for CV analysis. From the cyclic voltammograms of the CNT samples (Figure 4.3), it is obvious that out of the three CNT samples, only CNT-B produced a cathodic peak in the potential range where reduction of FePO$_4$ to Fe$^{2+}$ occurs. The findings for both CNT-pure and CNT-B were consistent with ICP–AES/MS results, which indicated that CNT-pure was virtually free of iron impurity while CNT-B contained 0.25 wt.% of iron, hence suggesting that CV might be useful in determining the presence of iron-based nanoparticles in CNT samples.
Figure 4.3 Cyclic voltammograms of CNT-pure, CNT-A and CNT-B (mass = 5 µg) deposited onto a GC electrode. The gray shaded region represents the potential range where reduction of FePO₄ to Fe²⁺ occurs. Conditions: 50 mM PBS buffer solution (N₂ purged; pH 4.0), scan rate, 0.1 V s⁻¹. Potential was measured against Ag/AgCl reference electrode.

On the other hand, CNT-A-modified GC electrodes did not exhibit the FePO₄ reduction signal even though the total iron content in CNT-A was found to be 0.33 wt.%. The absence of the cathodic peak might imply that the amount of redox available iron in the CNT-A is below the detection limit of this analytical technique. In an attempt to verify this assumption, we subjected the three CNT samples to different length of ultrasonication times (Figure 4.4). This is because it has been demonstrated in a previous report that the redox availability of metallic impurities in CNT could be affected by the duration of the ultrasonication step.³³ The cyclic voltammograms of CNT-A in Figure 4.4
clearly illustrate that the FePO$_4$ reduction signal became detectable only upon performing a longer period of ultrasonication, thus proving that the absence of this cathodic peak in the CNT-A sample (prepared from 15 min of ultrasonication treatment) was indeed the result of very low redox available iron content. We believe that the typical ultrasonication technique to disperse CNT in solution for further processing have probably caused the release/exposure of iron oxide impurities in CNT, which are otherwise encapsulated within the carbon shells as discussed in previous studies.$^{27, 34}$ Therefore, it is important to note the preparation procedures of the CNT suspensions, in particular the ultrasonication duration, when determining the amount of redox available iron-based nanoparticles in CNT with cyclic voltammetry as they can affect the measured value.

**Figure 4.4** Cyclic voltammograms of CNT-pure, CNT-A and CNT-B (mass = 5 µg) prepared under (A) 15 minutes, (B) 30 minutes and (C) 60 minutes of ultrasonication prior to deposition onto a GC electrode. This figure only shows a portion of the CV, in the potential range of $-0.4$ V to 0.1 V. The grey shaded region represents the potential range where reduction of FePO$_4$ to Fe$^{2+}$ occurs. Conditions: 50 mM PBS buffer solution (N$_2$ purged; pH 4.0), scan rate, 0.1 V s$^{-1}$. Potential was measured against Ag/AgCl reference electrode.
Figure 4.5 Plot of peak current (from CV measurements) corresponding to the reduction of FePO$_4$ against the equivalent mass of Fe added to the 5 µg of CNT-B deposited onto a GC electrode. $R^2 = 0.995$. Conditions: 50 mM PBS buffer solution ($N_2$ purged; pH 4.0), scan rate, 0.1 V s$^{-1}$. Potential was measured against Ag/AgCl reference electrode.

The next step in the study was to attempt to quantify the amount of redox-active iron-based impurity present in the CNT-B sample. We accomplished this through the use of standard addition method (Figure 4.5) to find out the mass and wt.% of iron in 5 mg of CNT-B sample. From the extrapolated linear plot of peak current against the equivalent mass of Fe added, the mass of redox-active iron-based nanoparticles in the CNT-B sample was found to be 11.8 ng or 0.24 wt.%.

In summary, cyclic voltammetry was demonstrated to be a rapid and sensitive technique for the quantification of redox-active iron-based nanoparticulate impurities in a CNT sample (CNT-B). The capability to do this is important as the bioavailable metallic impurities are the main contributing factor towards the toxicity of CNT and it is thus necessary to determine the amount of these nanoparticulate impurities.
4.3 Experimental

4.3.1 Materials

Iron (II,III) oxide nanoparticles (Fe₃O₄ NPs), N,N-dimethyl formamide (DMF), potassium phosphate dibasic, sodium phosphate monobasic, sodium chloride, potassium chloride, CNT-pure, and CNT-A were purchased from Sigma–Aldrich. CNT-B was obtained from BuckyUSA, USA. The CNT were characterised previously using inductively coupled plasma-atomic emission spectroscopy/mass spectrometry (ICP–AES/ICP–MS): CNT-A contains 0.33% (wt.) of Fe, CNT-pure contains Fe below detection limits, and CNT-B contains 0.25% (wt.) of Fe.

4.3.2 Apparatus

Autolab PGSTAT101 (Eco Chemie, The Netherlands) connected to a personal computer was used for all electrochemical measurements. The electrochemical experiments were carried out in a 10 mL voltammetric cell at room temperature (23 °C), while adopting a three-electrode configuration. The auxiliary electrode is a platinum electrode while the reference electrode is an Ag/AgCl electrode. A glassy carbon electrode (GC, diameter 3 mm) was purchased from CH Chemicals, USA and used as the working electrode.

4.3.3 Procedures

4.3.3.1 Electrochemical behaviour study of Fe₃O₄ NPs in PBS

1.0 mg mL⁻¹ pure Fe₃O₄ NPs (diameter < 50 nm) colloidal suspension was prepared in DMF and subjected to 15 min of ultrasonication for dispersion. The colloidal suspension of pure Fe₃O₄ NPs (1 mg mL⁻¹; 1 µL) was then drop-cast on the GC electrode and left to dry. Subsequently, cyclic voltammetry was then carried out in 50 mM PBS (N₂ purged) of different pH using the Fe₃O₄ NPs modified electrode. The potential was scanned from
Chapter 4: Determination of redox-active iron-based nanoparticles in carbon nanotubes

0.5 to −1.0 V (versus Ag/AgCl) and then reversed to 0.5 V for 3 cycles at a scan rate of 0.1 V s⁻¹.

4.3.3.2 Quantification of redox-active iron-based nanoparticles in carbon nanotubes

Colloidal suspensions of pure Fe₃O₄ NPs (diameter < 50 nm) of various concentrations (0.5–2.0 mg mL⁻¹) and pure CNTs (CNT-pure, CNT-A and CNT-B) of concentration 10 mg mL⁻¹ were prepared in DMF. The suspensions were then subjected to 15 min of ultrasonication for dispersion of the nanomaterials. Subsequently, mixtures containing 5 mg mL⁻¹ of CNT and different concentrations of Fe₃O₄ NPs were made and further ultrasonicated for 5 min. Following that, cyclic voltammetry was performed using either pure CNT or Fe₃O₄-NPs–CNT mixture modified electrode (1 µL drop-cast and dried on GC electrode) in PBS (N₂ purged; pH 4.0) for the determination of redox-active iron-based nanoparticles in the CNT.
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Chapter 4: Determination of redox-active iron-based nanoparticles in carbon nanotubes

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Chapter 5
Fate of engineered nanomaterials in natural waters I

Fate of silver nanoparticles in natural waters

5.1 Introduction

5.2 Results and Discussion

5.2.1 Transformation analysis using conventional techniques

5.2.2 Electrochemical analysis of AgNPs transformation

5.3 Experimental
Chapter 5: Fate of silver nanoparticles in natural waters

The results in this chapter were published in the following manuscript:

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Contributions:

The experiments were performed by W. Z. Teo. The project was conceptualised and directed by M. Pumera. All authors contributed to discussions and wrote the manuscript.
After affirming that cyclic voltammetric can be utilised as a facial and sensitive technique for the determination of metallic and metallic oxide nanoparticles, the focus of this thesis shifted towards investigating the transformation of ENMs in various types of natural waters. In addition, cyclic voltammetry was introduced as a new analytical technique to monitor the transformation of ENMs.

In Chapter 5, silver nanopowder was dispersed in ultrapure water, lake water, and seawater and stirred for a week to compare its transformation in these aqueous systems. Several analytical techniques such as SEM, ICP–MS, XPS, and CV were used to characterise and quantify the silver nanoparticles after one week of stirring. The transformation of three graphene oxides in ultrapure water, lake water, rainwater, and seawater over three months were then analysed in Chapter 6 with Raman spectroscopy, XPS, and CV. The graphene oxides were synthesised through different preparation methods, namely Hofmann, modified Hummers, and Staudenmaier method.

Chapters 5 and 6 are also part of the collective work in addressing Objective 1.
5.1 Introduction

Silver, a transition element with an attractive white metallic luster, has been widely used to make jewellery, currency coins and silverware since ancient times.\(^1\) Also, the antimicrobial capability of silver has earned the metal its place in medicinal uses and the first nanosilver ever used for medical applications was manufactured under the name "Collargol" since 1897,\(^2\) eight years after the initial report on the synthesis of citrate-stabilised silver colloid by M. C. Lea.\(^3\) In the few decades that follow, other nanosilver formulations were invented and the commercial sale of these silver colloidal containing medicinal products for the treatment of syphilis and other bacterial infections soon became widespread.\(^4\) Today, together with 120 years of the extensive knowledge about nanosilver and the advancement of nanotechnology, many consumer products available in the market have silver nanoparticles (AgNPs) incorporated in them and the numbers are growing exponentially. According to an online database collated by the team behind the Project on Emerging Nanotechnologies, the number of consumer products containing AgNPs has increased more than tenfold from about 27 products in March 2006 to 383 products in October 2013.\(^5\) These products include aerosol sprays, containers, dietary supplements, cosmetics, washing machines, detergents, lotions, soaps, clothes and socks.

When these items are utilised, washed or discarded, the AgNPs embedded within would inevitably be released into the environment. For instance, a study conducted by Benn and co-workers on six types of socks containing AgNPs showed that leaching of AgNPs and Ag ions was achieved in some of them simply through immersion and shaking the socks in water.\(^6\) In two other separate studies, it was found that AgNPs were released through the usage of an advertised AgNPs producing washing machine and
Chapter 5: Fate of silver nanoparticles in natural waters

from painted outdoor facades under ambient conditions respectively.\textsuperscript{7,8} The widespread use of these anthropogenic sources signifies that the amount of AgNPs in the environment would increase drastically in the years to come, posing potential health hazards to humans and other biological organisms.\textsuperscript{9-11} Since the toxicity and transport of the AgNPs can be altered by its transformation in the environment, it is of utmost importance to devise protocols or analytical methods that could allow the study of these changes and the quantification of individual species of the element.\textsuperscript{12} In fact, the impact of environmental conditions in aquatic media on AgNPs, such as pH, ionic strength, ionic composition, dissolved organic matter, and exposure to natural lights, have been examined and published in several literatures.\textsuperscript{13-15} Therefore, the objective of this study is to analyse and compare the transformation of AgNPs from a single source dispersed in various environmental waters after one week of stirring, to extend the investigation on the fate of AgNPs in water.
5.2 Results and Discussion

Silver nanopowder was weighed and dispersed in different aqueous media (ultrapure water, lake water and seawater) to obtain AgNPs suspensions with concentration of 0.1 mg mL\(^{-1}\) each. The suspensions were stirred continuously for 7 days before they were sampled for analysis using various techniques. SEM was performed to characterise the structure of the AgNPs; ICP–MS and XPS were carried out to determine the amount of Ag ions and particulate Ag present respectively; and lastly CV was used as an alternative method to compare the amount of AgNPs left in the three different types of AgNPs suspensions after one week of transformation. In addition, the chemical state of the solid Ag in the suspension was identified using XPS. AgNPs–ultrapure water suspension served as the controlled experiment in this work.

5.2.1 Transformation analysis using conventional techniques

Figure 5.1 SEM images of AgNPs, prepared and stirred in (A) & (B) ultrapure water, (C) & (D) Nanyang Lake water and (E) & (F) seawater for one week, at different magnifications. Scale bars are 100 nm for (A) to (D), 100 µm for (E) and 10 µm for (F). Foreign materials, suspected to be living organism were found to be adsorbed to the nanoparticles surface (C, circled). The cubic structures in (E) are salt crystals formed from the AgNPs seawater suspension.
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Figure 5.2 SEM images of AgNPs, dispersed in (A) & (B) ultrapure water and (C) & (D) Nanyang Lake water, prior to one week of stirring. The images are taken at different magnifications and the corresponding scale bars are 100 nm. Images of the AgNPs-seawater suspension are not shown as the nanoparticles could not be observed due to the presence of salt crystals and a layer of material, similar to those shown in Figure 5.1E and 5.1F.

The morphology of the AgNPs, after dispersing in different aqueous media and subjected to one week of stirring, was characterised using SEM so as to determine if the sizes and shapes of AgNPs are affected by the waters they resided in. Furthermore, the morphology of the AgNPs prior to the stirring process was also characterised. The SEM images are shown in Figure 5.1 and Figure 5.2. It is obvious from Figure 5.1A and C that aggregates of spherical AgNPs were formed in ultrapure water and lake water media. The aggregation might have happened while the nanoparticles were still suspended in the aqueous medium, as a consequence of drying the SEM sample preparation or a combination of both. The AgNPs dispersed in seawater might have experienced similar aggregation but these nanoparticles could not be observed under the SEM because they
might be hidden underneath the salt crystals (Figure 5.1E) or a layer of material (Figure 5.1F), believed to be sediments from the seawater. Further inspection of the AgNPs from the AgNPs–ultrapure water suspension (Figure 5.1B) revealed that these nanoparticles seemed to have two distinctive sizes; the smaller sized AgNPs are around 25 nm in diameter while the bigger sized ones are about 80 nm in diameter. On the other hand, the particle size of the AgNPs from the AgNPs–lake water suspension appeared to be more uniform (Figure 5.1D), with an average diameter of about 70 nm. Moreover, foreign materials, likely organic matter, were found to be adsorbed on the AgNPs aggregates (Figure 5.1C) from the AgNPs–lake water suspension. However, the shapes and sizes of these AgNPs were very similar when they were initially dispersed in the different waters (Figure 5.2). Therefore, it can be suggested that morphology of the AgNPs could be affected by the variation of the aqueous media, which in turn affects the transport and fate of these nanoparticles once they are released into the environment.

Owing to its ability to perform simultaneous multi-elemental analysis, high precision and sensitivity, ICP–MS is one of the most popular quantification technique for the analysis of nanoparticles in the environment. In order to achieve speciation analysis of the nanoparticles, pretreatment steps such as filtration, centrifugation or extraction are coupled to these methods to separate the different species before running the experiments. For instance, it has been reported that cloud point extraction of AgNPs from commercial products containing both AgNPs and Ag\(^+\) was accomplished using surfactant Triton X-114. The separated silver species were then sent for ICP–MS analysis to determine the concentration of AgNPs and Ag\(^+\) present in the products. For this study, simple filtration was carried out using Whatman Anopore™ inorganic membranes with pore sizes of 20 nm to separate Ag\(^+\) from the AgNPs which are much bigger in size as compared to the pores. The filtrates obtained from the different AgNPs
suspensions were then sent for ICP–MS analysis to determine the amount of Ag species in the aqueous medium and the results are shown in Table 5.1. Similar measurements were performed on the three types of aqueous media before the dispersion of AgNPs and the values were subtracted from that obtained from the AgNPs suspensions to show the amount of Ag species present in the filtrate that belonged to the added AgNPs. In addition, these final values could be assumed to be Ag ions species, which are formed as a result of transformation of the AgNPs added, as the SEM images in Figure 5.1 showed that the AgNPs in the suspensions were generally larger than 20 nm.

Based on the values shown in Table 5.1, it is evident that even though the amount of AgNPs dispersed in the different aqueous media was the same initially, the concentrations of the Ag ion species present in the suspensions after one week of stirring were very different. This suggests that the extent of transformation of AgNPs to Ag ions could be affected by the medium they are dispersed in. By comparing the aqueous media used in this study, the extent of transformation was the greatest for AgNPs dispersed in seawater, followed by AgNPs dispersed in lake water, and lastly the AgNPs dispersed in ultrapure water, which had a concentration of Ag ions around one order of magnitude lower than the value obtained from the AgNPs–seawater suspension.

**Table 5.1** ICP–MS analysis of Ag species in various aqueous media obtained after filtration using alumina membrane with pore size of 20 nm, before and after dispersing with $0.1 \text{ mg mL}^{-1}$ of AgNPs and stirring for 1 week.

<table>
<thead>
<tr>
<th>Aqueous Medium</th>
<th>[Ag]$_{\text{initial}}$ in filtrate (before dispersion) / ppb</th>
<th>[Ag]$_{\text{final}}$ in filtrate after dispersion and 1 week stirring / ppb</th>
<th>[Ag]$_{\text{final-initial}}$ / ppb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrapure water</td>
<td>0.049</td>
<td>218.04</td>
<td>217.99</td>
</tr>
<tr>
<td>Lake water</td>
<td>0.33</td>
<td>467.01</td>
<td>466.69</td>
</tr>
<tr>
<td>Seawater</td>
<td>413.74</td>
<td>2683</td>
<td>2269.26</td>
</tr>
</tbody>
</table>
The residue from the filtration of the various AgNPs suspensions were left on separate alumina membranes and they contained the metallic silver which remained in solid state after one week of stirring. These membranes were analysed using XPS, a semi-quantitative analysis method that gives the surface elemental composition and state of materials with high sensitivity. A XPS wide survey scan is able to allow users to identify elements that are found up to a depth of 10 nm from the surface of the materials as distinctive signals will be recorded at different binding energies for different elements.\(^{20}\) In addition, information on the chemical state of the elements can be gathered with high-resolution XPS-core level scans.

![Figure 5.3](image)

**Figure 5.3** (A) Wide survey scan X-ray photoelectron spectra and (B) Ag 3d core-level spectra of (bl) blank alumina membrane and AgNPs residue from (U) ultrapure water, (L) lake water and (S) seawater deposited on the alumina membrane.

Figure 5.3A shows the wide survey scans of the membranes that contained a layer of residue containing AgNPs, left behind during the filtration of the different AgNPs suspensions. The survey scan of a bare alumina membrane is also included in the figure to act as a blank so as to identify the elements that originated from the membrane itself. It is observed that the inorganic membrane was made up of C, O and Al. The additional peaks that appeared in the scans of AgNPs residue from ultrapure water (Figure 5.3A, U)
and lake water (Figure 5.3A, L) belonged to Ag. The alumina membrane obtained from filtration of AgNPs–seawater suspension contained two other elements, namely Na and Cl, on top of the elements already mentioned above. These two elements should have originated from the evaporation of the seawater that remained on the alumina membrane after the filtration process.

High-resolution XPS scans were performed on Ag 3d core-levels to find out the nature of silver element in the residue and the spectra obtained are shown in Figure 5.3B. As shown in the figure, all three residues have characteristic Ag 3d5/2 peak at 367.9 eV accompanied by an Ag 3d3/2 peak 6.0 eV apart, signifying the metallic nature of the Ag element in the AgNPs suspensions and suggesting that the residues consisted mainly the unreacted AgNPs. On top of that, the intensity of the peaks from the high-resolution scans can give a semi-quantitative comparison of the amount of AgNPs present in the suspensions. It can be seen from the figure that the intensities of the peaks corresponding to the different AgNPs suspensions were the highest for the AgNPs dispersed in ultrapure water, followed by lake water and lastly seawater, whose intensities were very low as compared to the other two. This trend is in agreement to the results obtained from the ICP–MS measurements as a high concentration of silver ion content measured from the ICP–MS analysis would translate to a low content of metallic silver under the XPS analysis, based on the fact that the initial amount of AgNPs added to the different aqueous media was the same.

The chemical transformation of AgNPs in ultrapure water is probably the result of the oxidation of the metallic silver on the surface of the AgNPs by the dissolved oxygen in the water, which subsequently dissolved to form Ag⁺ ions. This transformation has been observed in other studies and it was reported that the dissolution could be
Chapter 5: Fate of silver nanoparticles in natural waters

enhanced by low pH.\textsuperscript{22-24} The higher Ag ion content in lake water could possibly be due to higher amount of dissolved oxygen in the natural water. Furthermore, the exceptionally high Ag ion content in seawater can be explained by the presence of chloride (detected in the wide survey XPS scan) in the medium. It has been shown that the extent of dissolution of AgNPs and the ratio of Ag/Cl in a suspension containing Cl\textsuperscript{-} ions is inversely related.\textsuperscript{25} This is because anionic complexes of AgCl\textsubscript{2}\textsuperscript{-}, AgCl\textsubscript{3}\textsuperscript{2-} and AgCl\textsubscript{4}\textsuperscript{3-} instead of AgCl precipitates will form when the concentration of Cl\textsuperscript{-} in the suspension is very high.\textsuperscript{12} Since seawater contains high level of Cl\textsuperscript{-} ions (13 333 ppm in seawater versus 3.80 ppm in lake water),\textsuperscript{26, 27} dissolution of AgNPs in this aqueous medium will be enhanced as compared to the other two types of water, resulting in the observations mentioned above.

It has been predicted from thermodynamics simulations that the possible speciation of AgNPs in freshwater and seawater systems is different, with AgCl, Ag\textsubscript{2}S and Ag(0) species dominating in the former system and AgCl\textsubscript{2}\textsuperscript{-} and Ag(0) in the latter system.\textsuperscript{12} From the high-resolution Ag 3d core-level scans, it was concluded that the particulate species of silver in AgNPs–lake water and AgNPs–seawater suspensions, which are representative of freshwater and seawater systems respectively, was mainly Ag(0). On top of that, the high level of Ag ions recorded in the AgNPs–seawater suspensions shows the likelihood of AgCl\textsubscript{2}\textsuperscript{-} species. Therefore, we established a positive correlation between experimental results and theoretical calculations from this study. The absence of AgCl and Ag\textsubscript{2}S species in the AgNPs–lake water suspensions could be caused by low concentrations of chlorides and sulphides in the freshwater system where the lake water was collected.
5.2.2 Electrochemical analysis of AgNPs transformation

As the study on nanoparticles in the natural environment and its transformation using traditional techniques like ICP–MS and XPS are rather tedious and time-consuming, novel methods based on electrochemical measurements are emerging in this field.\textsuperscript{16} For example, Compton and co-workers demonstrated the use of anodic particle coulometry (APC) technique in the detection and sizing of AgNPs in seawater media.\textsuperscript{28} They discovered that the AgNPs have a tendency to aggregate in seawater, with a faster aggregation rate when the salt concentration of the water is higher. In another study, ion selective electrode analysis technique was employed for the speciation analysis of AgNPs with promising accuracy and precision.\textsuperscript{29} With a novel silver ion selective electrode, the concentration of free Ag\textsuperscript{+} in AgNPs solution was first measured. Subsequently, the amount of AgNPs was determined by finding the total Ag content after subjecting the AgNPs solution to H\textsubscript{2}O\textsubscript{2} oxidation. With these successful reports, it will therefore be interesting to find out if CV could aid in the investigation of the transformation of AgNPs after they are released into different aqueous environment.

Herein, we attempted to determine if similar observations obtained from time-consuming techniques such as ICP–MS and XPS can be achieved using this facile and rapid analytical method. The cyclic voltammogram responses of bare GC electrode and AgNPs-modified GC electrodes from the three AgNPs suspension sources (ultrapure water, lake water and seawater) in PBS (pH 7.2) are compared in Figure 5.4. An oxidation peak at around +0.12 V (versus Ag/AgCl), which did not appeared for the bare GC electrode (Figure 5.4 inset), was observed for the voltammograms corresponding to AgNPs-modified GC electrodes (Figure 5.4a–c), thus suggesting that this peak corresponded to the oxidation of the Ag(0) species to Ag\textsuperscript{+} on the surface of the modified
electrode. This hypothesis is further supported by independent studies involving the
determination of the electrochemical responses of AgNPs in PBS conducted
elsewhere.\textsuperscript{30, 31} Another observation from the cyclic voltammograms is that the
electrochemical reaction of the AgNPs on the electrode surface is irreversible as no
signal corresponding to the reduction of Ag\textsuperscript{+} back to Ag(0) was recorded in all cases.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cyclic_voltammograms.png}
\caption{Cyclic voltammograms of AgNPs-modified GC electrode from AgNPs suspensions of (a) ultrapure water, (b) lake water and (c) seawater after 1 week of stirring. Conditions: 50 mM PBS buffer solution, scan rate, 0.1 V s\textsuperscript{-1}. Insert: cyclic voltammogram of bare GC electrode under the same conditions and scan rate.}
\end{figure}

The magnitude of the Ag oxidation peak current is dependent on the amount of
the AgNPs on the surface of the modified electrode, which is in turn equivalent to the
concentration of the nanoparticles present in the suspension. As the initial amount of
AgNPs added into the different aqueous media was the same, comparison of the AgNPs’
oxidation peak current will enable us to decide if the type of aqueous medium in which
the nanoparticles are suspended affects the extent of metallic silver transformation to
silver ions. From the magnitude of the peak currents shown in Figure 5.4, it is clear that the amount of AgNPs left in the different AgNPs suspensions after one week of stirring varied vastly, with the AgNPs-modified electrode prepared from the AgNPs–seawater suspension having the lowest magnitude of 0.52 μA and the one from AgNPs–ultrapure water suspension having the highest magnitude of 49.2 μA. Therefore, it can be concluded that the transformation of metallic silver to its ionic species is the highest in seawater and lowest in ultrapure water. Similar CV experiments were conducted (data not shown) using bare GC electrodes immersed in solutions containing the respective AgNPs suspensions diluted ten times with PBS (pH 7.2). It was found that even though an anodic peak corresponding to the oxidation of the Ag(0) species could be detected, the magnitudes of the peak current measured were random with very high relative standard deviation (%RSD = 57–107%).

In summary, the trend obtained from the CV experiments correlated with that from the ICP–MS and XPS analysis, hence, showcasing the capability of this simple and fast method to monitor the transformation of nanoparticles dispersed in different environment. In addition, it was found that morphology of AgNPs and its extent of dissolution could be altered by the type of water they are placed in. The greatest dissolution occurred in seawater, followed by lake water, and the least in ultrapure water. The change from AgNPs to Ag ions could be explained by the presence of dissolved oxygen and Cl⁻ in the media, whose concentrations have an impact on the amount of Ag ions species formed. Lastly, XPS analysis of the different AgNPs-suspensions revealed that the solid Ag present in all three types of suspensions contained mainly Ag(0) species.
5.3 Experimental

5.3.1 Materials

Silver nanopowder (Product No.: 576832, diameter <100 nm), potassium phosphate dibasic, sodium phosphate monobasic, sodium chloride, and potassium chloride were purchased from Sigma-Aldrich and used as received, without further purification. Ultrapure water (18.2 MΩ cm) from a Millipore Milli-Q purification system, lake water (Nanyang Lake, Nanyang Technological University, Singapore), and seawater (Sentosa Beach, Singapore) were used to disperse the silver nanopowders (0.1 mg mL⁻¹). The pH of the waters were 6.87 (ultrapure water), 6.42 (lake water), and 8.19 (seawater) respectively and the prepared AgNPs suspensions were stirred inside a fumehood with an ambient temperature of 23 °C. While no salts were found in ultrapure water, ion chromatography measurements revealed that the lake water contained ions such as Na⁺ (2.49 ppm), K⁺ (0.58 ppm), Mg²⁺ (0.47 ppm), Ca²⁺ (6.32 ppm), F⁻ (0.028 ppm), Cl⁻ (3.80 ppm) and SO₄²⁻ (2.36 ppm). On the other hand, the major ion components in a typical Singapore seawater sample include Na⁺ (7522 ppm), K⁺ (265 ppm), Mg²⁺ (1212 ppm), Ca²⁺ (346 ppm), Br⁻ (74 ppm), Cl⁻ (13 333 ppm) and SO₄²⁻ (2155 ppm).

5.3.2 Apparatus

JEOL-7600F semi-in-lens FE-SEM was used for SEM analysis, with images being captured in SEM mode at a working distance of 7.4 mm and an accelerating voltage of 5 kV. Cyclic voltammetric measurements were carried out using Autolab PGSTAT101 (Eco Chemie, The Netherlands) connected to a personal computer. Three-electrode configuration was adopted for the electrochemical experiments, in a 10 mL voltammetric cell at room temperature (23 °C). The auxiliary electrode is a platinum electrode while the reference electrode is an Ag/AgCl electrode. A glassy carbon electrode (GC, diameter 3 mm) was
purchased from CH Chemicals, USA and used as a working electrode. For XPS measurements, a Phoibos 100 spectrometer and a monochromatic Mg X-ray radiation source (SPECS, Germany) were used. ICP–MS was performed with Agilent 7700 (Japan) instrumentation to determine the amount of Ag ions present in the suspensions and the original aqueous media.

5.3.3 Procedures

The silver nanopowder was weighed and added into ultrapure water, lake water, and seawater separately to prepare 0.1 mg mL⁻¹ of AgNPs suspensions. The suspensions were then subjected to one week of stirring followed by 15 minutes of ultrasonication.

5.3.3.1 Morphology analysis of AgNPs

Samples for SEM were prepared by drop-casting 1 µL of the AgNPs suspension on a piece of copper tape that is washed with acetone for removal of artifacts. The samples were then dried in the fumehood overnight before placing them under the microscope for analysis.

5.3.3.2 Elemental analysis of AgNPs

5 mL of the AgNPs suspensions were filtered using Whatman Anopore™ inorganic membranes of diameter 25 mm and pore size 0.02 µm. The residue on the membrane was dried in the fumehood overnight and then analysed using XPS. Both widesurvey and high-resolution Ag 3d spectra were collected. Subsequently, the filtrate containing Ag ions and AgNPs smaller than 20 nm in diameter was analysed using ICP–MS. On top of that, the amount of Ag species present in the original aqueous medium was also carried out using ICP–MS.
5.3.3.3 Electrochemical analysis of AgNPs

Prior to each electrochemical measurement, the AgNPs suspensions were ultrasonicated for 3 minutes to ensure homogeneity. 2 µL of the AgNPs suspension was drop-cast on a GC electrode and left to dry for 15 minutes. CV was then carried out in 50 mM PBS (pH 7.2) with the AgNPs-modified GC electrode as the working electrode. The measurements were taken by scanning the potential from −1.0 V to +1.1 V (versus Ag/AgCl) and reversing back to −1.0 V again for three cycles at a scan rate of 0.1 V s\(^{-1}\). For each AgNPs suspension, at least three replicates were performed to obtain reliable results.
References


Chapter 6
Fate of engineered nanomaterials in natural waters II

Fate of graphene oxides in natural waters

6.1 Introduction

6.2 Results and Discussion
   6.2.1 Transformation analysis using conventional techniques
   6.2.2 Electrochemical analysis of graphene oxide transformation

6.3 Experimental
The results in this chapter were published in the following manuscript:


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Contributions:

The experiments were performed by W. Z. Teo. The materials were synthesised by Z. Sofer. The project was conceptualised and directed by M. Pumera. W. Z. Teo and M. Pumera contributed to discussions and wrote the manuscript.
6.1 Introduction

Since the first isolation of graphene a decade ago, the interest in this two-dimensional carbon-based nanomaterial has grown exponentially.\textsuperscript{1} Research in graphene has been mainly fuelled by the superior physiochemical properties of the nanomaterial, which include high electrical and thermal conductivity, excellent mechanical toughness, and optical transparency.\textsuperscript{2-4} More recently, derivatives of graphene with enhanced properties were synthesised through chemical modifications of the nanomaterial.\textsuperscript{5, 6} Graphene oxide (GO) is an example of graphene derivative that exhibits excellent physiochemical properties and it is essentially the oxidised form of graphene that contains a range of reactive oxygen functional groups such as epoxy, hydroxyl, carboxyl and carbonyl groups. Being inherently electroactive, as opposed to graphenes, GO nanoplatelets were successfully applied as labels for biosensing.\textsuperscript{7-11} The novel approach involved the use of reductive signals from the reactive oxygen functional groups on the surface of the GO nanoplatelets as analytical signals for the sensing of oligonucleotide or protein-target binding. Besides having the potential to be utilised in the biomedical field, GO also found its application in areas like catalysis and hydrogen storage.\textsuperscript{12, 13}

Given that our exposure to both graphene and its derivatives are likely to increase in the future, many studies have been conducted to determine their toxicities.\textsuperscript{14-18} These nanomaterials were found to induce \textit{in vitro} cytotoxicity based on several factors: the shape, size and quantity of the nanomaterial, the duration of cells’ exposure to the nanomaterials, the types of cells tested, and the cell culture conditions.\textsuperscript{19-21} Furthermore, an investigation conducted recently on the toxicity of GO using human lung carcinoma epithelial cells suggested that its cytotoxicity was
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influenced by GO concentration and the oxidative methods used to produce the nanomaterials, as the latter would affect the oxygen content/functional groups present in the GO.\textsuperscript{22}

With the discovery that cytotoxicity of GO might be influenced by the amount and type of oxygen-containing functional groups present in the nanomaterial, it will be vital to investigate its transformation in natural waters and find out whether there are changes to their physiochemical properties over time. This is because environmental waters are one of the possible destinations where the released GO will reside and interact with the ecosystems in their transformed state. By understanding the changes in the structure of the GO, their oxygen content and the identity of the oxygen-containing functional groups after prolonged exposure to environmental waters, we will be able to have a better idea of their toxicity and the extent of damage they might cause by their accumulation in the waters. Therefore, in this study, GO were dispersed and stored in different natural waters with the primary objective of examining these changes as a function of residence time in these waters. In addition, three types of GO, prepared from commonly employed oxidative treatments of graphite, namely the Hofmann (GO-HO), modified Hummers (GO-HU), and Staudenmaier (GO-ST) methods were used for this study to investigate if GO synthesised through different oxidative treatments will result in differing transformations in the same aqueous media.
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6.2 Results and Discussion

GO-HO, GO-HU, and GO-ST suspensions (0.1 mg mL\(^{-1}\) each) prepared by dispersing the respective nanomaterials in different aqueous media (ultrapure water, lake water, rainwater, and seawater) were analysed with Raman spectroscopy, XPS, and CV 1 week, 1 month, 2 months, and 3 months from the date of dispersion. It is known that varying conditions and oxidants used (chlorates in the Hoffmann and Staudenmaier methods while permanganates in the modified Hummers methods) in the oxidation of graphite will result in varying degree of oxidation, as well as produce GO with intrinsically different oxygen functionalities.\(^{23}\)

6.2.1 Transformation analysis using conventional techniques

![Bar charts showing the \(I_d/I_g\) ratios (obtained from Raman spectra) of GO-HO, GO-HU and GO-ST dispersed in ultrapure water, lake water, rainwater and seawater for a period of 1 week (■), 1 month (▲), 2 months (▼) and 3 months (▲).](image)

Figure 6.1 Bar charts showing the \(I_d/I_g\) ratios (obtained from Raman spectra) of GO-HO, GO-HU and GO-ST dispersed in ultrapure water, lake water, rainwater and seawater for a period of 1 week (■), 1 month (▲), 2 months (▼) and 3 months (▲).

In graphene and related carbon-based nanomaterials, the presence of defects or sp\(^3\)-hybridised carbon atoms in the network is indicated by a D (disorder) band at
approximately 1350 cm\(^{-1}\) in Raman spectroscopic measurements, whereas a G (graphitic) band at around 1560 cm\(^{-1}\) signifies pristine sp\(^2\)-lattice carbon atoms in the network.\(^{24,25}\) The ratio of the intensities of the D and G bands (\(I_D/I_G\)) calculated represent the density of defects present in the nanomaterials and changes in the structure (i.e., the amount of defects) of the GO as a result of their interaction with the four different waters can be derived from the Raman spectra. Figure 6.1 shows the \(I_D/I_G\) ratios of the various GO suspensions obtained from the Raman spectra measured at fixed intervals after dispersion of the GO in the aqueous media.

In general, the \(I_D/I_G\) ratios of the GO-natural water suspensions after 1 week of residence were very similar to the control GO-ultrapure water suspension originating from the same GO, which suggests that the density of defects of the GO was not affected by the type of aqueous media they were dispersed in initially. The density of defects of GO-HU (≈ 0.95) and GO-ST (≈ 1.03) remained almost the same over the period of investigation, regardless of the type of aqueous media, which signifies that the structure of these GO (amount of defects) was not affected by their interaction with the waters. However, GO-HO experienced a slight increase in defects while residing in the aqueous media; the overall increase in the \(I_D/I_G\) ratios ranged from 7% (ultrapure water suspension) to 11% (rainwater suspension), and the most apparent rise in the number of defects occurred during the first month, with the exception of the GO-HO-rainwater suspension. An increase in the density of defects can be attributed to either a change in the degree of oxygen functionalisation or breakage of the GO to create more edges/edge-like defects, which results in more sp\(^3\)-hybridised carbon atoms in both cases.\(^{25,26}\) Therefore, the respective GO suspensions were subjected to XPS analysis to determine whether the amounts of oxygen content in the GO were altered after residing in the natural waters.
Figure 6.2 Bar charts showing the C/O ratios (obtained from XPS spectra) of GO-HO, GO-HU and GO-ST dispersed in ultrapure water, lake water, rainwater and seawater for a period of 1 week ( ), 1 month ( ), 2 months ( ) and 3 months ( ).

XPS is a surface-sensitive analysis method that allows users to find out the surface elemental composition of the GO and the chemistry of the elements present. Identification of the elements is achieved during a wide survey scan, in which distinctive signals corresponding to individual elements appear at specific binding energies if they exist in the nanomaterial up to a depth of 10 nm from the surface. In addition, the amount of these elements (in terms of atomic percentage, at.%) can be determined from the wide survey and the density of oxygen functional groups in the GO can be obtained by calculating the atomic percentage of the carbon to oxygen (C/O) ratio. The C/O ratios of the respective GO suspensions are displayed in Figure 6.2. From this, it can be observed that the C/O ratio of the same GO was varied after being exposed to different aqueous media for 1 week; the oxygen content of the GO extracted from the GO-lake water and GO-seawater suspensions was generally higher than those in the GO-rainwater suspension, and the latter had a similar C/O ratio as the control GO-ultrapure
water suspension. This illustrates that the natural waters might play a role in affecting the amount of oxygen-containing groups present in the GO. Over a period of 3 months, the oxygen content of the three GO from the GO-lake water suspensions gradually decreased while a mixture of trends were established in other waters, thus suggesting that GO prepared from different oxidative methods did not react in the same manner with these waters. As mentioned earlier, the C/O ratio of the GO might contribute to the density of defects in their structure, in which a lower C/O ratio will translate to a higher density of defects. This correlation, however, was not observed in this investigation as the rise in the C/O ratio of the GO from the GO-lake water suspensions was accompanied by either a slight increase (GO-HO) or negligible change (GO-HU and GO-ST) in the $I_D/I_G$ ratio. Hence, the change in the degree of oxygen functionalisation of the GO was not able to fully explain the variation in their amount of defects and there should be other contributing factors, which could not be identified in this study.

In addition to performing XPS wide survey scans to determine the surface elemental composition of the GO, high-resolution XPS core-level spectra of the C1s peak were obtained (Figures 6.3–6.6) to establish the presence of different carbon bonds and their relative amounts. This was made possible through careful fitting of the spectra with the use of relatively sensitive factors. Deconvolution of the spectra of all the GO showed that they consist of C=C, C–O, and C=O bonding and there were insignificant changes in the relative abundance of these bond types over the period of 3 months in the waters.
Figure 6.3 High-resolution core-level C1s X-ray photoelectron spectra of GO-HO, GO-HU and GO-ST nanomaterials in the respective GO-ultrapure water suspensions. Measurements were taken after 1 week, 1 month, 2 months and 3 months from the initial dispersion of the GO. Each spectrum consists of C=C (▬), C-O (▬) and C=O (▬) bonding.

Figure 6.4 High-resolution core-level C1s X-ray photoelectron spectra of GO-HO, GO-HU and GO-ST nanomaterials in the respective GO-lake water suspensions. Measurements were taken after 1 week, 1 month, 2 months and 3 months from the initial dispersion of the GO. Each spectrum consists of C=C (▬), C-O (▬) and C=O (▬) bonding.
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Figure 6.5 High-resolution core-level C1s X-ray photoelectron spectra of GO-HO, GO-HU and GO-ST nanomaterials in the respective GO-rainwater suspensions. Measurements were taken after 1 week, 1 month, 2 months and 3 months from the initial dispersion of the GO. Each spectrum consists of C=C (-----), C-O (----) and C=O (-----) bonding.

Figure 6.6 High-resolution core-level C1s X-ray photoelectron spectra of GO-HO, GO-HU and GO-ST nanomaterials in the respective GO-seawater suspensions. Measurements were taken after 1 week, 1 month, 2 months and 3 months from the initial dispersion of the GO. Each spectrum consists of C=C (-----), C-O (----) and C=O (-----) bonding.

6.2.2 Electrochemical analysis of graphene oxide transformation

CV was also employed in this study as a simple, efficient, and cost-effective analytical technique to investigate the change in amount of electroactive oxygen-containing groups in the GO with respect to the residence time in different natural
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waters. GO, which is inherently electroactive owing to the presence of electrochemically reductive groups (epoxy, peroxyl, and aldehyde groups) in the nanomaterial, displays voltammetric signals that correspond to the amount of electrochemically reducible oxygen-containing groups when subjected to CV measurements.\(^{29}\) The peak current measured will then give an indication of the quantity of these groups. Therefore, the amount of electrochemically reducible oxygen-containing groups in the GO from the GO suspensions over a period of 3 months can be compared with this property. The magnitudes of the reduction peak current measured from the various GO suspensions after 1 week, 1 month, 2 months, and 3 months of GO dispersion in the waters are illustrated in Figure 6.7A. Overall, GO-HO and GO-ST nanomaterials (except GO-HO-ultrapure water) underwent an increase in peak current magnitude, whereas GO-HU suffered a loss (except GO-HU-rainwater) in the amount of electroactive oxygen-containing groups over a period of 3 months in the waters.

The rise was most prominent in the GO-ST, with a 49.6 to 69.4% increase in peak currents over a period of 1 week to 3 months of residence in the waters. It is interesting to note that the waters had opposing effects on the GO prepared using different oxidative methods, but the exact reasons that caused this phenomenon are unknown at this juncture. Further study can be carried out in the future to establish better understanding of the oxygen-containing functional groups present in the different GO as well as the interactions between these functional groups and the waters.

Epoxy, peroxyl, and aldehyde moieties are electrochemically reduced at different potentials and this characteristic was utilised to find out whether there was a change in the oxygen-containing functional groups in the GO. Based on the CV measurements, a shift in the peak potential/narrowing of the peak was observed for the GO-ST (Figure
6.7B) after they had resided in the ultrapure and lake water for 3 months. This suggests that some of the electrochemically reducible groups are completely lost over this period in these waters.

Figure 6.7 (A) Bar charts showing the peak currents (obtained from CV measurements) of GO-HO, GO-HU and GO-ST dispersed in ultrapure water, lake water, and rainwater for a period of 1 week (■), 1 month (▲), 2 months (■) and 3 months (■). (B) Cyclic voltammograms of GO-ST dispersed in ultrapure water, lake water and rainwater for a period of 1 week (▬), 1 month (▬), 2 months (▬) and 3 months (▬).

In summary, the data acquired in this study have allowed us to draw a few deductions. Firstly, the structure of the GO, in particular the density of defects, remained relatively the same over the short time period (3 months) in natural waters. The intrinsic properties, such as the amount and types of oxygen-containing groups
present, however, changed after interacting with the waters. Furthermore, the changes depended on both the types of GO and the aqueous media used, and might affect the toxicity of the GO as their toxicological effects were demonstrated to be reliant on the identity and quantity of the oxygen-containing functional groups.\textsuperscript{22} Lastly, more studies should be carried out to comprehend the mechanisms behind the transformation of the GO and assess their risks to the environment.
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6.3 Experimental

6.3.1 Materials

Sulphuric acid (98 %, p.a.), nitric acid (68 %, p.a.), potassium chloride (99 %, p.a.), potassium permanganate (99.5%, p.a.), hydrogen peroxide (30%, p.a.), and hydrochloric acid (35%, p.a.) were obtained from PENTA, whereas sodium chloride, potassium chloride, dibasic potassium phosphate, and monobasic sodium phosphate were purchased from Sigma–Aldrich. The chemicals were used as received, without further purification. Graphite microparticles (< 50 mm) were obtained from KOH-I-NOOR GRAFIT, Czech Republic.

6.3.2 Apparatus

Raman spectra of the GO were obtained by using a confocal micro-Raman LabRam HR instrument (Horiba Scientific) in back-scattering geometry with a charge-coupled device (CCD) detector, a 100× objective mounted on an optical microscope (Olympus) and a 514.5 nm Ar laser. For XPS measurements, a Phoibos 100 spectrometer and a monochromatic Mg X-ray radiation source (SPECS, Germany) were used. CV measurements were carried out using Autolab PGSTAT101 (Eco Chemie, The Netherlands) connected to a personal computer. A three-electrode configuration was adopted for the electrochemical experiments, in a 10 mL voltammetric cell at room temperature (23 °C). The auxiliary electrode was a platinum electrode whereas the reference electrode was an Ag/AgCl (1M KCl) electrode. A glassy carbon electrode (GC, diameter 3 mm) was purchased from CH Chemicals, USA and used as the working electrode.
6.3.3 Preparation of graphene oxide

6.3.3.1 Synthesis of GO using Hofmann method

A reaction flask containing a mixture of sulphuric acid (17.5 mL, 98%) and nitric acid (9 mL, 68%) was prepared and cooled at 0 °C for 15 min. Graphite (1 g) was subsequently added to the mixture under vigorous stirring to obtain a homogeneous dispersion. Potassium chlorate (11 g) was then slowly added to the mixture (over 15 min) at 0 °C to prevent a sudden increase in temperature and the formation of the chlorine dioxide gas, which is explosive at high concentrations. The reaction flask was loosely capped after complete dissolution of the potassium chlorate to allow the evolution of gas, and the mixture was left to stir continuously at room temperature for 96 h. Upon completion of the reaction, the mixture was poured into ultrapure water (1 L) and filtered. GO was then redispersed and washed repeatedly in HCl solutions (5%) to remove sulphate ions. Following that, repeated washing of GO with ultrapure water was carried out to obtain a neutral pH of the filtrate. Finally, the GO slurry was dried in a vacuum oven at 50 °C for five days before use.

6.3.3.2 Synthesis of GO using modified Hummers method

Graphite (0.5 g) was stirred with sulphuric acid (23.0 mL, 98%) for 20 min at 0 °C prior to the addition of sodium nitrate (0.5 g) in portions. The mixture was then left to stir for another hour while adding potassium permanganate (3 g) in portions at 0 °C. The mixture was subsequently heated to 35 °C for 1 hour before the addition of water (40 mL) into the mixture, which caused the temperature of the mixture to rise up to 90 °C. The temperature was maintained at this temperature for 30 min. Additional water (100 mL) was added into the mixture, followed by a slow addition of hydrogen peroxide (10 mL). Then, the warm solution was filtered and washed with warm water (100 mL).
Subsequent washings of the reaction mixture were carried out using copious amounts of water until a neutral pH was obtained. Finally, the GO slurry was kept in the oven at 50 °C for five days prior to usage.

6.3.3.3 Synthesis of GO using Staudenmaier method

A reaction flask containing a mixture of sulphuric acid (17.5 mL, 98%) and nitric acid (9 mL, 98%) was prepared and cooled at 0 °C for 15 min. Graphite (1 g) was subsequently added to the mixture under vigorous stirring to obtain a homogeneous dispersion. Potassium chlorate (11 g) was then slowly added to the mixture (over 15 min) at 0 °C to prevent a sudden increase in temperature and the formation of the chlorine dioxide gas, which is explosive at high concentrations. The reaction flask was loosely capped after complete dissolution of the potassium chlorate to allow the evolution of gas, and the mixture was left to stir continuously at room temperature for 96 h. Upon completion of the reaction, the mixture was poured into ultrapure water (1 L) and filtered. GO was then redispersed and washed repeatedly in HCl solutions (5%) to remove sulphate ions. Following that, repeated washing of GO with ultrapure water was carried out to obtained a neutral pH of the filtrate. Finally, the GO slurry was dried in a vacuum oven at 50 °C for five days before use.

6.3.4 Procedures

The GO was weighed and added into ultrapure water (Milli-Q, 18.2 MW cm), lake water (Nanyang Lake, Nanyang Technological University, Singapore), rainwater, and seawater (Sentosa Beach, Singapore) separately and subjected to ultrasonication for 1 hour to prepare 0.1 mg mL⁻¹ GO suspensions. The pH of the waters were 6.59 (ultrapure water), 6.56 (lake water), 4.69 (rainwater), and 8.12 (seawater), respectively. The suspensions were kept inside a fumehood under laboratory conditions (T=23 °C) and were subjected
to analysis using Raman spectroscopy, XPS, and CV after 1 week, 1 month, 2 months, and 3 months.

6.3.4.1 Raman spectroscopy and XPS analysis

Filtration of the GO suspension (4 mL each) was carried out using Whatman Anopore inorganic membranes (d=25 mm; pore size= 0.02 µm) to obtain the GO residue for examination under Raman spectroscopy and XPS. The membrane containing the GO was dried in the fumehood overnight prior to the analysis. For the Raman spectroscopy analysis, the instrument was first calibrated with a silicon reference at 520 cm\(^{-1}\) and achieved a peak position resolution of less than 1 cm\(^{-1}\). Measurements were then performed using the GO to acquire their spectra (1000–3000 cm\(^{-1}\)) and average \(I_D/I_G\) ratios were subsequently calculated from five measurements per GO. The XPS measurements were carried out at 12.5 kV with the Mg X-ray source to collect both wide survey and high-resolution C1s spectra for each GO. Relative sensitivity factors were then used to evaluate the atomic C/O ratios and the relative amounts of carbon bonds present from the wide survey and high-resolution C1s spectra respectively.

6.3.4.2 Electrochemical analysis

The GO suspension was subjected to ultrasonication for 2 min to ensure homogeneity prior to the CV measurements. Then, the GO suspension (3 µL) was drop-cast on a GC electrode and left to dry for 15 min. This process was repeated three times to ensure maximum coverage of the GC electrode. With the GO-modified GC electrode as the working electrode, CV was then carried out in 50 mM PBS (pH 7.2). The measurements were taken by scanning the potential from 0 to +1.5 V (versus Ag/AgCl) and reversing it back to −1.8 V at a scan rate of 0.1 V s\(^{-1}\). Three replicates were performed for each set of CV measurements to ensure that the results were reliable.
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References


Chapter 7
Toxicity of engineered nanomaterials I
Cytotoxicity of non-two-dimensional fluorinated nanocarbons

7.1 Introduction

7.2 Results and Discussion
7.2.1 Materials characterisation
7.2.2 In vitro cell viability assessment
7.2.3 Fluorinated nanocarbon-induced interference on cell viability assays

7.3 Experimental
Chapter 7: Cytotoxicity of non-two-dimensional fluorinated nanocarbons

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Contributions:

Most of the experiments were performed by W. Z. Teo. C. K. Chua carried out X-ray photoelectron spectroscopy measurements. The materials were prepared by Z. Sofer. The project was conceptualised and directed by M. Pumera. W. Z. Teo and M. Pumera contributed to discussions and wrote the manuscript.
Nanocarbons have been explored for potential applications in various fields, and this trend has increased tremendously following the successful isolation of graphene. Functionalisation of nanocarbons can alter the physiochemical properties exhibited by the original nanomaterial, some of which made the functionalised nanocarbons more appealing for utilisation. For example, halogenated graphenes possess enhanced optical, electronic, thermal, magnetic, mechanical, biological, and chemical properties in comparison to their graphene counterparts. In addition, emerging two-dimensional analogues of graphene like transition metal dichalcogenides have attracted interest from scientific communities and industries for their superior properties.

Given that halogenated nanocarbons and two-dimensional transition metal dichalcogenides might be incorporated into consumer products in the near future, concerns have arisen over their acute and chronic toxicity under relevant exposure situations. However, little has been performed to investigate the toxicity of these nanomaterials. In view of that, this project examined the cytotoxicity of various halogenated nanocarbons in Chapters 7, 8, and 9 to elucidate whether they are safe for utilisation in real world applications. Chapter 7 investigated the effect of size and morphology of fluorinated nanocarbons on its cytotoxicity while Chapter 8 compared the level of cytotoxicity induced by fluorinated graphenes with varying fluorine content. Chapter 9 assessed other halogenated graphenes, namely chlorinated graphene, brominated graphene, and iodinated graphene, to determine the influence of halogen content on its cytotoxicity. Subsequently, cytotoxicity assessment of two-dimensional exfoliated MoS$_2$, WS$_2$, and WSe$_2$ was performed in Chapter 10 of this project.

Chapters 7, 8, 9, and 10 are part of a collective work in addressing Objective 2.
7.1 Introduction

The preparation of fluorinated nanocarbons (FNC) is of interest as the materials are mainly employed in solid lubricants and primary lithium batteries.\textsuperscript{1-5} Lithium primary batteries with carbon fluoride-based cathode material, in turn, are commonly used to power cameras, electrical lock, electronic counter, electronic measurement equipment, emergency power source, memory back-up and medical implants, as they demonstrate enhanced energy densities, reliability, durability and safety.\textsuperscript{6} With the exponential increase in our usage of lithium primary batteries in consumer appliances, the likelihood of releasing FNC into the environment upon disposal and degradation of the exhausted lithium primary batteries will escalate as well. In view of this situation, it is vital to examine the \textit{in vitro} cytotoxicity of FNC so that measures can be imposed to process these nanomaterials if they are found to be detrimental to our health. This is especially crucial because the strong carbon-fluoride bond in the FNC will cause the nanomaterials to become persistent global contaminants, bringing harm to wildlife should they be diagnosed as health hazards.

Therefore, the objective of this study is to investigate the cytotoxicity of four different FNCs towards mammalian cells and determine if variables such as size, shape and fluorine content in the FNC will affect their toxicological effects. Human lung carcinoma epithelial cell line (A549) was preferentially utilised for the cytotoxicity assessment of the FNC as the respiratory tract is one of the common passageway where nanomaterials will enter, reside and begin its interaction with the body.
Chapter 7: Cytotoxicity of non-two-dimensional fluorinated nanocarbons

7.2 Results and Discussion

7.2.1 Materials characterisation

Characterisation of the four non-two-dimensional FNC were carried out with SEM and XPS to determine their size, morphology and chemical composition. These physiochemical properties are usually, if not always, linked to the level of cytotoxicity that the nanomaterials display; therefore, it is important and necessary to perform these characterisation measurements to facilitate interpretation of the cell viability data.\(^7\)\(^,\)\(^8\)

![SEM images of FNC-1, FNC-2, FNC-3, and FNC-4.](image)

**Figure 7.1** SEM images of A) FNC-1, B) FNC-2, C) FNC-3, and D) FNC-4. Scale bars are 100 nm for A and B, 1 µm for C, and 10 µm for D.

SEM was utilised to examine the size and morphology of the FNC, and the images captured are presented in Figure 7.1. From the figure, it is obvious that FNC-1 and FNC-2 are spherical particles that are about 30 to 100 nm in diameter and that they existed as aggregates. FNC-3 and FNC-4, on the other hand, consist of flakes and fibers, respectively, and they are at least one order of magnitude larger than FNC-1 and FNC-2.
Table 7.1 Atomic percentage (at.%) of elements present in the FNC as acquired from XPS spectra.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Carbon, C</th>
<th>Fluorine, F</th>
<th>Oxygen, O</th>
</tr>
</thead>
<tbody>
<tr>
<td>FNC-1</td>
<td>71.4</td>
<td>22.9</td>
<td>5.7</td>
</tr>
<tr>
<td>FNC-2</td>
<td>62.7</td>
<td>35.4</td>
<td>1.9</td>
</tr>
<tr>
<td>FNC-3</td>
<td>61.6</td>
<td>36.8</td>
<td>1.6</td>
</tr>
<tr>
<td>FNC-4</td>
<td>61.0</td>
<td>37.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Figure 7.2 High resolution core-level C1s X-ray photoelectron spectra of A) FNC-1, B) FNC-2, C) FNC-3, and D) FNC-4.

The chemical composition of the FNC was analysed by using XPS, and the elemental composition findings are summarised in Table 7.1. The atomic percentages of the elements found in FNC-2, FNC-3, and FNC-4 were roughly the same, whereas FNC-1 contained one-third less fluorine. Deconvolution of the high-resolution core-level C1s XPS spectra of the four FNCs (Figure 7.2) revealed the presence of C−F₂ (291.0 eV), C−F (289.0 eV), and C−CF₂ (287.0 eV) in all the nanomaterials examined. The ratio of these bonding types were similar in FNC-2, FNC-3, and FNC-4. On the other hand, FNC-1 contained additional types of bonding like C−CF (285.7 eV) and C=C (283.9 eV) in relatively significant proportions. Oxygen-containing groups such as carbonyls and carboxylic acids (288.7 eV) were also found in FNC-1.
The difference in fluorine content between FNC-1 and FNC-2 enabled us to investigate the dependence of fluorine concentration on their toxicological effects on A549 cells, as the two nanomaterials are of similar shape and size. Also, the relationship between structural features and cytotoxicity of FNC could be studied owing to the similarities in both elemental composition and ratio of types of carbon–fluorine bonding present in FNC-2, FNC-3, and FNC-4.

### 7.2.2 In vitro cell viability assessment

The investigation of FNC-induced cytotoxicity effects was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and water-soluble tetrazolium salt (WST-8) assays, both of which contain an active reagent that can be reduced to coloured formazan upon accepting electrons from viable cells.\(^9\), \(^10\) The amount of metabolically active cells present will be proportional to the concentration of the coloured formazan generated as well as the colour intensity of the resultant assay liquid. The cytotoxicity of the FNC can therefore be deduced by calculating the relative absorbance of the resultant assay liquids obtained from FNC-incubated A549 cells to the control A549 cells (without FNC exposure). Performing assessments using two assays that function on similar mechanisms can ensure that the cytotoxicity findings are sound and reliable if the data acquired are coherent and complementary.

Figure 7.3A displays the percentage cell viability of A549 cells derived from MTT assay absorbance measurements which were taken after subjecting the cells to FNC exposure for 24 h. Generally, cell viability decreased with increasing concentration of nanomaterials incubated across all the four FNC tested, revealing a dose-dependent toxicological effect of FNC on the A549 cells. Of these, FNC-4 exhibited the highest cytotoxicity, resulting in approximately 40% loss in cell viability at a low concentration of
12.5 µg mL\(^{-1}\) and almost 60% loss in cell viability at the highest concentration of 400 µg mL\(^{-1}\). On the other hand, 75.9% of A549 cells remained viable after incubating with 400 µg mL\(^{-1}\) of FNC-3 for 24 h, indicating its low cytotoxicity. Overall, the degree of toxicological effects induced by the four FNC can be ranked in the order of FNC-4 > FNC-1 > FNC-2 > FNC-3 based on the MTT assay data, with FNC-4 being the most toxic and FNC-3 being the least toxic.

![Figure 7.3](image)

**Figure 7.3** Percentage cell viability of A549 cells calculated from absorbance readouts of A) MTT assay measurements, and B) WST-8 assay measurements, following 24 h exposure to different concentrations of FNC. The percentages derived are relative to the absorbance values from A549 control cells that were not treated with FNC and the numbers represent mean ± standard deviation of at least three repeat experiments, with four wells per treatment per experiment.

The WST-8 assay contains 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt as the active reagent that gets reduced in the presence of viable cells. Whilst both the MTT assay and the WST-8 assay function on similar mechanisms, the WST-8 assay is considered more sensitive because, unlike the MTT assay, the formazan products formed are soluble and thus do not require additional dissolution steps which could lead to sample loss in the process.

According to the percentage cell viability calculated using the WST-8 assay absorbance values obtained from the FNC-treated A549 cells (Figure 7.3B), few
Inferences could be drawn. First, the cytotoxicity of the FNCs examined in this study can be arranged in the order of FNC-4 > FNC-2 > FNC-3 > FNC-1, where FNC-4 and FNC-2 imparted a similar level of toxicity at the highest concentration of 400 µg mL⁻¹ and FNC-1 did not induce any significant reduction in cell viability across all concentrations. Second, likewise to the MTT assay results, FNC-3 appeared to have low cytotoxicity here, with over 87.5% of A549 cells remaining viable even at the highest concentration of 400 µg mL⁻¹. Third, the FNC, with the exception of FNC-1 which had no effect on the cell viability, seemed to induce a dose-dependent decline in cell viability, and A549 cells incubated with FNC-2 experienced a sharp decrease of about 40% viability from 200 µg mL⁻¹ to 400 µg mL⁻¹ of FNC-2 introduced. Lastly, it is noted that the toxicity profile of FNC-1 derived from WST-8 assay measurements, which showed its almost non-existent cytotoxicity, was incoherent with that acquired from MTT assay measurements where 35% loss in cell viability was detected at the highest concentration of 400 µg mL⁻¹. This contradiction led to a disagreement in the cytotoxicity ranking of the FNCs deduced from the two assays and in view of this, FNC-induced interference experiments were performed under cell-free conditions to determine if the conflicting cell viability data were affected by artifacts caused by the nanomaterials. The findings from the FNC-induced interference experiments will be discussed later.

As mentioned earlier, physiochemical properties such as chemical composition, morphology, size, and surface modification can influence the interaction between the materials and the cells and ultimately impact the degree of cytotoxicity induced by the material.⁷, ⁸ Since FNC-2, FNC-3, and FNC-4 have very similar chemical composition (Table 7.1), the differences in their cytotoxicities are likely to be attributed to their sizes and morphologies. Based on the toxicity profile trends and SEM images of the FNCs, FNC-4 fibrous materials were the most toxic, followed by FNC-2 spherical particles, with
the least toxic being FNC-3 flakes. Even though FNC-2 particles were much smaller than FNC-4 and hence more likely to induce higher cytotoxicity, our results suggested otherwise. This could be due to the aggregation effect of the FNC-2 particles which enlarged their sizes tremendously, or stemming from the fibrous nature of the FNC-4 materials, which enhanced their toxicological effect on the A549 cells. The variation in the fluorine content of the FNC might also play a significant role in contributing to the cytotoxicity of the nanomaterials as Group VII elements are known to be hazardous, and previous studies have demonstrated that organofluorine compounds exhibit a wide range of toxicities.\textsuperscript{11, 12} This factor was studied by comparing the cell viability data of FNC-1 and FNC-2, which differ only in their chemical composition, but no conclusive proposition can be reached at this juncture because the absorbance measurements collected from the two assays gave rise to completely opposite association between the fluorine concentration and cytotoxicity of the FNC. As such, additional fluorinated nanocarbons with varying amounts of fluorine should be sampled in the future to achieve a better understanding of this relationship.

\textbf{7.2.3 Fluorinated nanocarbon-induced interference on cell viability assays}

Numerous reports have demonstrated that some nanomaterials are able to interfere with cell viability results derived from absorbance readouts of colorimetric assays such as MTT, WST-1, and XTT.\textsuperscript{13-15} There are several pathways on how they can influence the absorbance values: through 1) absorbing or scattering the light used during the absorbance measurements, 2) reducing the viability markers present in the assays without the presence of viable cells, or 3) binding to the insoluble formazan product which inevitably leads to the removal of the latter after subsequent washing or centrifugation.\textsuperscript{14} These interferences ultimately give rise to false absorbance readings.
and an over- or under-estimation of the nanomaterials’ cytotoxicity. For example, titanium dioxide and zinc oxide nanoparticles commonly found in sunscreens have the ability to absorb and scatter light in the UV and visible light region, hence their presence in the final assay liquid may affect the data obtained. Certain carbon-based nanomaterials like carbon black, carbon nanotubes and graphene were found to be capable of reducing the MTT viability markers to the insoluble purple formazan product under cell-free conditions, resulting in an underestimation of the nanomaterials’ cytotoxic effects. In addition, carbon nanotubes have the tendency to adsorb to the MTT formazan crystals and get removed during the washing and centrifugation steps, causing the absorbance values to be lower than the actual readings. With regards to the likelihood of FNC inducing erroneous absorbance data, experiments were carried out by mixing varying concentrations of the FNC with either MTT or WST-8 assays in the absence of cells to determine whether there are any significant interactions between the two.

Previous studies have shown that carbon-based nanomaterials can interfere with the MTT assay measurements by either reacting with the viability markers or binding to the insoluble formazan product, thus two interference assessments were performed. In the first assessment, mixtures containing the MTT reagent with different concentrations of FNC were incubated at 37 °C for 3 h in order to test for reduction of the MTT reagent mediated by the FNC in the absence of cells. In the second assessment, ascorbic acid was subjected to an hour-long incubation with the cell-free, pre-incubated (3 h) MTT-FNC mixtures to induce the reduction of the MTT reagent into the formazan product so as to investigate if the insoluble formazan will be adsorbed onto the FNC and be removed following centrifugation. The absorbance data collected from the two experiments
were normalised against the absorbance values of the control (not exposed to FNC) and shown as a normalised percentage of formazan generated in Figure 7.4.

**Figure 7.4** Normalised percentage of formazan generated from the incubation of FNC in the MTT assay under cell-free conditions for the investigation on A) the extent of the MTT viability markers’ reaction with different concentrations of FNC, and B) examination of the binding magnitude between MTT/formazan product and the FNC. The absorbance of the blank control measured in both A and B is represented by the black dashed lines in the graphs.

Figure 7.4A illustrates clearly that the normalised percentages of all four FNC tested are lower than that of the control (60.3–99.9%), indicating that the nanomaterials did not reduce the MTT reagents to generate formazan products, which will otherwise result in an increase in the absorbance values and the normalised percentages to beyond 100%. The gradual decrease noticed in the normalised percentages of the FNC (with the exception of FNC-3) might be caused by the scattering effect of the FNC during the absorbance measurements. Even though the decline in approximately 30% to 40% of the normalised percentages at the highest concentration (400 µg mL⁻¹) examined may seem quite substantial and may give rise to an over-estimation of the nanomaterials’ cytotoxicity, the absolute drop in the absorbance values is considered insignificant as it amounts to a maximum of only 1.5% difference in the absorbance readings collected in the MTT cell viability experiments. In addition, thorough washings were carried out to remove the FNC before the introduction of the MTT assay during the cell viability
assessments. Therefore, it is believed that the scattering effect imparted by the FNC, if any, is minimal and does not affect the validity of the MTT assay data. The normalised percentages gathered from the investigation on the adsorption effects of the FNC in Figure 7.4B depict that there were only slight variations in either direction (approx. ±15%), thus suggesting that insignificant binding effects were detected and the credibility of the MTT cell viability results is maintained.

![Figure 7.5 Normalised percentage of formazan generated from the incubation of FNC in the WST-8 assay under cell-free conditions for the investigation on the extent of the WST-8 viability markers' reaction with different concentrations of FNC. The absorbance of the blank control measured is represented by the black dashed lines in the graphs.](image)

In the case of determining FNC-induced artifacts in WST-8 assay measurements, only one control experiment, which examines the possibility of reaction between the FNC and WST-8 viability markers to form the formazan product, needed to be conducted. This is because adsorption of the soluble WST-8 formazan product onto the FNC is deemed highly unlikely, so it was not necessary to perform any control experiments to determine this interference. Figure 7.5 shows the relative percentages of the WST-8 formazan produced, as calculated from the absorbance readings of the mixtures (incubated at 37 °C for 1 h) containing the WST-8 assay and varying concentrations of FNC. Negligible reduction of the WST-8 reagents was observed for all
the four nanomaterials, with the highest normalised percentage being 114.3%. Also, similar to the trend observed in Figure 7.4A, all four FNC experienced a gradual decrease in their normalised percentages with increasing concentration of FNC introduced, indicating that the light scattering effect of these nanomaterials disrupts the WST-8 absorbance measurements as well. The drop in both the normalised percentages and the absolute absorbance readings at the highest concentration are comparable to the ones acquired for Figure 7.4A; hence, it was concluded that the validity of the WST-8 assay data in this study is unlikely to be affected by this marginal decrease.

In summary, the cytotoxicity information of four different FNC gathered showed that cell viability of mammalian cells could be affected after incubation for 24h with the FNC, and the degree of cytotoxicity induced by the FNC was dependent on the dose, shape, size, and fluorine content of the nanomaterial. Furthermore, measurements from FNC-induced interference experiments suggested that there were insignificant interactions between the FNC and the cell viability assays (MTT and WST-8). This indicates that the presence of FNC in minute quantities after thorough processing is unlikely to affect the validity of the cell viability values. However, owing to conflicting cytotoxicity profiles of FNC-1 acquired from MTT and WST-8 assays, no concrete deduction could be established concerning whether an increase in severity of the nanomaterial’s cytotoxicity is the outcome of lower or higher fluorine content in the nanomaterial.

We will attempt to determine the relationship between fluorine content in fluorinated graphenes and their cytotoxicities in Chapter 8, using the working procedures established in this study. Investigation into the toxicity profiles of other halogenated graphenes in Chapter 9 and two-dimensional exfoliated transition metal
dichalcogenides in Chapter 10 will see the application of the same working procedures as well.
7.3 Experimental

7.3.1 Materials

Fluorinated carbons were obtained from Advance Research Chemicals (USA). FNC-1 and FNC-2 were produced by fluorination of carbon black; FNC-3 was produced by fluorination of petrol coke; and FNC-4 was produced by fluorination of carbon fibers. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was purchased from Sigma-Aldrich, water-soluble tetrazolium salt (WST-8) from Dojindo, and dimethyl sulfoxide (DMSO) from Tedia.

7.3.2 Characterisation of fluorinated nanocarbons

JEOL-7600F semi-in-lens FE-SEM, operating in gentle-beam mode with an accelerating voltage of 1–2 kV, was used to obtain SEM images at a working distance of 4.9–7.2 mm. X-ray photoelectron spectroscopy (XPS) wide survey and high-resolution C1s spectra measurements were carried out with a Phoibos 100 spectrometer and a monochromatic Mg X-ray radiation source (SPECS, Germany) at 12.5 kV.

7.3.3 Cell Culture

Complete cell culture medium, which consisted of Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum, (FBS; PAA Laboratories) and 1% penicillin-streptomycin liquid (PAA Laboratories), was used to culture the A549 cell line (Bio-REV Singapore) in an incubator (37 °C) containing 5% CO₂. The typical cell cycle time of A549 cell line is 22 h and these cells are commonly used in nanotoxicological studies. Prior to the introduction of the FNC, A549 cells (570 μL/well) with a cell density of 8.8 x 10⁴ cells mL⁻¹ were first seeded in 24-well plates and incubated at 37 °C for 24 h.
7.3.3.1 Incubation of cells with fluorinated nanocarbons

Upon seeding the A549 cells in the 24-well plates for 24 h, the medium was removed from each well and rinsed with 1x PBS (pH 7.2; Gibco). Following that, different concentrations of FNC suspensions (570 μL/well) were added to the cells and incubated at 37 °C for another 24 h. Wells that consisted of only the cells and the culture medium, without exposure to the FNC, served as control for the experiment.

7.3.3.2 Cell viability measurements with MTT Assay

The MTT reagent (1 mg mL⁻¹) used for cell viability measurements in this study was prepared by diluting the MTT stock solution (5 mg mL⁻¹ in 1x PBS) with complete cell culture medium. The cell viability measurements began with removing the FNC suspensions from the A549 cells in the 24-well plates after 24 h exposure to the FNC. The cells were then washed twice with 1x PBS before adding the MTT reagent (300 μL/well) and incubating the cells at 37°C and 5% CO₂ for 3 h. Subsequently, the MTT reagent was removed from the 24-well plates and replaced with dimethyl sulphoxide (DMSO; 300 μL/well) to dissolve the insoluble purple formazan crystals produced by the viable cells. The plates were gently agitated for 5 min before the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the FNC. Finally, 100 μL of supernatant from each eppendorf tube was transferred to a 96-well plate for absorbance measurements at 570 nm and 690 nm, where the latter acts as the background absorbance. Data were represented relative to the absorbance obtained from control cells that were not exposed to any FNC.

7.3.3.3 Particle interference on MTT Assay

The tendency for FNC-induced artifacts in the MTT assay measurement caused by reactions between the nanomaterial and MTT was determined by first mixing varying
concentrations of FNC with the MTT reagent (1 mg mL\(^{-1}\)). The mixtures (500 µL/well) were then added into 24-well plates under cell-free conditions and incubated at 37°C for 3 h. The MTT reagent (1 mg mL\(^{-1}\); 500 µL/well) was also added into separate wells as control. Following incubation, the mixtures were removed from the wells and replaced with DMSO (500 µL/well). The plates were gently agitated for 5 min before the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the FNC. Finally, the supernatant absorbance was measured at 570 nm, with 690 nm as the background absorbance.

In addition to possible reactions between FNC and the MTT reagent, FNC interference on MTT assay can be attributed to binding of the FNC to the MTT molecule or the formazan product, thus an extra control test was essential. The MTT-FNC mixture (200 µL/well), as well as the MTT reagent control, obtained from the 3 h incubation of the two respective materials as mentioned above were mixed with 4 mM ascorbic acid (160 µL/well) and gently agitated for 5 min before incubating at 37°C for 1 h. The ascorbic acid served as a reducing agent for the reduction of MTT to formazan in the absence of cells. Subsequently, DMSO (720 µL/well) was added to the MTT-FNC-ascorbic acid mixture in the ratio of 2:1 and placed in the incubator at 37°C for 10 min. Following incubation, the MTT-FNC-ascorbic acid mixtures were transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the FNC. Finally, the supernatant absorbance was measured at 570 nm, with 690 nm as the background absorbance. The data obtained in both tests were represented relative to the absorbance obtained from the respective control MTT reagent that was not exposed to any FNC.
7.3.3.4 Cell viability measurements with WST-8 Assay

The working WST-8 assay used for cell viability measurements in this study was prepared by diluting the WST-8 stock solution by a factor of 10 with complete cell culture medium. The cell viability measurements began with removing the FNC suspensions from the A549 cells in the 24-well plates after 24 h exposure to the FNC. The cells were then washed twice with 1x PBS before adding the working WST-8 assay (300 μL/well) and incubating the cells at 37°C and 5% CO₂ for 1 h. Subsequently, the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the FNC. Finally, 100 μL of supernatant from each eppendorf tube was transferred to a 96-well plate for absorbance measurements at 450 nm and 800 nm, where the latter acts as the background absorbance. Data were represented relative to the absorbance obtained from control cells that were not exposed to any FNC.

7.3.3.5 Particle interference on WST-8 Assay

Since the WST-8 assay uses a water-soluble tetrazolium salt, reactions between the FNC and the WST-8 reagent will be the only potential interference with the viability assessment. Consequently, the probability of FNC reacting directly with the WST-8 reagent was determined. In the absence of cells, varying concentrations of FNC were mixed with the WST-8 stock solution to obtain resulting mixtures containing 10% v/v of the working WST-8 reagent. The mixtures (300 μL/well) were then added into 24-well plates and incubated at 37°C for 1 h. The working WST-8 reagent (10% v/v; 300 μL/well) was also added into separate wells as control. Following incubation, the mixtures were transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the FNC. Finally, the supernatant absorbance was measured at 450 nm, with 800 nm as the background absorbance. Data were represented relative to the absorbance obtained from the control working WST-8 reagent not exposed to any FNC.
Chapter 7: Cytotoxicity of non-two-dimensional fluorinated nanocarbons

References


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Chapter 8
Toxicity of engineered nanomaterials II

Cytotoxicity of fluorinated graphenes

8.1 Introduction

8.2 Results and Discussion
  8.2.1 Materials characterisation
  8.2.2 In vitro cell viability assessment
  8.2.3 Fluorinated graphene-induced interference on cell viability assays

8.3 Experimental
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Contributions:

Most of the experiments were performed by W. Z. Teo. Z. Sofer, F. Šembera, and Z. Janoušek synthesised the materials and carried out some of the characterisation measurements. The project was conceptualised and directed by M. Pumera. W. Z. Teo and M. Pumera contributed to discussions and wrote the manuscript.
8.1 Introduction

Graphene and its family of derivatives like graphene oxide, nitrogen-doped graphene and halogenated graphene have attracted tremendous attention among researchers in recent years due to their interesting physiochemical properties. For instance, fluorinated graphenes (F-G) were reported to exhibit varying band gap energies with different degree of fluorination and some of these nanomaterials displayed prominent photoluminescence in the blue/ultraviolet region, thereby allowing them to be potentially applied in band gap engineering or optoelectronics. The F-G were also determined to possess high thermal stability and the chemical inertness of fully fluorinated graphene (C\textsubscript{1}F\textsubscript{1}) was discovered to be comparable to Teflon. Consequently, these nanomaterials will be expected to remain persistently in the environment when F-G are introduced commercially and consumers products containing this material are disposed of subsequently. This situation can be detrimental to our health if these nanomaterials are toxic, hence it is crucial to examine the toxicity of F-G before they are even incorporated into future consumer devices.

Many of the studies found in the literature involving F-G focused mainly on the synthetic methods and physiochemical properties of their synthesised nanomaterials; few discussed on their toxicity. In the previous chapter, it was demonstrated that cytotoxicity of non-two-dimensional fluorinated nanocarbons towards mammalian cells could be affected by the physiochemical properties of the nanomaterials. The objective of this work, therefore, is to conduct similar examinations on three different F-G in order to elucidate how the differences in their physiochemical properties, in particular the fluorine content, affect the nanomaterials’ cytotoxicity towards A549 cells.
Chapter 8: Cytotoxicity of fluorinated graphenes

8.2 Results and Discussion

8.2.1 Materials characterisation

Characterisation of the three F-G were performed by various analytical techniques to examine the physiochemical properties of the nanomaterials. SEM was carried out to determine the morphology of the F-G. Elemental composition was investigated with a combination of combustible elemental analysis (CHN−O) and ion-selective electrode (ISE) measurement for fluorine concentration determination. Furthermore, XPS was performed to investigate the types of carbon bonding present in the nanomaterials. The structure and chemical composition was investigated using XRD and FTIR. The information obtained from the characterisation of the F-G will enable us to better interpret and understand the cytotoxicity data collected from the cell viability measurements as physiochemical properties of a nanomaterial are the primary factors that affect its toxicity.\textsuperscript{1,2}

Table 8.1 Atomic percentage (at.%) of elements present in the F-G as acquired from combustible elemental analysis (for C, H, N, and O) and ion-selective electrode (for F) data.

<table>
<thead>
<tr>
<th>Material</th>
<th>Carbon, C</th>
<th>Hydrogen, H</th>
<th>Nitrogen, N</th>
<th>Oxygen, O</th>
<th>Fluorine, F</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-G1</td>
<td>74.6</td>
<td>8.1</td>
<td>0.0</td>
<td>15.7</td>
<td>1.5</td>
</tr>
<tr>
<td>F-G2</td>
<td>46.7</td>
<td>7.3</td>
<td>0.1</td>
<td>3.2</td>
<td>42.6</td>
</tr>
<tr>
<td>F-G3</td>
<td>36.4</td>
<td>2.8</td>
<td>4.9</td>
<td>5.2</td>
<td>50.7</td>
</tr>
</tbody>
</table>

Table 8.1 summarises the atomic percentages (at.%) of all elements found in the three F-G. From the values, it was clear that both F-G2 and F-G3 nanomaterials were highly fluorinated, with as much as 50.7 at.% F found in F-G3. F-G1, on the other hand, can be described as fluorine-doped graphene nanomaterial as only 1.5 at.% of F is present in the nanomaterial.
Figure 8.1 SEM images of three different fluorinated graphenes (F-G1, F-G2 and F-G3). Scale bar at the bottom right of each image represents 100 nm (top) and 1 µm (bottom) respectively.

Figure 8.2 XRD spectra of three different fluorinated graphenes (F-G1, F-G2 and F-G3).

SEM images of the three F-G (Figure 8.1) revealed that the degree of exfoliation of F-G2 and F-G3 is much higher than that of F-G1. This is in agreement to the synthesis procedure of the three F-Gs, where F-G1 was prepared directly from the fluorination of graphite oxide while in the synthesis of F-G2 and F-G3, the graphite oxide were microwave exfoliated first before the fluorination procedure. Also, increasing amounts
of charging were observed from F-G1 to F-G3, which could be due to the nanomaterials becoming more insulated as more fluorine atoms are present in the nanomaterial. The structural properties were further investigated by XRD. The significant increase of interlayer spacing and broadening of reflections can be seen with increasing fluorine content. The interlayer spacing increased from 0.369 nm for F-G1 on 0.706 nm and 0.719 nm for F-G2 and F-G3, respectively. The results of XRD are shown in Figure 8.2.

Further analysis of the F-G chemical composition through deconvolution of their high-resolution core-level C1s X-ray photoelectron spectra (Figure 8.3A) revealed that all three nanomaterials contain C=C (284.5 eV) \( \text{C}^-\text{F} \) (289.0 eV) and \( \text{C}^-\text{F}_2 \) (291.9 eV) bond types in different amounts. In addition, other carbon-fluorine bond types were found in F-G2 and F-G3, namely \( \text{C}^-\text{CF} \) (286.1 eV; F-G2 only), \( \text{C}^-\text{CF}_2 \) (287.3 eV), \( \text{CF}^-\text{CF}_2 \) (290.5 eV), and \( \text{C}^-\text{F}_3 \) (293.5 eV; F-G3 only).\(^{13, 14}\) Besides fluorine-containing groups, oxygen-containing groups such as epoxy/hydroxyl (C–O; 286.0 eV), carbonyls (C=O; 287.3 eV), and carboxylic acids (O–C=O; 288.7 eV) were detected in F-G1 which contained at least thrice the at.% of oxygen as compared to F-G2 and F-G3. The FTIR spectroscopy measurement gives more information's about the chemical bonds in F-G. The FTIR spectra of F-G2 and F-G3 spectra are dominated by C–F vibration band located around 1150 cm\(^{-1}\). The C–F band is significantly weaker in F-G1 with lower concentration of fluorine. The results of FTIR spectroscopy are shown in Figure 8.3B.
As graphene are functionalised with increasing number of fluorine atoms (from F-G1 to F-G3), the planar structure becomes increasingly puckered as the carbon atoms change from sp$^2$ to sp$^3$ hybridisation. Consequently, the F-G will adopt different structural arrangements like chair, stirrup, boat or twist conformations, and it has been reported that while the chair configuration is the most stable conformer for fully fluorinated graphene, the stirrup configuration played a significant role in a mixed
fluorinated graphene sample.\textsuperscript{15, 16} Therefore, F-G2 and F-G3 are likely to possess the chair conformation, whereas F-G1 is expected to adopt the stirrup configuration.

With a better knowledge on the elemental content and types of carbon bonds available in these three F-G, we would be able to establish the relationship between these features and the nanomaterials’ cytotoxicity, if any.

### 8.2.2 In vitro cell viability assessment

We examined the cytotoxicity of F-G towards A549 cells through the use of two cell viability assays, namely 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and water-soluble tetrazolium salt (WST-8) assays. Both MTT and WST-8 assays function on similar principles, hence we could ensure the reliability of the cell viability assessment if both assays show identical trends.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure84.png}
\caption{Percentage cell viability of A549 cells calculated from absorbance readouts of A) MTT assay measurements, and B) WST-8 assay measurements, following 24 h exposure to different concentrations of F-G. The percentages derived are relative to the absorbance values from A549 control cells that were not treated with F-G and the numbers represent mean ± standard deviation of at least three repeat experiments, with four wells per treatment per experiment.}
\end{figure}

Absorbance readouts of the MTT assay after incubating with A549 cells (F-G exposed) were acquired and shown as percentage cell viability in Figure 8.4A. It is observed from the figure that all three F-G induced dose-dependent cytotoxicity on the
A549 cells, as a trend of decreasing A549 cell viability with increasing dosage of F-G introduced can be seen across all three nanomaterials tested. This dose-dependent toxicological effect is especially evident for F-G2, where the percentage cell viability changed from 95.8% at the lowest concentration of 3.125 µg mL\(^{-1}\) to 22.5% at the highest concentration of 400 µg mL\(^{-1}\) F-G2 exposure. At the highest concentration of F-G exposure, cell viability of the A549 cells incubated with F-G1, F-G2 and F-G3 were 76.8%, 22.5% and 73.0% respectively. Hence, based on the MTT assay assessment, the order of the level of cytotoxicity exhibited by the three F-G is F-G2 > F-G3 > F-G1; F-G2 is the most toxic while F-G1 is the least toxic.

Figure 8.4B displays the percentage cell viability derived from A549 cells (F-G exposed) which were incubated with WST-8 assay. Similar to the trend depicted in Figure 8.4A, decreasing A549 cell viability with increasing concentration of F-G exposure was noted in Figure 8.4B for all three nanomaterials examined, and again F-G2 showed the steepest drop in cell viability, changing from 100% at the lowest concentration to 6.8% at the highest concentration. In addition, likewise to the MTT assay data, the cytotoxicity of the three F-G is in the order of F-G2 > F-G3 > F-G1. In both assays, the least toxic F-G1 induced very low cytotoxicity to A549 cells, as the loss in cell viability was only 23.2% (MTT assay) and 13.0% (WST-8 assay) at a very high dosage of 400 µg mL\(^{-1}\) F-G exposure.

Since the toxicity profile trends of the F-G acquired from the MTT assay and WST-8 assay were coherent and consistent, we moved on to look into the factors that attributed to the differences in the degree of cytotoxicity induced by the three F-G. Judging from the elemental composition of the three F-G (Table 8.1), even though we believed that the amount of fluorine found in the nanomaterial plays a role in affecting
the cytotoxicity of F-G, it might be harsh to simply conclude that an increase in fluorine content in the F-G will contribute to higher cytotoxicity of the nanomaterial as F-G3 which has the highest at.% of F available (50.7 at.%) is at least 3 times less toxic than F-G2, which contains 42.6 at.% of F. In-depth analysis of the types of carbon–fluorine bonding found in the F-G (Figure 8.3A), however, might shed some light on the toxicity profile trends observed in this study. Between F-G2 and F-G3, although both contain large quantity of fluorine atoms, the amount of individual carbon–fluorine bonds in the two nanomaterials were largely different; the carbon atoms in F-G2 were mainly mono-substituted with fluorine while F-G3 has more carbon atoms which were di-substituted/tri-substituted with fluorine. This contrast could possibly be the determining factor of their cytotoxicity profile as it had been reported in the past that lower level of toxicity in fluoro-substituted alkanes/alkenes was associated with increasing number of fluorine atoms in the molecule.\(^\text{17}\) In addition, there is also a likelihood that F-G1 exhibited low cytotoxicity as a result of being less exfoliated than the other two F-G. This positive correlation between the level of exfoliation of a material and its cytotoxicity has been observed in other two-dimensional nanomaterials such as MoS\(_2\).\(^\text{18}\)

Besides that, it had been demonstrated in a previous study that the intermediate product, HO-GO (graphene oxide prepared by the Hofmann method), induced a dose-dependent toxicity on A549 cells, with approximately 43% (from MTT assay) and 50% (from WST-8 assay) of the cells remaining viable after incubating the cells with 400 \(\mu\)g mL\(^{-1}\) of HO-GO for 24 hours.\(^\text{19}\) By comparing the cytotoxicity profiles between HO-GO and the three F-G, we inferred that the fluorination procedure have altered the cytotoxicity of the final products, either by making them less toxic (in the case of F-G1 and F-G3) or more toxic (in the case of F-G2). Consequently, it was believed that the
absolute amounts of fluorine found in the nanomaterial, as well as the extent of fluoro-substitution are likely to be the critical factors influencing the trends observed.

8.2.3 Fluorinated graphene-induced interference on cell viability assays

As mentioned in the previous chapter, particles in the nanometre scale range might cause the absorbance measurements of cell viability assays such as MTT, WST-1 and XTT to be erroneous, leading to false estimation of the nanomaterials' cytotoxicity. In view of the likelihood of F-G inducing similar artifacts on the MTT assay, we performed two assessments to determine whether there are significant interactions between the F-G and the MTT assays in the absence of cells.

Different concentrations of F-G were mixed with the MTT reagent under cell-free conditions and incubated at 37 °C for 3 h in the first assessment to determine if the MTT viability markers will be reduced by F-G. Figure 8.5A shows the normalised percentage of formazan generated and clearly no reduction of the MTT viability markers by F-G took place as the normalised percentages recorded were all ≤100% (71.9–100%) across all three F-G. However, it was noted from the figure that there was a gradual decrease in the normalised percentages of all the three F-G, which could probably be the result of scattering effect caused by the nanomaterials during the absorbance measurements. A similar trend was observed in Chapter 7 involving the cytotoxicity study of fluorinated nanocarbons. Since the drop in the normalised percentages were less than 20% (except at 400 µg mL⁻¹ F-G) and substantial washings were carried out to remove most of the F-G before incubating the F-G exposed-cells with the MTT assay during the cytotoxicity assessments, this scattering interference by the F-G on the MTT assay was considered insignificant and would not render the MTT cell viability data invalid.
Figure 8.5 Normalised percentage of formazan generated from the incubation of F-G in the MTT assay under cell-free conditions for the investigation on A) the extent of the MTT viability markers’ reaction with different concentrations of F-G, and B) examination of the binding magnitude between MTT/formazan product and the F-G. The absorbance of the blank control measured in both A and B is represented by the black dashed lines in the graphs.

The second assessment was conducted to examine if the insoluble MTT formazan product will bind to the F-G and be removed subsequently during centrifugation. By subjecting ascorbic acid to the cell-free, pre-incubated (3 h) MTT–F-G mixtures for an hour of incubation at 37 °C, the MTT reagent will be reduced into the formazan product. The mixtures were then centrifuged before performing absorbance measurements and the normalised percentages of formazan formed are shown in Figure 8.5B. The normalised percentages recorded showed only slight variation from 100% (approx. ±10%), thus indicating that there were no interference arising from binding between the F-G and the MTT formazan product, and the MTT cell viability results in Figure 8.4A can be deemed as interference-free.

WST-8 assay has also been shown to be able to react with nanomaterials without the presence of viable cells to produce soluble formazan product. Therefore, we investigated the possibility of WST-8 formazan generation by F-G in the absence of viable cells. The relative percentages of the WST-8 formazan produced, calculated from the absorbance readings of the mixtures containing WST-8 assay and varying
concentrations of F-G, are illustrated in Figure 8.6. Similar to the data obtained in Figure 8.5A, no reduction of WST-8 viability markers were detected across all three F-G. Instead, they experienced gradual decrease in the normalised percentages with increasing amounts of F-G, indicating that the light scattering effect induced by these nanomaterials disrupts the WST-8 absorbance measurements as well. Although the drop in the normalised percentages from the WST-8 measurements (52.9–100%) is relatively higher as compared to the values from the MTT measurements, we believed that the credibility of the WST-8 assay data in Figure 8.4B will be maintained as the absolute drop in the absorbance values only constituted to a marginal decrease in the absorbance readouts from the WST-8 cell viability experiments.

Figure 8.6 Normalised percentage of formazan generated from the incubation of F-G in the WST-8 assay under cell-free conditions for the investigation on the extent of the WST-8 viability markers’ reaction with different concentrations of F-G. The absorbance of the blank control measured is represented by the black dashed lines in the graphs.

In summary, we investigated the cytotoxicity of three F-Gs with differing elemental content in this study. Based on the cell viability assessments results, it seemed that F-G with higher amounts of fluorine atoms, especially those rich in monofluoro-substituted groups, imparted higher toxicological effects on A549 cells. However, it might be beneficial to screen more F-Gs before a more conclusive statement is made.
Nonetheless, manufacturers who are producing these nanomaterials should indicate clearly the elemental composition and types of fluorine-containing groups present in the future. Lastly, absorbance data acquired from control experiments suggested that the F-G did not interact with both MTT and WST-8 assays significantly to create any distortion in the cell viability values.
8.3 Experimental

8.3.1 Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay were purchased from Sigma-Aldrich, water-soluble tetrazolium salt (WST-8) from Dojindo, and dimethyl sulfoxide (DMSO) from Tedia. High purity microcrystalline graphite (2–15 mm, 99.9995%) was obtained from Alfa Aesar, Germany. Sulphuric acid (98 wt.%), nitric acid (68 wt.%), potassium chlorate (>99%). Hydrochloric acid (37%), were obtained from Penta (Czech Republic). Fluorine (20 vol% in N₂) was obtained from Solvay, Belgium. Hydrogen (99.9999%) and nitrogen (99.9999%) were obtained from SIAD (Czech Republic).

8.3.2 Preparation of fluorinated graphenes

8.3.2.1 Synthesis of graphite oxide

The graphite oxide prepared by the Hofmann method was termed ‘HO-GO’. Concentrated sulphuric acid (87.5 mL) and nitric acid (27 mL) were added to a reaction flask containing a magnetic stir bar. The mixture was then cooled at 0 °C, and graphite (5 g) was added. The mixture was vigorously stirred to avoid agglomeration and to obtain a homogeneous dispersion. While keeping the reaction flask at 0 °C, potassium chlorate (55 g) was slowly added to the mixture in order to avoid a sudden increase in temperature and the consequent formation of explosive chlorine dioxide gas. Upon the complete dissolution of the potassium chlorate, the reaction flask was then loosely capped to allow the escape of the evolved gas and the mixture was continuously vigorously stirred for 96 h at room temperature before being poured into deionised water (3 L) and decanted. The graphite oxide was first redispersed in HCl (5%) solutions.
to remove sulphate ions and then repeatedly centrifuged and redispersed in deionised water until all chloride and sulphate ions were removed. The graphite oxide slurry was then dried in a vacuum oven at 50 °C for 48 h before use.

8.3.2.2 Microwave assisted exfoliation in hydrogenation plasma

Further, 1 g of HO-GO was placed in quartz glass microwave reactor. The reactor was repeatedly evacuated and purged with high purity nitrogen. The exfoliation was performed using 2.45 GHz/1 kW power for 3 minutes under hydrogen atmosphere (50 mL min\(^{-1}\)) at reduced pressure (10 mbar). During the exfoliation, a nitrogen plasma was formed which further accelerated the exfoliation and reduction of graphite oxide. The reduced graphite oxide was further used for fluorination.

8.3.2.3 Fluorination procedure

The fluorination was performed in Teflon lined Monel autoclave using a nitrogen-fluorine mixture (20 vol% F\(_2\)) from a dedicated fluorine line.\(^\text{28}\) An amount of 1 g of graphene or graphite oxide was placed in the Teflon liner, the autoclave was evacuated and filled with N\(_2\)/F\(_2\) mixture under 3 bar pressure. Various times and temperatures of fluorination were applied to investigate influence of different starting material (graphene and graphite oxide) as well as different reaction times. F-G1 was prepared by direct fluorination of graphene oxide at 180 °C for 4 days. F-G2 and F-G3 were prepared by direct fluorination of graphene synthesised by microwave exfoliation in hydrogen plasma. F-G2 was treated for 24 hours while F-G3 was treated for 4 days.

8.3.3 Characterisation of fluorinated graphenes

Scanning electron microscopy (SEM) was performed with JEOL-7600F semi-in-lens FE-SEM in gentle-beam mode at a working distance of 5.8–8.5 mm, and an accelerating voltage of 0.5–2 kV. Combustible elemental analysis (CHN–O) was performed with a PE
2400 Series II CHN/O Analyser (Perkin Elmer, USA). In CHN operating mode (the most robust and interference free mode), the instrument employs a classical combustion principle to convert the sample elements to simple gases (CO$_2$, H$_2$O and N$_2$). The PE 2400 analyser performs automatically combustion and reduction, homogenisation of product gases, separation and detection. A microbalance MX5 (Mettler Toledo) was used for precise weighing of samples (1.5–2.5 mg per single sample analysis). The accuracy of CHN determination is better than 0.30% abs. Internal calibration is performed using N-phenyl urea. For the measurement of fluorine concentration, the samples were decomposed for analysis according to Schöniger method. The exact amount of sample (about 2 mg) was wrapped in an ash free paper, burned in pure oxygen atmosphere and leached out with deionised water and total ionic strength adjustment buffer (TISAB) was added subsequently. The concentration of fluorine was determined by potentiometric measurement with an ion-selective electrode (ISE). XPS wide survey and high-resolution C1s spectra measurements were performed using a Phoibos 100 spectrometer and a monochromatic Mg X-ray radiation source (SPECS, Germany) at 12.5 kV. XRD was performed with a Bruker D8 diffractometer in Bragg–Brentano parafocusing geometry using CuKα radiation. Diffraction patterns were collected for 2θ values from 5° to 80°. FTIR spectra were measured using an iS50R FTIR spectrometer (Thermo Scientific, USA). The measurement was performed using diamond ATR crystal and KBr beamsplitter.

**8.3.4 Cell Culture**

Complete cell culture medium, which consisted of Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum, (FBS; PAA Laboratories) and 1% penicillin-streptomycin liquid (PAA Laboratories), was used to culture the A549 cell line (Bio-REV Singapore) in an incubator (37 °C) containing 5% CO$_2$. The typical cell cycle
time of A549 cell line is 22 h and these cells are commonly used in nanotoxicological studies. Prior to the introduction of the F-G, A549 cells (570 μL/well) with a cell density of 8.8 x 10^4 cells mL⁻¹ were first seeded in 24-well plates and incubated at 37 °C for 24 h.

8.3.4.1 Incubation of cells with fluorinated graphenes

Upon seeding the A549 cells in the 24-well plates for 24 h, the medium was removed from each well and rinsed with 1x PBS (pH 7.2; Gibco). Following that, different concentrations of F-G suspensions (570 μL/well) were added to the cells and incubated at 37 °C for another 24 h. Wells that consisted of only the cells and the culture medium, without exposure to the F-G, served as control for the experiment.

8.3.4.2 Cell viability measurements with MTT Assay

The MTT reagent (1 mg mL⁻¹) used for cell viability measurements in this study was prepared by diluting the MTT stock solution (5 mg mL⁻¹ in 1x PBS) with complete cell culture medium. The cell viability measurements began with removing the F-G suspensions from the A549 cells in the 24-well plates after 24 h exposure to the F-G. The cells were then washed twice with 1x PBS before adding the MTT reagent (300 μL/well) and incubating the cells at 37°C and 5% CO₂ for 3 h. Subsequently, the MTT reagent was removed from the 24-well plates and replaced with dimethyl sulphoxide (DMSO; 300 μL/well) to dissolve the insoluble purple formazan crystals produced by the viable cells. The plates were gently agitated for 5 min before the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the F-G. Finally, 100 μL of supernatant from each eppendorf tube was transferred to a 96-well plate for absorbance measurements at 570 nm and 690 nm, where the latter acts as the background absorbance. Data were represented relative to the absorbance obtained from control cells that were not exposed to any F-G.
8.3.4.3 Particle interference on MTT Assay

The tendency for F-G-induced artifacts in the MTT assay measurement caused by reactions between the nanomaterial and MTT was determined by first mixing varying concentrations of F-G with the MTT reagent (1 mg mL⁻¹). The mixtures (500 μL/well) were then added into 24-well plates under cell-free conditions and incubated at 37°C for 3 h. The MTT reagentant (1 mg mL⁻¹; 500 μL/well) was also added into separate wells as control. Following incubation, the mixtures were removed from the wells and replaced with DMSO (500 μL/well). The plates were gently agitated for 5 min before the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the F-G. Finally, the supernatant absorbance was measured at 570 nm, with 690 nm as the background absorbance.

In addition to possible reactions between F-G and the MTT reagent, F-G interference on MTT assay can be attributed to the binding of F-G to the MTT molecule or the formazan product, thus an extra control test was essential. The MTT-F-G mixture (200 μL/well), as well as the MTT reagent control, obtained from the 3 h incubation of the two respective materials as mentioned above were mixed with 4 mM ascorbic acid (160 μL/well) and gently agitated for 5 min before incubating at 37°C for 1 h. The ascorbic acid served as a reducing agent for the reduction of MTT to formazan in the absence of cells. Subsequently, DMSO (720 μL/well) was added to the MTT-F-G-ascorbic acid mixture in the ratio of 2:1 and placed in the incubator at 37°C for 10 min. Following incubation, the MTT-F-G-ascorbic acid mixtures were transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the F-G. Finally, the supernatant absorbance was measured at 570 nm, with 690 nm as the background absorbance. The data obtained in both tests were represented relative to the
absorbance obtained from the respective control MTT reagent that was not exposed to any F-G.

8.3.4.4 Cell viability measurements with WST-8 Assay

The working WST-8 assay used for cell viability measurements in this study was prepared by diluting the WST-8 stock solution by a factor of 10 with complete cell culture medium. The cell viability measurements began with removing the F-G suspensions from the A549 cells in the 24-well plates after 24 h exposure to the F-G. The cells were then washed twice with 1x PBS before adding the working WST-8 assay (300 μL/well) and incubating the cells at 37°C and 5% CO₂ for 1 h. Subsequently, the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the F-G. Finally, 100 μL of supernatant from each eppendorf tube was transferred to a 96-well plate for absorbance measurements at 450 nm and 800 nm, where the latter acts as the background absorbance. Data were represented relative to the absorbance obtained from control cells that were not exposed to any F-G.

8.3.4.5 Particle interference on WST-8 Assay

Since the WST-8 assay uses a water-soluble tetrazolium salt, reactions between the F-G and the WST-8 reagent will be the only potential interference with the viability assessment. Consequently, the probability of F-G reacting directly with the WST-8 reagent was determined. In the absence of cells, varying concentrations of F-G were mixed with the WST-8 stock solution to obtain resulting mixtures containing 10% v/v of the working WST-8 reagent. The mixtures (300 μL/well) were then added into 24-well plates and incubated at 37°C for 1 h. The working WST-8 reagent (10% v/v; 300 μL/well) was also added into separate wells as control. Following incubation, the mixtures were transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in
order to discard the F-G. Finally, the supernatant absorbance was measured at 450 nm, with 800 nm as the background absorbance. Data were represented relative to the absorbance obtained from the control working WST-8 reagent not exposed to any F-G.
Chapter 8: Cytotoxicity of fluorinated graphenes

References


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Chapter 9
Toxicity of nanomaterials III

Cytotoxicity of other halogenated graphenes

9.1 Introduction

9.2 Results and Discussion

9.2.1 Materials characterisation

9.2.2 In vitro cell viability assessment

9.2.3 Halogenated graphene-induced interference on cell viability assays

9.3 Experimental
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Contributions:

Most of the experiments were performed by W. Z. Teo. E. L. K. Chng helped out during cell viability measurements. The materials were synthesised by Z. Sofer. The project was conceptualised and directed by M. Pumera. W. Z. Teo, E. L. K. Chng, and M. Pumera contributed to discussions and wrote the manuscript.
9.1 Introduction

Besides fluorinated graphene, immense research has been performed on other halogenated graphenes owing to the enhanced properties they exhibit as compared to their graphene counterparts. In a recent study, iodine-doped graphene showed an excellent oxygen reduction reaction (ORR) electrocatalytic activity, long-term stability, as well as an exceptional tolerance to crossover effects for ORR, thus making the material a suitable candidate to replace current commercial Pt/C catalysts.\(^1\) Another halogenated graphene fabricated by co-grafting of halogens and aryl/oxygen functional groups on different sides of the two-dimensional graphene starting material yielded a Janus-like nanomaterial with fascinating surface properties, allowing it to be used as novel sensors, actuators and surfactants.\(^2\) The studies highlight the possible commercialisation of halogenated graphenes in the future, leading to large-scale production of these nanomaterials and thereby increasing the likelihood of releasing them into the environment during manufacture, usage and disposal of halogenated graphene containing products. However, toxicity studies performed on halogenated graphenes other than fluorinated graphene are unheard of. In lieu of this situation, it is thus necessary to investigate the \textit{in vitro} cytotoxicity of halogenated graphene to mammalian cells.

This present study will systematically examine the toxicological effects of three different types of halogenated thermally reduced graphene oxide nanomaterials, each containing either chlorine (Cl-G), bromine (Br-G), or iodine (I-G) on A549 cell line through \textit{in vitro} cell viability assessment. The data derived from each nanomaterial will be compared to determine if the halogen content of the nanomaterial will influence the cytotoxicity of halogenated graphenes.
Chapter 9: Cytotoxicity of other halogenated graphenes

9.2 Results and Discussion

9.2.1 Materials characterisation

The cytotoxicity of nanomaterials is usually, if not always, linked to their physicochemical properties. It is therefore important to characterise the halogenated graphenes used in this work in order to assist with the interpretation of the results obtained from the cell viability assessment. The halogenated graphenes were prepared using the method of thermal exfoliation/reduction of graphite oxide in various different halogen gas atmospheres.\(^3\) Table 9.1 shows a summary of the selected properties acquired from a previous report, where the detailed characterisation of the halogenated graphenes used in this study had been carried out.

Table 9.1 Summarised materials properties of halogenated graphenes as characterised by Raman spectroscopy, combustible elemental analysis, and XPS.\(^3\)

<table>
<thead>
<tr>
<th>Material</th>
<th>Crystallite size(^a) /nm</th>
<th>Amt. of halogen(^b) (ref. 4) (Cl, Br or I) at.%</th>
<th>C/O ratio(^c) / at.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl-G</td>
<td>14.4</td>
<td>2.1 (Cl)</td>
<td>16.77</td>
</tr>
<tr>
<td>Br-G</td>
<td>15.4</td>
<td>1.6 (Br)</td>
<td>20.37</td>
</tr>
<tr>
<td>I-G</td>
<td>22.3</td>
<td>0.2 (I)</td>
<td>11.75</td>
</tr>
</tbody>
</table>

\(^a\) As evaluated by Raman spectroscopy. \(^b\) As determined by combustible elemental analysis. \(^c\) As calculated based on data obtained from XPS analysis.

The crystallite sizes depicted in Table 9.1 were calculated based on Raman spectroscopic measurements and the values correspond to the undisturbed sp\(^2\) lattice size of the material.\(^4\) The smaller crystallite size indicates the presence of defects in the graphene structure, which is usually correlated with a higher degree of functionalisation of the graphene material with heteroatoms.\(^5\) From the figures, it is obvious that the extents of functionalisation of Cl-G and Br-G are similar and higher than that of I-G, suggesting that the latter might consist of a lesser amount of halogen atoms. This proposition was confirmed by the data obtained from combustible elemental analysis,
where the calculated atomic percentage (at.%) of iodine (0.2%) in I-G is found to be one order of magnitude lower than that of chlorine and bromine in Cl-G (2.1%) and Br-G (1.6%) respectively. The difference in the amount of available halogen atoms in the graphene material might influence its toxicity towards the A549 cells as the Group VII elements are known to be hazardous. Another property that could contribute to the toxicity of the halogenated graphene is the oxygen content of the material. In a previous study conducted to determine the toxicity of graphene oxides, it was reported that the level of oxidation of the graphene oxides, which is likely to be associated with the oxygen content and the quantity of carbonyl groups present, influenced the cytotoxic effects on the A549 cells. Therefore, the C/O ratio of the halogenated graphene materials used in this study is determined from wide survey XPS spectra measurements to examine whether the oxygen content in the halogenated graphenes could also play a significant role in the toxicity imparted by the nanomaterial. One shall note that the C/O ratio of thermally reduced graphene prepared from exfoliation of graphite oxide in the inert gas atmosphere is similar to the values published in Table 9.1.

9.2.2 In vitro cell viability assessment

The cytotoxicity of the halogenated graphenes was investigated through the use of two well-established cell viability assays that produce coloured formazan in the presence of living cells: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and water-soluble tetrazolium salt (WST-8) assay which uses 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt as the active reagent. Both MTT and WST-8 function on similar principles, hence we could ensure the reliability of the cell viability assessment if both assays show identical trends. The results obtained from these assays are summarised and discussed as follows.
Figure 9.1 Percentage cell viability of A549 cells calculated from absorbance readouts of A) MTT assay measurements, and B) WST-8 assay measurements, following 24 h exposure to different concentrations of halogenated graphene. The percentages derived are relative to the absorbance values from A549 control cells that were not treated with halogenated graphene and the numbers represent mean ± standard deviation of at least three repeat experiments, with four wells per treatment per experiment.

Figure 9.1A shows the cytotoxicity data of the three types of halogenated graphenes (Cl-G, Br-G and I-G) prepared from the thermal exfoliation of graphite oxide in different gaseous halogen atmospheres (chlorine, bromine, or iodine respectively), measured with the MTT viability assay. As depicted in the figure, the halogenated graphenes have a dose-dependent toxic effect on the A549 cells, with a significant loss of approximately 60–75% cell viability when the nanomaterials were introduced to the cells for 24 h at the highest concentration of 200 µg mL⁻¹. Based on the viability data obtained from the MTT assay, it can also be concluded that Cl-G induced the highest cytotoxicity to the cells while Br-G and I-G seemed to be of similar toxicity with an
exception at 200 µg mL\(^{-1}\) of the respective halogenated graphenes. Furthermore, cells exposed to Cl-G experienced more than 50% of cell death following exposure to a rather low concentration of the Cl-G at 12.5 µg mL\(^{-1}\), whereas a similar toxic response was only observed at a concentration of at least 100 µg mL\(^{-1}\) with the other two halogenated graphenes (Br-G and I-G). Therefore, it is strongly indicative that Cl-G is highly toxic as compared to its bromine or iodine-doped counterparts. According to the characterisation data presented in Table 1, we hypothesise that the variation in their toxicity profile can be attributed to the difference in the amount of halogen found in the halogenated graphenes. The halogen content in the halogenated graphenes can be placed in the order of Cl-G (2.1 at.% of Cl) > Br-G (1.6 at.% of Br) > I-G (0.2 at.% of I).

However, it is interesting that 12.5 µg mL\(^{-1}\) of Cl-G could induce more than 50% cell death while it took 100 µg mL\(^{-1}\) for Br-G, whose concentration is 8 times higher, to impart a similar effect when the atomic percentage of the two halogens are similar. It is also observed that Br-G imparted a similar degree of toxicity effect to I-G even though the atomic percentage of the corresponding halogen found in Br-G is almost 10 times more than that found in I-G. A possible explanation could be that the Br atoms in Br-G were less readily bioavailable to the cells as compared to the Cl and I atoms in Cl-G and I-G respectively.\(^9\)

Apart from the contribution of toxicity by the halogens in the nanomaterial, the amount of oxygen present in the halogenated graphenes could also play a part in inducing cytotoxic effects on the human lung epithelial cells, as evidenced by a similar study conducted using graphene oxide samples.\(^7\) In that study, it was demonstrated that lower oxygen content (higher C/O ratio) would lead to higher cytotoxicity. Therefore, based on this understanding, the degree of toxicity in the halogenated graphenes is expected to be in the order of Br-G > Cl-G > I-G. However, the level of toxicity induced by
Cl-G was significantly higher than that of Br-G, thereby suggesting that even though the oxygen content in the nanomaterials plays a role in affecting the cell viability, it is not a critical factor in the case of halogenated graphenes. The amount of halogen present in the halogenated graphene is the main determining factor for its cytotoxicity properties.

The A549 cell viability data measured from the WST-8 assay, at 24 h post-exposure to the three different halogenated graphenes, are presented in Figure 9.1B. On the whole, the WST-8 data illustrate that the cytotoxic effects imparted on the A549 cells by the three halogenated graphenes are dose-dependent and the degree of cytotoxicity presented by the halogenated graphenes is in the order of Cl-G > Br-G > I-G. At the highest dosage of 200 µg mL\(^{-1}\) Cl-G, Br-G and I-G, 25.7%, 29.5% and 54.4% of the A549 cells remained viable, respectively. The conclusions drawn from the WST-8 data were coherent with the findings from the MTT assay measurements, thus, the trend of the cytotoxic effects observed in this figure can again be attributed to the difference in the amount of halogen found in the halogenated graphene nanomaterials. In addition, the toxicity profile observed from the WST-8 data is in better agreement with the atomic percentage of halogen found in the halogenated graphenes, that is, I-G exhibited much lesser toxicity than Cl-G and Br-G since the amount of halogen in I-G was one order of magnitude lower than the other two (refer to Table 9.1).

Even though similar general deductions were derived from the MTT and WST-8 assays, there are some interesting differences between the data obtained. Firstly, I-G showed slightly less toxicity effects in the WST-8 data as compared to the MTT assay. Secondly, Br-G exhibited higher toxicity at dosages of 3.125 to 100 µg mL\(^{-1}\) in the WST-8 assay than with the MTT assay. There appears to be a drastic difference in the cytotoxicity profile of Br-G, whereby it seemingly exhibited a similar toxicity to Cl-G.
according to the WST-8 data (Figure 9.1B), whereas in the MTT data presented, Br-G shared a similar toxicity to I-G (Figure 9.1A). More work needs to be carried out in the future using a variety of toxicity assessment assays such as the LDH assay, Annexin V assay, GSH assay, comet assay to better understand the observed discrepancy.

9.2.3 Halogenated graphene-induced interference on cell viability assays

As with previous chapters, we performed control experiments to investigate whether there will be significant interactions between halogenated graphene and the cell viability assays in the absence of cells, which would result in false readings.\textsuperscript{10-12}

Possible interactions in MTT assay include the reduction of the MTT reagent due to the presence of the nanomaterial, adsorption to the reagent preventing its reduction, or binding to the insoluble formazan product resulting in its removal after rinsing or centrifugation. Therefore, two particle interference control experiments were performed for the MTT assay. First, the possibility of cell-free reaction between the halogenated graphenes and the MTT reagent was investigated. The MTT reagent was mixed with different concentrations of the halogenated graphenes and incubated for 3 h to examine the generation of formazan by the reaction of the two materials. The data obtained are illustrated in Figure 9.2A. From the figure, a slight increment (max. 20% at the highest concentration of 200 µg mL\textsuperscript{-1}) in the amount of the formazan product can be observed for all the halogenated graphenes, suggesting a possible interaction between the nanomaterials and the MTT reagent. However, since the increase is considerably small and thorough washings prior to the addition of the MTT reagent were ensured in this work, it is sufficient to say that the effective concentration of halogenated graphene that came into contact with the MTT reagent is much less than 200 µg mL\textsuperscript{-1}. Thus, it is
clear that the halogenated graphenes are unable to sufficiently interfere with the viability results via the spontaneous reduction of the MTT reagent by the nanomaterials.

**Figure 9.2** Normalised percentage of formazan generated from the incubation of halogenated graphenes in the MTT assay under cell-free conditions for the investigation on A) the extent of the MTT viability markers’ reaction with different concentrations of halogenated graphene, and B) examination of the binding magnitude between MTT/formazan product and the halogenated graphene. The absorbance of the blank control measured in both A and B is represented by the black dashed lines in the graphs.

Next, the extent of adsorption of the insoluble formazan crystals onto the halogenated graphenes was determined. The halogenated graphene suspensions of varying concentrations were first incubated with the MTT reagent for 3 h. Then ascorbic acid, which is an organic compound capable of reducing MTT into formazan in the absence of cells, was added to the mixture and incubated for another hour and the final concentration of formazan was measured after centrifugation to remove the nanomaterials. **Figure 9.2B** shows the percentage of the amount of formazan generated by the reduction of MTT reagent with ascorbic acid in mixtures containing different concentrations of the halogenated graphene, which were normalised to the formazan generated in the absence of halogenated graphenes. It can be seen that all the halogenated graphenes brought about minor reductions in the formazan measured, with a value of approximately ≥ 75% of the halogenated graphene-free absorbance at the
highest concentration of 200 $\mu$g mL$^{-1}$ for all the three nanomaterials, which can be considered comparable to the normalised amount of formazan (black dashed line in Figure 9.2B).

Based on the results obtained from the cell-free control experiments, two conclusions can be drawn: (1) all three halogenated graphenes evaluated showed evidence of minor chemical interactions with the MTT reagent to produce the formazan crystals after incubation for 3 h and these interactions are considered insignificant and are unlikely to affect the validity of the MTT assay data; (2) even though a decrease in the measured formazan concentration was observed from subsequent reduction by ascorbic acid, we believe it to be insignificant, thereby suggesting that there was no substantial binding between the halogenated graphenes and the insoluble MTT-formazan crystal.

![Figure 9.3 Normalised percentage of formazan generated from the incubation of halogenated graphenes in the WST-8 assay under cell-free conditions for the investigation on the extent of the WST-8 viability markers’ reaction with different concentrations of halogenated graphene. Inset: Relative percentage of formazan generated from a control experiment to determine the amount of reduction in formazan production attributed by serial dilution preparation. The solutions used are prepared by first mixing ultrapure water and WST-8 solution (10% v/v) in a 1:4 ratio and subsequently through serial dilution with WST-8 (10% v/v) in a 1:1 ratio. The black dashed lines in the graphs represent the absorbance of the blank control.]
Cell-free control experiments were also carried out with the WST-8 assay to verify that the measurements were not affected by the interference of the halogenated graphene with the viability maker, the WST-8 reagent. As the product of the WST-8 assay is water soluble, it is not of interest to determine whether the halogenated graphene would adsorb onto the formazan crystal and be removed in the centrifugation step. Consequently, the only control experiment required for the WST-8 assay was to find out if halogenated graphenes could react directly with the WST-8 reagent to produce a coloured formazan product in the absence of cells, thereby indicating a false non-toxic response.

WST-8 was incubated with the halogenated graphene suspensions for 1 h and the data are presented in Figure 9.3. The nanomaterial-induced interference control experiments showed that instead of reacting with the WST-8 reagent to form the coloured WST-8 formazan product, the halogenated graphene caused a reduction in the amount of formazan generated and the reduction could be as much as 57% in the mixture containing 200 µg mL\(^{-1}\) of Br-G. This reduction could be partly attributed to the dilution factor that was introduced during the preparation of the mixture of WST-8 and halogenated graphene suspension using serial dilution. At the highest concentration of 200 µg mL\(^{-1}\), the mixture consisted of the stock nanomaterial dispersed in ultrapure water and the WST-8 solution (10% v/v) in a 1 : 4 ratio and half of this mixture was subsequently added to an equal volume of WST-8 solution (10% v/v) to obtain 100 mg mL\(^{-1}\) of halogenated graphene-WST-8 mixture. This process of serial dilution proceeded in the same manner until the final concentration of 3.125 µg mL\(^{-1}\) was obtained, thus resulting in a mixture of diluted WST-8 reagent with different halogenated graphene concentrations; with the highest concentration containing the least amount of WST-8 reagent, giving rise to the trend observed in Figure 9.3. To verify this speculation, an
experiment was conducted and the data are shown in Figure 9.3 as an inset. It is obvious from the graph that the relative percentage of formazan measured in the cell-free control experiment is affected by the process of serial dilution during the preparation step, and the reduction in the value measured was as much as 21%. Despite the obvious reduction in the absorbance reading when compared with the normalised blank which consists of only the WST-8 reagent, we believe that the cell viability data obtained from the WST-8 assay in Figure 9.1B in this study are not likely to be affected as extensive washings were carried out to remove most of the halogenated graphene nanomaterials prior to the addition of the WST-8 reagent for incubation. As such, it is important to note that the effective concentration of halogenated graphenes when the WST-8 reagent is introduced is sufficiently low (less than 25 µg mL⁻¹) enough to not result in any significant reduction in formazan produced. That is to say, both the MTT and WST-8 assays data obtained throughout this study are supported and verified by the appropriate cell-free control experiments, ensuring the reliability of the cell viability data we have obtained.

In summary, the data obtained from both assays evidenced a dose-dependent toxicological effect in all the halogenated graphenes and the degree of cytotoxicity of can generally be placed in the order of Cl-G > Br-G > I-G, with Cl-G being the most toxic and I-G being the least toxic. Based on the trends obtained from the cell viability assessments, it is suggested that the halogen content plays a crucial role in the toxicity induced by the halogenated graphenes. Control experiments performed by incubating the nanomaterials with either assays in the absence of cells showed that there are slight nanomaterial-induced interferences. However, the effect is considered insignificant as thorough washings were performed prior to the cell viability assessment to remove most, if not all, of the halogenated graphenes during the study.
Chapter 9: Cytotoxicity of other halogenated graphenes

9.3 Experimental

9.3.1 Materials

Chlorine (99.8%) was obtained from Linde, ascorbic acid, bromine (99.5%), iodine (99.8%) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, nitric acid, sulphuric acid, hydrochloric acid, and potassium chlorate were obtained from PENTA, water-soluble tetrazolium salt (WST) from Dojindo, and dimethyl sulphoxide (DMSO) from Tedia. Graphite used for the synthesis of the halogenated graphene was obtained from KOH-I-NOOR GRAFIT.

9.3.2 Preparation of halogenated graphenes

To synthesise the halogenated graphenes, oxidation of the graphite starting material was first carried out, followed by exfoliation of the graphite oxide product in a halogen (chlorine, bromine or iodine) atmosphere. The detailed procedures for the oxidation and subsequent exfoliation are as described in the following paragraphs.

9.3.2.1 Synthesis of graphite oxide

The Hofmann method was adopted for the preparation of the graphite oxide.\textsuperscript{14} Graphite (5 g) was added to a mixture containing sulphuric acid (98%, 87.5 mL) and nitric acid (68%, 27 mL). The resulting mixture was cooled in an ice bath and maintained at 0 °C while potassium perchlorate (55 g) was slowly added to it before stirring the reaction mixture for 96 h at room temperature. Upon completion of the reaction, the mixture was poured into deionised water (3 L) and decanted. Subsequently, 5% hydrochloric acid solution was used for the dispersion of the resulting graphite oxide and repeated centrifugation was carried out to remove chloride and sulphate ions. The graphite oxide slurry was then left to dry in a vacuum oven at 60 °C for 48 h before use.
9.3.2.2 Synthesis of halogenated graphenes

The synthesis of the halogenated graphenes was carried out in a vacuum tight quartz glass reactor under a controlled atmosphere. The reactor has a porous quartz glass capsule with a magnetic manipulator connected to it, and this setup allowed production of a temperature gradient of over 1000 °C min⁻¹. The reaction conditions for all syntheses were 1000 °C under a pressure of 100 kPa for 12 min each. Graphite oxide (100 mg) was first added into a porous quartz glass capsule and the reactor was flushed with high purity nitrogen several times. Subsequently, the sample was introduced into the preheated furnace under the desired halogen atmosphere. For instance, a flow of 1000 mL min⁻¹ chlorine and 1000 mL min⁻¹ nitrogen was used to produce the Cl-G. For the synthesis of the Br-G, a flow of 1000 mL min⁻¹ of high purity nitrogen saturated with 0.6 × 10⁻³ mol min⁻¹ of bromine vapor supplied by a bromine-filled bubbler was used. Nitrogen flow through the bubbler was kept at 100 mL min⁻¹ at 100 kPa in order to achieve this. Furthermore, the bromine-filled bubbler was kept at 10 °C to prevent condensation of bromine vapor in the gas line. In order to prepare I-G, additional steps of mixing graphite oxide with iodine in a 1 : 1 mass ratio, dispersion in acetone, and evaporation to dryness at room temperature were taken before placing the iodine–graphite oxide mixture (100 mg) in a quartz glass porous capsule and flushing the reactor several times with high purity nitrogen. This is because the intrinsic low vapor pressure of iodine resulted in transportation of only 4.2 × 10⁻⁶ mol min⁻¹ iodine vapor from the iodine-filled bubbler, maintained at 15 °C and 100 kPa, when 1000 mL min⁻¹ of nitrogen flowed through it. To obtain the I-G final product, exfoliation of the iodine–graphite oxide mixture was performed with a 1000 mL min⁻¹ flow of high purity nitrogen to remove reaction byproducts.
9.3.3 Cell Culture

Complete cell culture medium, which consisted of Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum, (FBS; PAA Laboratories) and 1% penicillin-streptomycin liquid (PAA Laboratories), was used to culture the A549 cell line (Bio-REV Singapore) in an incubator (37 °C) containing 5% CO₂. The typical cell cycle time of A549 cell line is 22 h and these cells are commonly used in nanotoxicological studies. Prior to the introduction of the halogenated graphenes, A549 cells (570 μL/well) with a cell density of 8.8 x 10⁴ cells mL⁻¹ were first seeded in 24-well plates and incubated at 37 °C for 24 h.

9.3.3.1 Incubation of cells with halogenated graphenes

Upon seeding the A549 cells in the 24-well plates for 24 h, the medium was removed from each well and rinsed with 1x PBS (pH 7.2; Gibco). Following that, different concentrations of halogenated graphene suspensions (570 μL/well) were added to the cells and incubated at 37 °C for another 24 h. Wells that consisted of only the cells and the culture medium, without exposure to the halogenated graphenes, served as control for the experiment.

9.3.3.2 Cell viability measurements with MTT Assay

The MTT reagent (1 mg mL⁻¹) used for cell viability measurements in this study was prepared by diluting the MTT stock solution (5 mg mL⁻¹ in 1x PBS) with complete cell culture medium. The cell viability measurements began with removing the halogenated graphene suspensions from the A549 cells in the 24-well plates after 24 h exposure to the halogenated graphenes. The cells were then washed twice with 1x PBS before adding the MTT reagent (300 μL/well) and incubating the cells at 37°C and 5% CO₂ for 3 h. Subsequently, the MTT reagent was removed from the 24-well plates and replaced...
with dimethyl sulfoxide (DMSO; 300 μL/well) to dissolve the insoluble purple formazan crystals produced by the viable cells. The plates were gently agitated for 5 min before the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the halogenated graphenes. Finally, 100 μL of supernatant from each eppendorf tube was transferred to a 96-well plate for absorbance measurements at 570 nm and 690 nm, where the latter acts as the background absorbance. Data were represented relative to the absorbance obtained from control cells that were not exposed to any halogenated graphenes.

9.3.3.3 Particle interference on MTT Assay

The tendency for halogenated graphene-induced artifacts in the MTT assay measurement caused by reactions between the nanomaterial and MTT was determined by first mixing varying concentrations of halogenated graphenes with the MTT reagent (1 mg mL\(^{-1}\)). The mixtures (500 μL/well) were then added into 24-well plates under cell-free conditions and incubated at 37°C for 3 h. The MTT reagentant (1 mg mL\(^{-1}\); 500 μL/well) was also added into separate wells as control. Following incubation, the mixtures were removed from the wells and replaced with DMSO (500 μL/well). The plates were gently agitated for 5 min before the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the halogenated graphenes. Finally, the supernatant absorbance was measured at 570 nm, with 690 nm as the background absorbance.

In addition to possible reactions between halogenated graphenes and the MTT reagent, halogenated graphenes interference on MTT assay can be attributed to binding of the halogenated graphene to the MTT molecule or the formazan product, thus an extra control test was essential. The MTT-halogenated graphene mixture (200 μL/well), as well
as the MTT reagent control, obtained from the 3 h incubation of the two respective materials as mentioned above were mixed with 4 mM ascorbic acid (160 μL/well) and gently agitated for 5 min before incubating at 37°C for 1 h. The ascorbic acid served as a reducing agent for the reduction of MTT to formazan in the absence of cells. Subsequently, DMSO (720 μL/well) was added to the MTT-graphene-ascorbic acid mixture in the ratio of 2:1 and placed in the incubator at 37°C for 10 min. Following incubation, the MTT-graphene-ascorbic acid mixtures were transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the halogenated graphenes. Finally, the supernatant absorbance was measured at 570 nm, with 690 nm as the background absorbance. The data obtained in both tests were represented relative to the absorbance obtained from the respective control MTT reagent that was not exposed to any halogenated graphenes.

9.3.3.4 Cell viability measurements with WST-8 Assay

The working WST-8 assay used for cell viability measurements in this study was prepared by diluting the WST-8 stock solution by a factor of 10 with complete cell culture medium. The cell viability measurements began with removing the halogenated graphene suspensions from the A549 cells in the 24-well plates after 24 h exposure to the halogenated graphenes. The cells were then washed twice with 1x PBS before adding the working WST-8 assay (300 μL/well) and incubating the cells at 37°C and 5% CO₂ for 1 h. Subsequently, the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the halogenated graphenes. Finally, 100 μL of supernatant from each eppendorf tube was transferred to a 96-well plate for absorbance measurements at 450 nm and 800 nm, where the latter acts as the background absorbance. Data were represented relative to the absorbance obtained from control cells that were not exposed to any halogenated graphenes.
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9.3.3.5 Particle interference on WST-8 Assay

Since the WST-8 assay uses a water-soluble tetrazolium salt, reactions between the halogenated graphenes and the WST-8 reagent will be the only potential interference with the viability assessment. Consequently, the probability of halogenated graphenes reacting directly with the WST-8 reagent was determined. In the absence of cells, varying concentrations of halogenated graphenes were mixed with the working WST-8 reagent (10% v/v) and the resulting mixtures (300 μL/well) were added into 24-well plates to be incubated at 37°C for 1 h. The working WST-8 reagent (10% v/v; 300 μL/well) was also added into separate wells as control. Following incubation, the mixtures were transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the halogenated graphenes. Finally, the supernatant absorbance was measured at 450 nm, with 800 nm as the background absorbance. Data were represented relative to the absorbance obtained from the control working WST-8 reagent that was not exposed to any halogenated graphenes.
References


Chapter 10
Toxicity of nanomaterials IV

Cytotoxicity of exfoliated MoS$_2$, WS$_2$ and WSe$_2$ (transition metal dichalcogenides nanosheets)

10.1 Introduction
10.2 Results and Discussion
  10.2.1 Materials characterisation
  10.2.2 In vitro cell viability assessment
  10.2.3 Exfoliated TMD-induced interference on cell viability assays
10.3 Experimental
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Contributions:

Most of the experiments were performed by W. Z. Teo. E. L. K. Chng helped out during cell viability measurements. The materials were synthesised by Z. Sofer. The project was conceptualised and directed by M. Pumera. W. Z. Teo and M. Pumera contributed to discussions and wrote the manuscript.
10.1 Introduction

Inorganic analogues of graphene such as transition metal dichalcogenides (TMDs) have been placed under the limelight in the recent decade due to their superior electronic and structural properties.\textsuperscript{1,2} They have a general formula of MX\textsubscript{2}, where M represents any transition metal element from group IV, V or VI (e.g. Ti, Zr, V, Nb, Mo and W) in the periodic table and X represents a chalcogen (S, Se or Te).\textsuperscript{3}

It has been reported that the thickness (from multilayers to monolayer) of the semiconducting MoX\textsubscript{2} and WX\textsubscript{2} can have an influence on their band gap which will result in their transition from indirect band gap to direct band gap semiconductors.\textsuperscript{4,5} With direct and sizable band gaps between 1 to 2 eV, these two-dimensional TMDs can be used as field-effect transistors that possess high on/off current ratios and decent carrier mobility.\textsuperscript{3,6,7} In addition, monolayer MoS\textsubscript{2} was found to display emit photoluminescence and the luminescence intensity is dependent on the thickness of the material as well, making them applicable in light-emitting diodes and photosensors.\textsuperscript{5,8} Another important feature of two-dimensional TMDs is that they are highly elastic, as established by studies conducted on MoS\textsubscript{2} nanosheets (single to few-layers).\textsuperscript{9,10} These semiconducting materials will be required in next generation electronics that are flexible, and in composite films as reinforcing agents.\textsuperscript{11} As the research on TMDs nanomaterials progresses, it is vital to initiate the study on toxicological effects of this group of nanomaterials so that we can be informed of the health hazards that they may pose if they are commercialised in the future. Therefore, the aim of this study is to investigate the \textit{in vitro} cytotoxicity of three different exfoliated TMDs (exTMDs), namely MoS\textsubscript{2}, WS\textsubscript{2}, and WSe\textsubscript{2}, towards A549 cell line. Furthermore, we compared their cytotoxicities with graphene derivatives such as graphene oxides (GO) and halogenated graphenes (X-G).
10.2 Results and Discussion

10.2.1 Materials characterisation

Prior to assessing the cytotoxicity of exTMDs, characterisation of the nanomaterials were carried out with SEM, TEM, AFM, XRD, and SEM-EDX. This step was necessary because the toxicity of nanomaterials, in general, are associated with their physicochemical properties.

![Image](image1.png)

**Figure 10.1** SEM (on the left) and TEM (on the right) images of MoS$_2$, WS$_2$, and WSe$_2$ nanosheets. The scale bars represent 1 µm and 20 nm for SEM and TEM respectively.

Figure 10.1 shows the SEM and TEM images of the MoS$_2$, WS$_2$, and WSe$_2$ nanosheets. From the figure, it can be observed that all three types of exTMDs have similar morphology and are arranged in a disorderly manner. Examination of the nanomaterials by using TEM reveals that they are likely to be few-layered structures. Furthermore, AFM measurements (Figure 10.2) shows that the nanosheets have
thickness ranging from approximately 4.5–20 nm, which is typical of few-layered TMDs,\textsuperscript{12} thus indicating that the exfoliation of the bulk TMDs was successful. XRD patterns (Figure 10.3) demonstrated that MoS\textsubscript{2} underwent the highest degree of exfoliation out of the three TMDs. This is in agreement with the AFM data in which the thinnest sheets were observed for MoS\textsubscript{2}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{afm_images.png}
\caption{AFM images of few-layered A) MoS\textsubscript{2}, B) WS\textsubscript{2}, and C) WSe\textsubscript{2} nanosheets and their associated height profile (nm).}
\end{figure}
Elemental composition analysis of the exTMDs revealed that the ratio of M/X in MoS$_2$, WS$_2$, and WSe$_2$ was 1:1.9, 1:1.7, and 1:1.7, respectively. In addition, trace elements Ti (1.12 at. %), Ca (0.96 at. %), W (0.23 at. %), and Se (0.08 at. %) were found in the MoS$_2$ nanosheets, whereas Fe (1.50 at. %) was found in the WSe$_2$ nanosheets; WS$_2$ nanosheets was free of impurities.

### 10.2.2 In vitro cell viability assessment

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and water-soluble tetrazolium salt (WST-8) assay, which utilises the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt as the active reagent, were selected to investigate the cytotoxicity of the exTMDs. Since MTT and WST-8 function on similar principles, the reliability of the cell viability assessment could be ensured if both assays show identical trends. Data collected from the MTT and WST-8 assays are illustrated in Figure 10.4.

As depicted in the figure, it can be seen that the percentage cell viability of both MoS$_2$ and WS$_2$ remained relatively high ($\geq 80\%$), up to concentrations of 200 and 400 µg mL$^{-1}$ of the nanosheets incubated, respectively, indicating that these two nanomaterials induce low toxicological effects on A549 cells. This finding is in good agreement with toxicity experiments conducted on IF-MoS$_2$ and IF-WS$_2$, which demonstrated that these
zero-dimensional TMDs were non-toxic. Furthermore, polyethylene-glycolated (PEGylated) MoS$_2$ and WS$_2$ nanosheets have also been recently reported to exhibit high biocompatibility with no observable \textit{in vitro} and \textit{in vivo} toxicity. WSe$_2$, on the contrary, exhibited significant cytotoxicity towards A549 cells, causing approximately 70% cell death (at the highest concentration of 400 µg mL$^{-1}$) according to the results of the WST-8 assay. The cytotoxicity of WSe$_2$ appears to be dose-dependent and slightly more than 100 µg mL$^{-1}$ of the material was sufficient to result in at least 50% cell mortality. Therefore, the degree of cytotoxicity exhibited by the exTMDs examined in this study can be ranked in the order of WSe$_2$ > MoS$_2$ > WS$_2$, the most toxic being WSe$_2$ and the least toxic being WS$_2$.

![Figure 10.4 Percentage cell viability of A549 cells calculated from absorbance readouts of A) MTT assay measurements, and B) WST-8 assay measurements, following 24 h exposure to different concentrations of exTMDs. The percentages derived are relative to the absorbance values from A549 control cells that were not treated with exTMDs and the numbers represent mean ± standard deviation of at least three repeat experiments, with four wells per treatment per experiment.](image)

Even though the trends attained from the two cell viability assays are similar, it was noted that at higher exTMDs concentrations, the percentage cell viability measured from the MTT assay tend to be higher than the values derived from the WST-8 assays.
This phenomenon could be caused by induced interference of exTMDs on the viability markers, which will be discussed in detail later.

The level of toxicity imparted by a nanomaterial can be influenced by several factors, including its size, morphology, chemical composition, and the presence of surface coating on the nanomaterial.\(^{17, 18}\) Here we address the toxicity of the exTMDs without any surface modification. Amongst these, we believed that the chemical composition and the identity of the chalcogen present in the exTMDs was probably the main contributing factor attributing to the variation in their toxicity profile in this study. This hypothesis is evident from the cell viability measurements, which generally showed a small difference in percentage cell viability between WS\(_2\) and MoS\(_2\) nanosheets and a relatively big difference between WS\(_2\) and WSe\(_2\) nanosheets. A possible reason for this could be that the chalcogens are on the exterior of each TMDs layer, allowing more interaction with the cells as compared to the transition metal. Likewise to the case between hydrogen selenide and hydrogen sulphide in which H\(_2\)Se is 15 times more toxic than H\(_2\)S,\(^{19}\) selenium might be more hazardous than sulphur in exTMDs, thus explaining the observed values. However, in depth investigations need to be carried out in the future to ensure that this speculation is valid.

Apart from assessing the cytotoxicity of exTMDs, it will be interesting to compare the results with those obtained from similar studies on graphene and its family of derivatives. This is because exTMDs, which are dubbed as the inorganic analogues of graphene, are expected to be employed in applications similar to the family of graphenes. Table 10.1 shows the selected cytotoxicity data of graphene derivatives such as GO and X-G obtained from previous studies conducted by our group using the same cell line and conditions.\(^{20, 21}\)
Table 10.1 Normalised percentage of viable cells measured using MTT/WST-8 assays, after 24 h exposure with either GO (125 µg mL⁻¹), synthesised using the Hoffmann (GO-HO) and Hummers method (GO-HU), or 200 µg mL⁻¹ of chlorinated graphene (Cl-G), iodinated graphene (I-G), and exTMDs.

<table>
<thead>
<tr>
<th>Cell viability (%)</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GO-HO</td>
</tr>
<tr>
<td>MTT assay</td>
<td>40.0</td>
</tr>
<tr>
<td>WST-8 assay</td>
<td>35.0</td>
</tr>
</tbody>
</table>

*Data of GO and X-G are obtained from reference 20 and 21.

Even though the concentration of MoS₂ and WS₂ nanosheets tested was 1.6 times higher than that of GO, the percentage of viable cells of the former were at least 2 times higher in both assays, with the exception of MoS₂ data in MTT assay. Similarly, MoS₂ and WS₂ nanosheets are comparatively less toxic than X-G, with cell viability values ≥ 1.5 times than those from the incubation of X-G (same concentration) with A549 cells. Therefore, it is evident that exfoliated MoS₂ and WS₂ are less cytotoxic than GO and X-G and they might be more biocompatible candidates for potential applications. On the other hand, the cytotoxicity of WSe₂ seems to be on the same level as the GO and X-G, thus suggesting that the health and safety issue is unlikely to be one of the criteria for replacing these graphene derivatives with WSe₂ nanosheets in the future.

10.2.3 Exfoliated TMD-induced interference on cell viability assays

Nanomaterials can interact with the viability markers in MTT and WST-8 assays either by reducing the MTT or WST-8 reagent in the absence of viable cells, or in the case of MTT assay, binding to the insoluble formazan product, which will lead to its removal in subsequent washing or centrifugation steps. These interactions will interfere with the cell viability results, giving rise to false readings. In view of possible exTMD-induced interferences on both MTT and WST-8 assays, control experiments were performed under cell-free conditions to find out whether the exTMDs used in this study would interact with the viability markers significantly to produce invalid viability data.
Chapter 10: Cytotoxicity of exfoliated MoS$_2$, WS$_2$, and WSe$_2$

Figure 10.5 Normalised percentage of formazan generated from the incubation of exTMDs in the MTT assay under cell-free conditions for the investigation on A) the extent of the MTT viability markers’ reaction with different concentrations of exTMD, and B) examination of the binding magnitude between MTT/formazan product and the exTMD. The absorbance of the blank control measured in both A and B is represented by the black dashed lines in the graphs.

In the case of MTT assay, two different control experiments were carried out. First, generation of MTT formazan in the absence of cells through reaction between the exTMDs and the MTT reagent was tested. This is done by mixing the MTT reagent with different concentrations of the exTMDs and incubating the mixture at 37 °C for 3 h. As presented in Figure 10.5A, the absorbance data collected were normalised against the measurements of the control with zero exTMDs added. It is clear that the reaction between exTMDs and MTT reagent produced the formazan crystals in excess of that generated by the control (250–500%) at the highest exTMDs concentration of 400 µg mL$^{-1}$ examined. Below this concentration, no considerable reduction of the MTT reagent by WS$_2$ was found. However, the results from WSe$_2$ and MoS$_2$ showed that their reaction with MTT reagent was dose-dependent and the increment became significant at concentrations 12.5 and 100 µg mL$^{-1}$, respectively. Thus, MTT assay might be an unsuitable kit for the cytotoxicity investigation of exTMDs, especially for WSe$_2$ and MoS$_2$, even if washings were performed in attempt to remove these nanomaterials before adding the MTT reagent as the cell viability data will be overestimated, giving false impression of lower cytotoxicity.
Next, the second control experiment was conducted to investigate the effects of binding between the exTMDs and the insoluble formazan crystal on the absorbance intensity using the following procedure: MTT-exTMDs mixtures (varying concentrations) were incubated at 37 °C for 3 h prior to addition of ascorbic acid, an organic compound that can reduce MTT reagent into formazan under cell-free conditions, to the mixture. Then, the MTT-exTMD-ascorbic acid mixtures were incubated for another hour before measuring the final concentration of formazan. Figure 10.5B illustrates the normalised percentage of the generated formazan that were not bound to the exTMDs. From Figure 10.5B, it is obvious that there were no reductions in the formazan concentrations, thus suggesting that either no adsorption of exTMDs to the formazan occurred or the binding effects were negligible. In fact, slight increases in the amount of formazan were recorded in almost all the samples, possibly due to the reduction of MTT reagent by the exTMDs, which adds to the formazan generated from the ascorbic acid reaction.

![Figure 10.6](image)

**Figure 10.6** Normalised percentage of formazan generated from the incubation of exTMDs in the WST-8 assay under cell-free conditions for the investigation on the extent of the WST-8 viability markers’ reaction with different concentrations of exTMD. The absorbance of the blank control measured is represented by the black dashed lines in the graphs.

As the formazan product of the WST-8 assay is soluble, only one control experiment that examines a possible reduction of the WST-8 reagent by exTMDs without the presence of viable cells is required. WST-8 assay was mixed with various
concentrations of exTMDs suspensions and incubated at 37 °C for 1 h. Figure 10.6 shows the relative percentage of formazan generated from the exTMDs in the WST-8 assays under cell-free conditions. It can be observed that with the exception of 400 µg mL\(^{-1}\) WSe\(_2\) (= 150 %), there was an insignificant reduction of the WST-8 assay by the exTMDs; the amount of formazan generated by the 1 h incubation of these exTMDs with WST-8 reagent were around ±15% of the quantity measured from the control without exTMDs. Hence, it was concluded that whereas MoS\(_2\) and WS\(_2\) were free from nanomaterial-induced interference on the WST-8 assay, WSe\(_2\) did react with WST-8 reagent, resulting in a substantial increase in the formazan produced at the highest WSe\(_2\) concentration tested (400 µg mL\(^{-1}\)) and an underestimation of its potential \textit{in vitro} cytotoxicity. However, as the exTMDs were washed off thoroughly from the cells before introducing the WST-8 assay, the effective concentration of exTMDs that came into contact with the WST-8 reagent was believed to be at least below 200 µg mL\(^{-1}\), thereby not likely to distort the cell viability results in this study, particularly for WSe\(_2\).

Based on the results obtained from the cell-free control experiments, it can thus be concluded that the slightly inflated percentage cell viability values detected in the MTT assay measurements at the higher exTMDs concentrations (Figure 10.4) are most likely the effects of interaction between the MTT reagent and exTMDs, generating the formazan product in excess of the genuine amount. In other words, the WST-8 assay ought to be the preferred choice for future \textit{in vitro} cytotoxicity assessments of exTMDs as it has been verified to be almost interference-free, ensuring that reliable data can be acquired.

In summary, from the cell viability data collected, it was concluded that WSe\(_2\) is the most toxic nanomaterial among the exTMDs tested, followed by MoS\(_2\) and WS\(_2\).
nанослоев, оба из которых вызывают низкое токсикологическое воздействие на A549 клетки до 200 μg mL⁻¹ MoS₂/WS₂ экспозиции. Этот тренд указывает, что идентичность халькогена, составляющего exTMDs, может играть важную роль в определении токсичности наноматериала. Учитывая, что оба exfoliated MoS₂ и WS₂ в целом менее токсичны, чем органические аналоги, такие как графен оксид и хлорированые графены, они могут быть использованы в качестве более безопасных альтернатив в будущих приложениях. Результаты контрольных экспериментов показали, что при отсутствии клеток WST-8 реагент не взаимодействует с exTMDs, но exTMDs могут снизить MTT реагент при отсутствии клеток, что приводит к ложно-направленной оценке токсичности exTMDs. В свете этого, MTT assay следует избегать в будущих исследованиях в vitro токсичности exTMDs.
10.3 Experimental

10.3.1 Materials

Molybdenum (IV) disulphide (MoS$_2$) bulk powder (99% purity; < 2 μm), n-butyllithium (n-BuLi; 1.6 M in hexane) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay were purchased from Sigma-Aldrich, tungsten (IV) selenide (WSe$_2$) bulk powder (99.8% purity) and tungsten (IV) sulphide (WS$_2$) bulk powder (99.8% purity) from Alfa Aesar, water-soluble tetrazolium salt (WST-8) from Dojindo, dimethyl sulphoxide (DMSO) from Tedia, hexane from Lach-ner, and argon (99.9999% purity) from SIAD.

10.3.2 Preparation of exTMDs

Exfoliation of the TMDs was carried out as follows: First, 3 g of the TMD bulk powder were suspended in 20 mL of n-BuLi. Then, the suspension was stirred for 72 h at 25 °C under argon atmosphere in order to intercalate the TMDs with Li. Subsequently, the Li-intercalated material was separated by suction filtration under argon atmosphere and washed thoroughly with hexane (dried over Na). Repeated centrifugation (18 000 g) was carried out after placing the separated TMD with intercalated Li in water (100 mL). Finally, the exfoliated TMD was dried in vacuum oven at 50 °C for 48 hours before use.

10.3.3 Characterisation of exTMDs

JEOL-7600F semi-in-lens FE-SEM, operating in gentle-beam mode with an accelerating voltage of 2 kV, was used to obtain SEM images at a working distance of 4.9 mm. SEM-EDX data were obtained with JEOL-7600F semi-in-lens FE-SEM operating in SEM mode, at a working distance of 14.9 mm and an accelerating voltage of 15 kV. XRD data were collected at room temperature with an X'Pert PRO θ-θ powder diffractometer with parafocusing Bragg-Brentano geometry using CuKα radiation ($λ = 1.5418Å$, $U = 40$ kV, $I =$
30 mA). Data were scanned with an ultrafast detector X'Celerator over the angular range 5-80° (2θ) with a step size of 0.0167° (2θ) and a counting time of 20.32 s step⁻¹. Data evaluation were performed in the software package HighScore Plus. AFM was carried out on NT-MTD Ntegra Spectra from NT-MDT in a tapping mode with Si tips under ambient condition with scan rate of 1 Hz and 512 scan lines. A JEM 2100F field emission transmission electron microscope (JEOL, Japan) operating at 200 kV was employed to obtain TEM images.

10.3.4 Cell Culture

Complete cell culture medium, which consisted of Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum, (FBS; PAA Laboratories) and 1% penicillin-streptomycin liquid (PAA Laboratories), was used to culture the A549 cell line (Bio-REV Singapore) in an incubator (37 °C) containing 5% CO₂. The typical cell cycle time of A549 cell line is 22 h and these cells are commonly used in nanotoxicological studies. Prior to the introduction of the exTMDs, A549 cells (570 μL/well) with a cell density of 8.8 x 10⁴ cells mL⁻¹ were first seeded in 24-well plates and incubated at 37 °C for 24 h.

10.3.4.1 Incubation of cells with exTMDs

Upon seeding the A549 cells in the 24-well plates for 24 h, the medium was removed from each well and rinsed with 1x PBS (pH 7.2; Gibco). Following that, different concentrations of exTMDs suspensions (570 μL/well) were added to the cells and incubated at 37 °C for another 24 h. Wells that consisted of only the cells and the culture medium, without exposure to the exTMDs, served as control for the experiment.
10.3.4.2 Cell viability measurements with MTT Assay

The MTT reagent (1 mg mL⁻¹) used for cell viability measurements in this study was prepared by diluting the MTT stock solution (5 mg mL⁻¹ in 1x PBS) with complete cell culture medium. The cell viability measurements began with removing the exTMDs suspensions from the A549 cells in the 24-well plates after 24 h exposure to the exTMDs. The cells were then washed twice with 1x PBS before adding the MTT reagent (300 μL/well) and incubating the cells at 37°C and 5% CO₂ for 3 h. Subsequently, the MTT reagent was removed from the 24-well plates and replaced with dimethyl sulphoxide (DMSO; 300 μL/well) to dissolve the insoluble purple formazan crystals produced by the viable cells. The plates were gently agitated for 5 min before the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the exTMDs. Finally, 100 μL of supernatant from each eppendorf tube was transferred to a 96-well plate for absorbance measurements at 570 nm and 690 nm, where the latter acts as the background absorbance. Data were represented relative to the absorbance obtained from control cells that were not exposed to any exTMDs.

10.3.4.3 Particle interference on MTT Assay

The tendency for exTMDs-induced artifacts in the MTT assay measurement caused by reactions between the nanomaterial and MTT was determined by first mixing varying concentrations of exTMDs with the MTT reagent (1 mg mL⁻¹). The mixtures (500 μL/well) were then added into 24-well plates under cell-free conditions and incubated at 37°C for 3 h. The MTT reagent (1 mg mL⁻¹; 500 μL/well) was also added into separate wells as control. Following incubation, the mixtures were removed from the wells and replaced with DMSO (500 μL/well). The plates were gently agitated for 5 min before the assay liquid was transferred into individual eppendorf tubes for centrifugation at 8000 rpm for
10 min in order to discard the exTMDs. Finally, the supernatant absorbance was measured at 570 nm, with 690 nm as the background absorbance.

In addition to possible reactions between TMDs and the MTT reagent, exTMDs interference on MTT assay can be attributed to the binding of the exTMDs to the MTT molecule or the formazan product, thus an extra control test was essential. The MTT-exTMDs mixture (200 μL/well), as well as the MTT reagent control, obtained from the 3 h incubation of the two respective materials as mentioned above were mixed with 4 mM ascorbic acid (160 μL/well) and gently agitated for 5 min before incubating at 37°C for 1 h. The ascorbic acid served as a reducing agent for the reduction of MTT to formazan in the absence of cells. Subsequently, DMSO (720 μL/well) was added to the MTT-exTMD-ascorbic acid mixture in the ratio of 2:1 and placed in the incubator at 37°C for 10 min. Following incubation, the MTT-exTMD-ascorbic acid mixtures were transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the exTMDs. Finally, the supernatant absorbance was measured at 570 nm, with 690 nm as the background absorbance. The data obtained in both tests were represented relative to the absorbance obtained from the respective control MTT reagent that was not exposed to any exTMDs.

**10.3.4.4 Cell viability measurements with WST-8 Assay**

The working WST-8 assay used for cell viability measurements in this study was prepared by diluting the WST-8 stock solution by a factor of 10 with complete cell culture medium. The cell viability measurements began with removing the exTMDs suspensions from the A549 cells in the 24-well plates after 24 h exposure to the exTMDs. The cells were then washed twice with 1x PBS before adding the working WST-8 assay (300 μL/well) and incubating the cells at 37°C and 5% CO₂ for 1 h. Subsequently, the assay liquid was
transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the exTMDs. Finally, 100 μL of supernatant from each eppendorf tube was transferred to a 96-well plate for absorbance measurements at 450 nm and 800 nm, where the latter acts as the background absorbance. Data were represented relative to the absorbance obtained from control cells that were not exposed to any exTMDs.

### 10.3.4.5 Particle interference on WST-8 Assay

Since the WST-8 assay uses a water-soluble tetrazolium salt, reactions between the exTMDs and the WST-8 reagent will be the only potential interference with the viability assessment. Consequently, the probability of exTMDs reacting directly with the WST-8 reagent was determined. In the absence of cells, varying concentrations of exTMDs were mixed with the WST-8 stock solution to obtain resulting mixtures containing 10% v/v of the working WST-8 reagent. The mixtures (300 μL/well) were then added into 24-well plates and incubated at 37°C for 1 h. The working WST-8 reagent (10% v/v; 300 μL/well) was also added into separate wells as control. Following incubation, the mixtures were transferred into individual eppendorf tubes for centrifugation at 8000 rpm for 10 min in order to discard the exTMDs. Finally, the supernatant absorbance was measured at 450 nm, with 800 nm as the background absorbance. Data were represented relative to the absorbance obtained from the control working WST-8 reagent that was not exposed to any exTMDs.
References


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Chapter 11
Towards real-world application of self-propelled micro-/nanomotors for environmental remediation I

Platinum-free bubble-propelled tubular micromotors

11.1 Introduction
11.2 Results and Discussion
   11.2.1 Micromotors Characterisation
   11.2.2 Bubble propulsion in H₂O₂ study
11.3 Experimental
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Contributions:

The experiments were performed by W. Z. Teo. H. Wang assisted in the synthesis of the micromotors. The project was conceptualised and directed by M. Pumera. W. Z. Teo and M. Pumera contributed to discussions and wrote the manuscript.
Micro-/nanomotors have been touted as revolutionary devices that could undergo self-propulsion and be utilised in drug delivery, biosensing, and environmental remediation. Many proof-of-concept studies have showcased the capability of self-propelled micro-/nanomotors to improve the efficiency of pollutants removal and degradation. However, several challenges need to be resolved before these small scale motors can be employed in real-world environment.

First, most of the current micro-/nanomotors required scarce and costly platinum as catalyst to decompose hazardous hydrogen peroxide fuel for propulsion, and this could be a problem in real-world applications where large-scale quantities of micro-/nanomotors are needed. Nonetheless, there are already reports that demonstrated the use of alternative catalysts to power the propulsion. In Chapter 11, silver-catalyst based tubular micromotors were fabricated and its locomotion was examined to determine its potential of replacing platinum with an alternative catalyst without affecting the speed and lifetime of the micromotor. The second challenge is the difficulty to prepare micro-/nanomotors in a simple, low cost, and truly large-scale (kilograms/tons) manner using current fabrication methods to meet the demands for real-world application. Chapter 12 will attempt to address this issue by investigating the capability of Fe(0) nanomotors, which were fabricated in huge quantities at low cost, in enhancing azo-dye pollutant degradation. Lastly, current proof-of-concept studies need to be scaled-up to simulate real-world scenario to verify whether similar enhancement in environmental remediation can be observed at the macroscale level. This will be examined in Chapter 13 by observing the impact of bubbles formation and micromotors motion on mechanical mixing at the macroscale level.

Chapters 11, 12, and 13 are part of a collective work in addressing Objective 3.
11.1 Introduction

The development of synthetic micro- and nanomotors which are able to perform autonomous propulsion through the catalytic conversion of fuel to mechanical energy is progressing at an alarming rate over the past few years.\textsuperscript{1} These small devices displayed promising applications in many areas such as environmental remediation,\textsuperscript{2-7} natural resource discovery,\textsuperscript{8} drug delivery,\textsuperscript{9, 10} and sensing.\textsuperscript{11-17} As bubble-propelled micro-/nanomotors have been demonstrated to exhibit high velocity and power, they are frequently chosen for environmental remediation studies. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is the most commonly utilised fuel where the surface of the micro-/nanomotors (typically platinum) catalyses its decomposition to generate water (H\textsubscript{2}O) and oxygen (O\textsubscript{2}) bubbles.\textsuperscript{17-21} As platinum metal is scarce and expensive, alternative metals such as silver (Ag) and manganese oxide (MnO\textsubscript{2}) were used in place of platinum in the fabricated micro-/nanomotors to catalyse the decomposition of H\textsubscript{2}O\textsubscript{2} and the synthesised micro-/nanomotors showed promising results.\textsuperscript{22-27} However, these studies reported the use of Janus particles or require complex micromotor preparation steps which limited the ease of fabricating them elsewhere to attain similar outcomes. Besides relying on metal-based catalysis of H\textsubscript{2}O\textsubscript{2}, Sanchez and co-workers described the use of an enzyme for the decomposition reaction to generate motion for the enzyme-filled micromotor.\textsuperscript{28} Nonetheless, their limited lifetime made the micromotors less attractive to be implemented in large-scale.

In this study, we established a simple and straightforward preparation method to produce silver-catalyst based (platinum free) bubble-propelled tubular micromotors with long lifetime and excellent bubble-powered motion.
11.2 Results and Discussion

Ag is known to be able to catalyse the decomposition of H$_2$O$_2$ to form H$_2$O and O$_2$ bubbles. The Ag-based tubular micromotors were prepared by sequentially electrodepositing copper (Cu) and Ag onto the pores of a cyclopore polycarbonate membrane template (see Experimental section for details). When Cu/Ag segmented bimetallic tubular micromotors are placed in a solution containing H$_2$O$_2$, O$_2$ bubbles will be generated and released at the Ag end of the micromotor. The continuous detachment of O$_2$ bubbles from one end of the Cu/Ag micromotor will then result in the propulsion of the latter in the direction towards the Cu end. Figure 11.1 shows a schematic illustration of the Cu/Ag segmented bimetallic tubular micromotors’ propulsion in H$_2$O$_2$.

![Diagram of Cu/Ag segmented bimetallic tubular micromotors](image)

**Figure 11.1** Schematic representation of the Cu/Ag segmented bimetallic tubular micromotors’ reaction with H$_2$O$_2$ (in the presence of a surfactant) to generate O$_2$ bubbles for propulsion. The red arrow depicts the direction of motion of the Cu/Ag micromotors, which is towards the Cu end with respect to the stationary fluid.

11.2.1 Micromotors characterisation

SEM images were obtained to characterise the morphology of the Cu/Ag micromotors and shown in Figure 11.2A & B. The images reveal that the Cu/Ag micromotors have an average length of 10–15 µm and the metallic surface of the
micromotors is relatively thick and uneven. In addition, the Cu/Ag micromotors are slightly conical in shape, with pore sizes of approximately 3 µm and 3.5 µm at respective ends. Elemental characterisation of the Cu/Ag micromotors under SEM-EDX (Figure 11.2C) verified that the fabricated micromotors consisted of both Cu and Ag elements which are segmented. The Cu/Ag micromotors were subsequently subjected to running solutions containing different amounts of H₂O₂ (0.5–3%) to investigate the dependence of their average velocity on this variable.

![SEM images](image)

**Figure 11.2** (A) & (B) SEM images showing the structure of the Cu/Ag segmented bimetallic tubular micromotors. (C) Elemental characterisation of the Cu/Ag micromotors using SEM-EDX. Scale bars in (A) & (B) represent 1 µm while scale bars in (C) represent 5 µm.

### 11.2.1 Bubble propulsion in H₂O₂ study

Bubble propulsion of the Cu/Ag micromotors was observed and recorded under an optical microscope to analyse their motion in H₂O₂ solutions (with 1% SDS surfactant). Figure 11.3 shows the time-lapsed images of a Cu/Ag micromotor in 1.5% H₂O₂ solution. From the images, it was clear that bubbles are released vigorously from
one end of the Cu/Ag micromotor, driving the movement of the micromotor in the \( \text{H}_2\text{O}_2 \) fluid. Interestingly, \( \text{O}_2 \) bubbles were not generated at the outer wall of the Ag segment of the Cu/Ag micromotor even though it consisted of the Ag catalyst as well. This observation is likely to be caused by the combined effects of heterogeneous nucleation energy and asymmetric distribution of oxygen concentration between the concave surface (inner wall) and convex surface (outer wall) of the micromotor, leading to \( \text{O}_2 \) molecules nucleating, growing into bubbles and ejecting preferentially from the hollow portion (concave surface) of the micromotor.\textsuperscript{29, 30} Different kinds of motion pathways were also observed for the bubble-propelled Cu/Ag micromotors (see example, Figure 11.3 inset), namely self-rotating, curved, and circular trajectories. These trajectories were typical of tubular micromotors reported in the literature, which are affected by the size, shape and geometry of the moving micromotor.\textsuperscript{31}

**Figure 11.3** Time-lapsed images illustrating the bubble propulsion of the Cu/Ag micromotors in a solution containing 1.5% \( \text{H}_2\text{O}_2 \) and 1% SDS (surfactant). Scale bars in the images represent 50 \( \mu \text{m} \). The inset at the bottom right of the figure shows the motion trajectory of the Cu/Ag micromotor. The green arrows in the inset represent the direction of the motion.
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Figure 11.4 SEM images of Cu/Ag micromotors before (A) & (B) and after (C) propelling in 3 % H₂O₂ running solution. Elemental mapping was carried out on the Cu/Ag micromotor, which was immersed in the H₂O₂ running solution, using SEM/EDX (D) – (F) to determine if Ag was oxidised while in the H₂O₂ solution. Scale bars in (A) – (C) represent 1 μm while (D) – (F) represent 10 μm.

It has been previously reported that the Ag surface might undergo partial dissolution in H₂O₂ (fuel) solution. Therefore, we examined the Cu/Ag micromotors under SEM (Figure 11.4) before and after placing them in the running solution for 30 minutes. It was noticed that the Ag segment of the Cu/Ag micromotor had become grainy after propelling for 30 minutes (Figure 11.4C), which was a probable indication that Ag had partially dissolved. However, this slight corrosion of Ag is unlikely to be significant enough to limit the lifetime of Cu/Ag micromotors. Besides dissolving into the running solution, Ag can be easily oxidised, thereby forming a layer of oxide which could in turn ‘poison’ the Cu/Ag micromotor and reduce its lifetime by preventing Ag from catalysing H₂O₂ decomposition. Elemental mapping of the corroded Cu/Ag micromotor (Figure 11.4D–F) showed that the Ag segment of the micromotor is almost free of oxygen, thus indicating that the Ag surface underwent little oxidation and would not affect the lifetime of the Cu/Ag micromotor.
A positive correlation between the velocity of the Cu/Ag micromotors and the H$_2$O$_2$ fuel concentration was determined from the average velocity data acquired. As displayed in Figure 11.5, the average velocity of the propelling Cu/Ag micromotors increased with the amount of H$_2$O$_2$ fuel present in the running solution, from 13.1 µm s$^{-1}$ (approx. 1 body length s$^{-1}$) in 0.5% H$_2$O$_2$ to 252.4 µm s$^{-1}$ (approx. 20 body lengths s$^{-1}$) in 3% H$_2$O$_2$. Furthermore, the average speed exhibited by the Cu/Ag micromotors in 3% H$_2$O$_2$ is comparable to that of Cu/Pt concentric bimetallic micromotors (Figure 11.5, inset), which are similar in shape and size, reported previously by our group.$^{17,25}$ In addition, the Cu/Ag micromotors have demonstrated about 5 times higher mobility as compared to Ag micromotors (microparticles) described in another study.$^{25}$ Lastly, the average efficiency of the Cu/Ag micromotors in 0.5% H$_2$O$_2$ was determined to be 2.52 × 10$^{-9}$, based on the calculation method previously described in the literature.$^{33}$
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In summary, we have successfully fabricated segmented bimetallic tubular micromotors (consisting of Cu and Ag metals) that are powered by the catalytic decomposition of H$_2$O$_2$ fuel by the Ag end of the Cu/Ag micromotors. The motion of these Pt-free micromotors were achieved via the bubble propulsion mechanism and they exhibited high mobility and speed which are analogous to Cu/Pt bimetallic concentric micromotors of similar sizes, suggesting that the Cu/Ag micromotors could be efficient and effective alternatives to the popular Pt-containing micromotors. It will thus be advantageous to utilise the catalytic properties of Ag metal for the development of more micro-/nanomotors in the future to reduce our reliance on the scarce and expensive Pt metal so that they can be synthesised in large quantities at an affordable cost. The Cu/Ag micromotors fabricated in this study could find potential application for removal of biological threats (eg. bacterium) in water. In addition, the Cu/Ag micromotors could be modified to include a layer of graphene oxide on its exterior for efficient removal of heavy metals like Pb$^{2+}$ from water.$^{34}$
11.3 Experimental

11.3.1 Materials

Cyclopore polycarbonate membranes with conical-shaped pores of 3 μm in diameter were purchased from Whatman, USA (Cat. no. 7060-4712), hydrogen peroxide (H₂O₂; 35%) from Alfa Aesar, Singapore, methylene chloride and ethanol from Tedia, USA, platinum electrode (1 mm diameter) and Ag/AgCl/1M KCl electrode from CH instruments, USA, copper (II) sulphate (CuSO₄·5H₂O, 98%), and sodium dodecyl sulphate (SDS, Product No.: L3771) from Sigma–Aldrich, platinum-plating solution from Technic Inc., USA, and silver sputtering target from Electron Microscopy Sciences, USA. The chemicals were used as received and ultrapure water (18.2 MΩ cm) from a Millipore Milli-Q purification system was used throughout the experiments.

11.3.2 Apparatus

Autolab PGSTAT 101 electrochemical analyser (Eco Chemie, Utrecht, The Netherlands) connected to a computer and controlled by NOVA version 1.8 software (Eco Chemie) was used for electrochemical deposition of Cu and Ag on polycarbonate membrane. A three electrode configuration was adopted for the deposition procedure, in a customised electrochemical deposition cell at room temperature (23 °C). The auxiliary electrode was a platinum electrode while the reference electrode was an Ag/AgCl electrode. A JEOL JFC-1600 Auto Fine Coater was utilised to sputter the polycarbonate membrane. Ultrasonication process was carried out with a Fisherbrand FB 11203 ultrasonicator, and centrifugation was carried out with a Beckman Coulter Allegra 64R centrifuge. Video recordings were obtained using Nikon Eclipse 50i optical microscope, which in turn were analysed with Nikon NIS Elements software. SEM images were captured using JEOL-7600F semi-in-lens FE-SEM, in SEM mode with an accelerating
voltage of 15 kV, at a working distance of 7.2 mm. SEM-EDX data were measured with JEOL-7600F semi-in-lens FE-SEM, in SEM mode with an accelerating voltage of 30 kV, at a working distance of 15.2 mm.

11.3.3 Preparation of Cu/Ag segmented bimetallic microtubes

Cu/Ag segmented bimetallic microtubes were synthesised through an electrochemical deposition method with a cyclopore polycarbonate template. Prior to the electrochemical deposition experiment, the polycarbonate template was sputtered with silver (15 nm) on one side of the membrane and subsequently attached to a piece of copper tape. The template, which will serve as the working electrode, was then assembled into a customised electrochemical deposition cell. A layer of Cu was first deposited along the pores of the template by adding 1M CuSO$_4$ electrolyte into the cell after rinsing the template thoroughly with ultrapure water (18.2 MΩ cm). The electrochemical deposition of Cu was carried out galvanostatically at $-5$ mA for 900 s. Subsequently, the CuSO$_4$ electrolyte was removed, the cell rinsed thoroughly with ultrapure water and replaced with commercial silver-plating solution. Following that, the silver segment was electrodeposited at $-5$ mA for 1200s. Upon completion of the deposition of microtubes, the template was removed from the electrochemical cell and washed with ultrapure water. Methylene chloride was then used to dissolve the template and ultrasonication was performed to enhance the dissolution of the template. The Cu/Ag microtubes were then collected by centrifuging the solution at 6000 rpm for 3 min. The process of ultrasonication and centrifugation was repeated 3 times with methylene chloride to ensure complete dissolution of the template. Finally, impurities in the Cu/Ag microtubes solution were removed by washing with ethanol and water for 2
times each, with 3 min of centrifugation at 6000 rpm following each washing step. The Cu/Ag microtubes were stored in water at room temperature.

### 11.3.4 Operation of micromotors

The Cu/Ag micromotors were dispersed in aqueous solutions containing different concentrations of hydrogen peroxide (0.5–3%) and 1 % SDS surfactant for the motion study experiments. At the start of each experiment, the micromotor mixture was placed on a freshly cleaned glass slide. Then, the glass slide is mounted on Nikon Eclipse 50i optical microscope where videos and optical images of the bubble propulsion process were taken. Finally, the recordings were processed and analysed with the Nikon NIS-Elements software.
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References


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Chapter 12

Towards real-world application of self-propelled micro-/nanomotors for environmental remediation II

Efficient large-scale synthesis of Fe(0) nanomotors for environmental remediation

12.1 Introduction

12.2 Results and Discussion

12.2.1 Nanomotors characterisation

12.2.2 Bubble propulsion in citric acid study

12.2.3 Degradation of azo-dyes

12.3 Experimental
The results in this chapter were published in the following manuscript:

1. Wei Zhe Teo, Radek Zboril, Ivo Medrik, Martin Pumera. Fe\textsuperscript{0} Nanomotors in Ton Quantities (10\textsuperscript{20} Units) for Environmental Remediation Chem. – Eur. J. 2016, 22, 4789.

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Contributions:

W. Z. Teo performed most of the experiments. R. Zboril and I. Medrik fabricated the materials and carried out the HAADF/EDS and \textsuperscript{57}Fe zero-field Mössbauer spectrum measurements. The project was conceptualised and directed by M. Pumera. W. Z. Teo and M. Pumera contributed to discussions and wrote the manuscript.
12.1 Introduction

Despite the progress in the design of micro-/nanomotors and their locomotion control, it remained a challenge for large-scale application of these devices for environmental remediation. This is because of the inability to fabricate the micro-/nanomotors in a truly large-scale (kilograms/tons) quantity at low cost with the current fabrication techniques such as template electrodeposition and rolled-up technology.\(^1\),\(^2\) The latter required clean room and advanced equipment for the synthesis of the micro-/nanomotors. Micro-/nanomotors prepared for pollutant degradation studies typically contain a catalytic surface which reacts with a fuel (usually H\(_2\)O\(_2\)) to undergo bubble propulsion, and the rapid and continuous motion of these micro-/nanomotors had been reported as the main factor that accelerated the rate of the degradation process via enhanced diffusion.\(^3\)–\(^9\)

As the research in self-propelled micro- and nanomotors progresses towards real-world application in the field of environmental remediation, the issues of the use of non-toxic alternative fuel and mass production of the micro-/nanomotors need to be addressed.\(^8\)–\(^11\) While there are already studies that reported the use of water or acid as alternative fuel to power micro-/nanomotors,\(^12\)–\(^17\) the issue on mass production still seemed largely unexplored.

In the present study, Fe(0) nanoparticles that can be easily fabricated in industrial scale quantities were investigated for their ability to utilise weak acid as fuel to power its motion, and to act as remediation agents for the degradation of azo-dyes.
12.2 Results and Discussion

Metallic iron is known to be reactive with acids and hydrogen gas bubbles will be released in the process.\textsuperscript{18} In this study, we sought to utilise this acid–metal reaction to generate sufficient propulsion by hydrogen bubbles, from the reaction between Fe(0) nanoparticles and a weak acid (citric acid) in the presence of a surfactant, for continuous propulsion of Fe(0) nanomotors (Figure 12.1). Subsequently, we examined the feasibility of enhancing azo-dye degradation rate through the employment of the Fe(0) nanomotors.

The Fe(0) nanoparticles, unlike other micro/nanomotors which generally require a template and tedious steps for fabrication, can be easily synthesised in huge quantities (industrial scale), hence they can be easily applied in large-scale decontamination works at a lower cost.\textsuperscript{11} In addition, the utilisation of environmentally friendly fuel as compared to the commonly used hydrogen peroxide ensured that the remediation process is less likely to leave a negative impact on the environment.\textsuperscript{10}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure12.1.png}
\caption{Schematic depicting the reaction between citric acid and Fe(0) nanoparticles to generate H\textsubscript{2} bubbles for propulsion of the Fe(0) nanomotor.}
\end{figure}
12.2.1 Nanomotors characterisation

SEM images of the Fe(0) nanoparticles in Figure 12.2A illustrated that the nanoparticles have size ranging from 100–400 nm and they form aggregates with various shapes and sizes in the micrometre range. In addition, elemental mapping of the aggregates through SEM-EDX in Figure 12.2B confirmed that the aggregates are predominantly made up of Fe(0).

Figure 12.2 (A) SEM images showing Fe(0) nanoparticle aggregates. (B) Element mapping of the Fe(0) nanoparticle aggregates using SEM/EDX. The red color in the figure denotes Fe element. The scale bars in both (A) and (B) represents 100 nm.

Figure 12.3 (a) HAADF/EDS chemical mapping of individual nanoparticles showing the ultrathin iron oxide shell on the Fe(0) surface. (b) Room temperature Mössbauer spectrum of Fe(0) nanoparticles (red sextet spectrum) stabilised with approx. 5 wt.% of iron(III) oxide (yellow doublet).
For real applications of such Fe(0) nanomotors, the presence of a suitable stabilisation surface preventing their burning in the air is another important requirement in addition to the large-scale production. Zero-valent iron nanoparticles react with water very slowly in anaerobic conditions due to the self-stabilisation by the formed oxidic shell. The detailed description of kinetics and mechanism of reaction of these nanoparticles with water were described in a previous study. Thus, we explored the surface of the individual Fe(0) nanoparticles by HAADF/EDX chemical mapping (Figure 12.3a). This analysis clearly proved the presence of an ultrathin iron oxide layer (yellow map corresponds to oxygen) covering the elemental iron core (red colour). In addition, the Mössbauer spectrum in Figure 12.3b demonstrated that this oxidic phase corresponds to iron(III) oxide as proven by the isomer shift parameter (0.37 mm s\(^{-1}\)) of the doublet component, which is typical for Fe(III). The dominant sextet (95% of the total spectrum area) is assignable to Fe(0) with a characteristic zero value of the isomer shift parameter.

### 12.2.2 Bubble propulsion in citric acid study

The Fe(0) nanoparticles were added to weak citric acid solutions (varying wt.%) containing 1 wt.% SDS (surfactant) to determine the minimum amount of acid fuel required to propel the Fe(0) nanomotors. Observations of the Fe(0) nanomotors under an optical microscope revealed that while they produced bubbles in solutions with as little as 0.42 wt.% of citric acid, motion was only detected in the presence of at least 3.38 wt.% of citric acid. As the Fe(0) nanoparticles form aggregates with diverse sizes, the velocities of the Fe(0) nanomotors in 3.38 wt.% of citric acid ranges from 7 to 20 μm s\(^{-1}\) and their speed did not differ significantly in citric acid solutions between 3.38 and 27.1 wt.%. Figure 12.4 shows bubble-propelled motion of the Fe(0) nanomotors in 27.1
wt.% of citric acid. Interestingly, it was noted from the snapshots (Figure 12.4B–D) that the Fe(0) nanomotors would adsorb smaller stationary Fe(0) nanoparticles, which were found along their motion pathway. Since we have ascertained that bubble propulsion of Fe(0) nanoparticle aggregates does occur in weak citric acid with 1 wt.% SDS, we proceeded to examine the possibility of enhancing the rate of azo-dye degradation with moving Fe(0) nanomotors.

Figure 12.4 Snapshots of a video recording showing bubble-propelled Fe(0) nanomotors in a solution containing 27.1 wt.% of citric acid (fuel) and 1 wt.% of SDS (surfactant). The snapshots (A–D) are taken at 5 s intervals and the scale bars represent 10 µm. Adsorption of a small Fe(0) nanoparticle aggregate onto the Fe(0) nanomotors as the latter travelled towards the aggregate can be observed from snapshots B to D.

12.2.3 Degradation of azo-dyes

Azo-dyes, a group of synthetic colourants which generally contains an azo group (-N=N-) in the compound, constitute approximately 70% of organic commercial dyes produced today and they are being widely used in the textile industry to colour the fabrics. It is estimated that, during the dyeing process, about 15% of the dyes end up
in wastewaters which are inevitably discharged into the environment subsequently.\textsuperscript{21, 22} Some of these azo-dyes are known to be highly toxic and carcinogenic, thus their presence in environmental waters pose deadly consequences to the ecosystem and our health.\textsuperscript{23-26} Besides being toxic, azo-dyes can damage aquatic environment by limiting light penetration and eventually photosynthetic activity.\textsuperscript{26} Extensive researches have been carried out to develop ways to decolourise or degrade the harmful azo-dyes and the use of zerovalent iron (ZVI) micro-/nanoparticles as remediation agents is one of them.\textsuperscript{21, 27-32} For instance, it was revealed that rapid decolourisation/degradation of methyl orange (MO) could occur in the presence of nanoscale zerovalent iron particles and the process is favoured in acidic medium.\textsuperscript{21, 27} A very recent article also showed that Fe(0) nanoparticles could result in the degradation of methylene blue (MB) and the authors proposed a possible removal mechanism for the reaction.\textsuperscript{28}

Therefore, MO and MB are used as model compounds in this study to investigate the effect of bubble propulsion of Fe(0) nanoparticles in weak acid on the degradation of azo-dyes. Spectrophotometry was performed to determine the extent of decontamination. Mixtures containing predetermined amounts of either MO or MB, 6.8 wt.% of citric acid and 1 wt.% of SDS were first prepared to give the absorbance spectra of MO/MB in these mixtures. Subsequently, Fe(0) nanoparticles were added (final concentration of Fe(0) nanoparticles = 1 mg mL\textsuperscript{-1}) to the mixtures to measure the absorbance of MO/MB at fixed time intervals after the addition of Fe(0) nanoparticles, for a total duration of 15 min. Figure 12.5A shows the absorbance spectra of MO/MB in their respective mixtures. From the absorbance spectra collected, it was clear that the amount of MO/MB decreased within the 15 min period, which suggests that the azo-dyes were indeed degraded by the Fe(0) nanomotors.
Figure 12.5 (A) Absorbance spectra of methyl orange (MO) and methylene blue (MB) taken at 0, 5, 10, and 15 min from the addition of Fe(0) nanomotors into mixtures containing 6.8 wt.% of citric acid, 1 wt.% SDS, and the respective azo-dye. (B) Bar graphs showing absolute change in peak absorbance of methyl orange (MO) and methylene blue (MB) over 15 min from the addition of Fe(0) nanomotors into different mixtures (1 wt.% SDS—black; 6.8 wt.% citric acid—red; 6.8 wt.% citric acid+1 wt.% SDS—blue) containing the respective azo-dye.

Control experiments were conducted to ensure that continuous self-powered movement of the Fe(0) nanomotors did affect the extent of MO/MB degradation. The absolute changes in peak absorbance of the azo-dyes in different MO/MB mixtures are displayed in Figure 12.5B. From the figure, it was evident that in the presence of only 1 wt.% SDS (no citric acid added), there were negligible (almost zero) changes in the peak absorbance within the 15 min interaction between the Fe(0) nanoparticles and both azo-dyes. In the presence of 6.8 wt.% citric acid only (under no bubble propulsion by Fe(0) nanoparticles), however, changes in peak absorbance in both MO/MB were observed, with an average decrease in 1.19 a.u. (RSD=7.8%, n=3) for MO and 0.649 a.u. (RSD=7.0
% (n=3) for MB, respectively. The data from the two control experiments indicated that acidic conditions are required for initiation of the degradation of MO/MB by Fe(0) nanoparticles and the existence of SDS alone would not cause any degradation reaction to occur. Furthermore, the comparison between the absolute change in MO/MB peak absorbance of citric acid mixtures with and without the SDS surfactant (Figure 12.5B red and blue bars) revealed that the presence of moving Fe(0) nanoparticles enhanced the rate of MO/MB degradation, as the average decrease in peak absorbance over the 15 min interaction period was raised from 1.19 to 1.63 a.u. (RSD=10.8 %, n=3) for MO and from 0.649 to 1.05 a.u. (RSD= 9.0 %, n=3) for MB, respectively. This increase in the rate of azo-dye pollutant removal by bubble-propelled Fe(0) nanomotors is likely the result of a mechanical mixing effect by the bubble ejection and continuous motion of the nanomotors.

In summary, it was demonstrated that Fe(0) nanoparticles that can be fabricated readily in large quantities were able to undergo autonomous propulsion by utilising weak citric acid to power the motion. In addition, an improvement in the rate of azo-dye pollutants degradation was observed from the use of the environmentally friendly and controllably stabilised Fe(0) nanomotors. Such study will facilitate the real-world applications of nanomotors in environmental remediation or waste water treatment in the future.
12.3 Experimental

12.3.1 Materials

Sodium dodecyl sulphate (SDS, Product No.: L3771) and methyl orange (MO, Product No.: 68250) were purchased from Sigma–Aldrich while citric acid monohydrate (99.5+%, Product No.: 22869) and methylene blue (MB, Product No.: A18174) from Alfa Aesar. The chemicals were used as received and ultrapure water (18.2 MW cm) from a Millipore Milli-Q purification system was used throughout the experiments.

12.3.2 Preparation of Fe(0) nanoparticles and their characterisation

12.3.2.1 Fabrication of Fe(0) nanoparticles

Fe(0) nanoparticles were prepared by thermally induced solid-state reduction of iron(III) oxyhydroxide precursor. Briefly, goethite nanoparticles were placed into the vacuum furnace at room temperature and reduced by hydrogen at 400 °C for 7 h (the heating of 10 °C min⁻¹) at applied overpressure of 0.2 bars. Then, the sample was cooled down to room temperature under a nitrogen atmosphere. This process resulted in pure Fe(0) nanoparticles. Immediately after the nanoparticles had cooled down, the sample was treated in a gas mixture consisting of 2% of oxygen and 98% of nitrogen for 2 h, which resulted in the formation of an ultrathin iron(III) oxide layer (5 wt.%) onto the Fe(0) nanoparticle surface. A similar product stabilised by an ultrathin oxidic layer is available in tons quantities (which corresponds to ≈ 10²⁰ nanomotors units per ton) and produced by Nanoiron company, Czech Republic.

12.3.2.2 Characterisation of Fe(0) nanoparticles

The SEM images of Fe(0) nanoparticles were collected using JEOL-7600F semi-in-lens FE-SEM, in SEM mode with an accelerating voltage of 5 kV, at a working distance of 5.2 mm.
SEM-EDX data were measured with JEOL-7600F semi-in-lens FE-SEM, in SEM mode with an accelerating voltage of 30 kV, at a working distance of 15.5 mm. The SEM samples were prepared by drop-casting an aqueous suspension of the Fe(0) particles on a silicon wafer. The transmission $^{57}$Fe zero-field Mössbauer spectrum of iron nanoparticles was measured at room temperature employing a MS2007 Mössbauer spectrometer based on virtual instrumentation technique, operating at a constant acceleration mode and equipped with a 50 mCi $^{57}$Co(Rh) source. The values of the derived hyperfine Mössbauer parameters are referred to the metallic iron (α-Fe) at room temperature. Microscopic images of Fe nanoparticles were obtained by HRTEM TITAN 60–300 with X-FEG type emission gun, operating at 80 kV. This microscope is equipped with Cs image corrector and a STEM high-angle annular dark-field detector (HAADF). The point resolution is 0.06 nm in HRTEM mode. The elemental mappings were obtained by STEM Energy Dispersive X-ray Spectroscopy (EDX) with an acquisition time of 20 min.

### 12.3.3 Operation of nanomotors

A Nikon Eclipse 50i optical microscope was used to record videos and images of the Fe(0) nanomotors, which in turn were analysed with Nikon NIS-Elements software. Motion of the Fe(0) nanomotors was studied by dispersing the Fe(0) nanoparticles in aqueous solutions containing SDS (1 wt.%) and varying percentages of weak citric acid (0.21–27.1 wt.%). For each experiment involving the determination of Fe(0) nanomotors’ motion, a mixture (10 mL) consisting of the Fe(0) nanomotors, SDS (1 wt.%) and the citric acid fuel (0.21–27.1 wt.%) was placed on a clean glass slide for observation under the optical microscope.
12.3.4 Degradation of methyl orange (MO) and methylene blue (MB)

UV/Vis measurements of MO and MB were performed using Cary 100 Bio UV/Vis spectrophotometer (Varian, Inc.). MO and MB were weighed and dissolved in different aqueous mixtures to prepare solutions containing 25.0 mg mL\(^{-1}\) of MO and 13.6 mg mL\(^{-1}\) of MB, respectively. The degradations of MO and MB were analysed through UV/Vis experiments that measured the respective azo-dyes’ absorption peaks with respect to time. At the start of each UV/Vis experiment, 3 mg of Fe(0) nanoparticles were added to 3 mL of a MO/MB aqueous mixture in a glass cuvette before placing the cuvette into the spectrophotometer. The absorbance spectrum of the mixture was then recorded at fixed intervals over a period of 15 min to observe the change in the absorption peak intensity.

A total of three different aqueous mixtures were used to prepare the MO/MB solutions: (A) SDS (1 wt.%) only, (B) citric acid (6.8 wt.%) only, and (C) SDS (1 wt.%) and citric acid (6.8 wt.%).
References

Chapter 12: Efficient large-scale synthesis of Fe(0) nanomotors for environmental remediation


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Chapter 13
Towards real-world application of self-propelled micro-/nanomotors for environmental remediation

Gating effect by bubble-propelled micromotors at macroscale levels

13.1 Introduction
13.2 Results and Discussion
13.3 Experimental
The results in this chapter were published in the following manuscript:

1. Wei Zhe Teo, Hong Wang, Martin Pumera. The gating effect by thousands of bubble-propelled micromotors in macroscale channels *Nanoscale* 2015, 7, 11575.

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**Contributions:**

The experiments were performed by W. Z. Teo. H. Wang assisted in the fabrication of the micromotors. The project was conceptualised and directed by M. Pumera. All authors contributed to discussions and wrote the manuscript.
Chapter 13: Gating effect by bubble-propelled micromotors at macroscale levels

13.1 Introduction

Over the past few years, studies have demonstrated that rapid and efficient degradation of pollutants could be achieved with the introduction of micro-/nanomotors into the polluted water.\textsuperscript{1-4} However, these were mainly proof-of-concepts studies where experiments were carried out in small volumes of solution. As we advance towards the real-world application of self-propelled micro-/nanomotors for environmental remediation, it is crucial to determine how the mechanical agitation instigated by micro-/nanomotors propulsion and bubbling will affect mixing in the macroscale level.

Therefore, the effect of adding varying amounts of active bubble-propelled micromotors on the rate of sodium hydroxide (NaOH) dissolution and dispersion across long distances (> 30 cm) was investigated herein. This was carried out by placing NaOH pellets at one end of a solution-filled channel and recording the time taken for pH to change at the opposite end. The solutions contained known numbers of propelling tubular bimetallic Cu/Pt micromotors, H\textsubscript{2}O\textsubscript{2}, SDS and a pH indicator to observe the change in pH. We sought to infer, from this study, how bubbles formation and micromotors’ motion could have an impact on the duration required for a remediation agent to reach target pollutants located tens of centimeters away in order to develop suitable protocols for micro-/nanomotors assisted clean-up of pollutants in real systems.
13.2 Results and Discussion

We studied the consequence of having large numbers (tens of thousands) of active bubble-propelled micromotors on the dissolution and dispersion rate of the NaOH solid in the present study. The SEM image in Figure 13.1 displays a typical Cu/Pt concentric bimetallic micromotor used in this study. It can be observed from the image that the micromotors are conical in shape, measuring approximately 1 μm and 1.5 μm in diameters at the extreme ends, and about 8 μm in length.

![Figure 13.1 SEM image of the Cu/Pt micromotor. Debris found on the bottom right of the image belonged to the graphite ink which was applied to the polycarbonate template before the electrochemical deposition process. Scale bar represents 1 μm.](image)

A system of channels, in which the NaOH solid dissolution and dispersion measurements were carried out, consisted of a straight channel (length = 21 cm) connected to a circular channel (diameter = 15 cm) at one end created on a Teflon plate. The channels are 1 cm in both width and depth and they were filled with 25 mL of fresh pre-mixed solution, comprising of 4% H₂O₂, 1% SDS, 500 μL bromothymol blue solution (BTB; pH indicator) and known numbers of tubular bimetallic Cu/Pt micromotors, prior to the start of each experiment. Figure 13.2A shows the set-up of the system of channels before the addition of a NaOH pellet (average mass = 90.1 mg) at the bottom end of the system.
Upon introducing the solid NaOH pellet into the system, a colour change from yellow to blue in the pH indicator, which corresponded to an increase in pH of the solution, was first observed in the solution nearer to the pellet and spread towards the opposite end as the OH\(^-\) ions released from the dissolution of NaOH were dispersed by convection and diffusion due to the build-up of an OH\(^-\) concentration gradient and density flux during the dissolution process. The rate of colour change decreased as time progressed, signifying a retardation in the OH\(^-\) dispersion process as the NaOH pellet became completely dissolved. Based on the experimental results, an average time of approximately 10 minutes was needed for the whole solution to turn from yellow to blue (sufficient OH\(^-\) ions to reach the top of the system) in the absence of bubble-propelled micromotors. The final alkaline solution obtained is illustrated in Figure 13.2B.

With the incorporation of bubble-propelled micromotors into the pre-mixed solution, it was expected that the NaOH dissolution and dispersion process will be
accomplished in a shorter time frame as demonstrated in previous studies.\textsuperscript{1-4} However, in contrast, we noticed a drastic reduction in the rate of colour change of the solution which contained around $4.5 \times 10^4$ active micromotors after 2 minutes from the addition of the NaOH pellet. The bubble-propelled micromotors seemed to have a gating effect on the OH\textsuperscript{-} ions, impeding their dispersion along the channels, down the concentration gradient. As depicted in Figure 13.3, the solution at top end of the system of channels (29.1 ± 6.5%, n = 3) remained yellow even after 1 hour.

![Images illustrating the change in pH of the pre-mixed solution containing 4% H\textsubscript{2}O\textsubscript{2}, 1% SDS, 500 μL BTB and $4.5 \times 10^4$ active micromotors as OH\textsuperscript{-} ions dispersed from the bottom of the system following NaOH(s) dissolution. The images were taken at 2, 10, 30 and 60 minutes from the addition of a NaOH pellet at the bottom of the system. An increase in pH was indicated by the change in the colour of the solution from yellow to blue. Width of channels = 1 cm.](image)

**Figure 13.3** Gating effect of the propelling micromotors in macroscale channels. Images illustrating the change in pH of the pre-mixed solution containing 4% H\textsubscript{2}O\textsubscript{2}, 1% SDS, 500 μL BTB and $4.5 \times 10^4$ active micromotors as OH\textsuperscript{-} ions dispersed from the bottom of the system following NaOH(s) dissolution. The images were taken at 2, 10, 30 and 60 minutes from the addition of a NaOH pellet at the bottom of the system. An increase in pH was indicated by the change in the colour of the solution from yellow to blue. Width of channels = 1 cm.

We investigated this phenomenon further by altering the amount of active micromotors present in the pre-mixed solution and images of the system at various times of the experiment are displayed in Figure 13.4. The amount of propelling micromotors present in the freshly pre-mixed solution in samples a to e illustrated in the figure is 0 (a), $0.75 \times 10^4$ (b), $1.50 \times 10^4$ (c), $2.25 \times 10^4$ (d), and $4.50 \times 10^4$ (e) respectively.
Figure 13.4 Gating effect of micromotors as a function of active micromotors’ concentration and time. Snapshots of the pre-mixed solutions comprising 4% H$_2$O$_2$, 1% SDS, 500 µL BTB and various numbers of micromotors, taken at various times of the experiment, starting from 0.5 min after the addition of a NaOH pellet at the bottom of the system. The pre-mixed solutions contained (a) zero, (b) 0.75 × 10$^4$, (c) 1.50 × 10$^4$, (d) 2.25 × 10$^4$ and (e) 4.50 × 10$^4$ propelling micromotors respectively. Changes in the colour of the solution corresponded to an increase in pH of the solution. Width of channels = 1 cm.

From the data collected, it can be seen that in the first minute of the experiment, the rate of NaOH dissolution and dispersion was similar in all the samples. However, subsequent snapshots taken after 2 minutes revealed that the area of yellow solution remaining (did not experience an increase in pH) in the channels started to differ between the samples, especially between the control (sample a) and samples c to e. It was found that the percentage of yellow solution remaining 5 minutes after placing the NaOH pellet increased from 3.8% (a) to 7.9% (c), 14.7% (d) and 40.8% (e) respectively. These observations suggest that the micromotors were responsible for the gating effect on the dispersion of OH$^-$ ions and the intensity of the impedance was dependent on the amount of active micromotors available in the solution. In addition, the concentration
gradient and density flux present during the first minute of the experiment might be too strong, resulting in insignificant retardation of OH\textsuperscript{−} dispersion by the active micromotors. On the other hand, the NaOH dissolution and dispersion process underwent a slight acceleration in the solution which contained \(0.75 \times 10^4\) of propelling micromotors, with 100% change in solution colour observed after 5 minutes, suggesting that an optimal amount of active micromotors will result in favourable mixing of a solution in the macroscale level.

![Figure 13.5](image)

**Figure 13.5** Close-up pictures showing portion of the system containing pre-mixed solutions with (i) \(0.75 \times 10^4\), (ii) \(2.25 \times 10^4\) and (iii) \(4.50 \times 10^4\) propelling micromotors. Formation of a layer of bubbles on top of the solution is circled in red. The photographs were taken following the addition of NaOH pellets at the bottom of the system, at 5 minute mark for (i) and at 10 minute mark for (ii) and (iii). Width of channels = 1 cm.

A comprehensive examination of the photographs acquired revealed that very small bubbles started to form a layer on top of the solution as the experiments proceeded and they often covered the entire width of the channel at the top of the system after a few minutes (Figure 13.5). In addition, the bubbles tend to accumulate above the area with little or no NaOH present (yellow solution) and their quantity decreased with the number of active bubble-propelled micromotors available in the solution as expected. This observation led us to believe that higher amounts of bubbling per unit area of solution and the subsequent formation of a more extensive layer of bubbles in solutions containing larger numbers of propelling micromotors attributed to the successful retardation of OH\textsuperscript{−} ion dispersion.
In summary, the presence of active bubble-propelled bimetallic Cu/Pt micromotors in large numbers (tens of thousands) was found to slow down the dissolution and dispersion of the NaOH pellet in the macroscale level and this gating effect depended on the quantity of propelling micromotors available in the solution. The ejection of bubbles by propelling micromotors and the resulting bubble layer generated are probably the major contributors to the retardation of OH⁻ ion dispersion and they might affect the dispersion of remediation agents as well. More in-depth studies should be performed in the future to elucidate the exact mechanism which caused the gating effect so that we could improvise on the real-world applications of self-propelled micro-/nanomotors for environmental remediation.
Chapter 13: Gating effect by bubble-propelled micromotors at macroscale levels

13.3 Experimental

13.3.1 Materials

Cyclopore polycarbonate membranes with conical-shaped pores of 2 μm in diameter were purchased from Whatman, USA (Cat. no. 7060-2511), colloidal graphite (isopropanol base) from Ted Pella, Inc. (Lot no. 12009-2, USA), hydrogen peroxide (H₂O₂; 35%) from Alfa Aesar, Singapore, methylene chloride and ethanol from Tedia, USA, platinum electrode (1 mm diameter) and Ag/AgCl/1 M KCl electrode from CH instruments, USA, copper(II) sulphate (CuSO₄·5H₂O, 98+%), sodium hydroxide (NaOH, Product No. S8045) and sodium dodecyl sulphate (SDS, Product No. L3771) from Sigma-Aldrich, platinum-plating solution from Technic Inc., USA, and bromothymol blue (BTB, Product No. A17746) from Alfa Aesar. The chemicals were used as received and ultrapure water (18.2 MΩ cm) from a Millipore Milli-Q purification system was used throughout the experiments.

13.3.2 Apparatus

Electrochemical deposition was carried out using an Autolab PGSTAT 101 electrochemical analyser (Eco Chemie, Utrecht, The Netherlands) connected to a computer and controlled by NOVA version 1.8 software (Eco Chemie). A three-electrode configuration was adopted for the deposition procedure, in a customised electrochemical deposition cell at room temperature (23 °C). The auxiliary electrode was a platinum electrode while the reference electrode was an Ag/AgCl electrode. The ultrasonication process was carried out with a Fisherbrand FB 11203 ultrasonicator, and centrifugation was carried out with a Beckman Coulter Allegra 64R centrifuge. A Casio HD video-recorder was utilised for video recordings which were subsequently analysed using Nikon NIS-Elements software. An optical microscope Nikon Eclipse 50i was utilised
to observe and count the number of propelling micromotors. SEM images were obtained using a JEOL-7600F semi-in-lens FE-SEM in SEI mode with an accelerating voltage of 30.0 kV, at a working distance of 7.2 mm.

13.3.3 Preparation of Cu/Pt concentric bimetallic micromotors

Synthesis of the Cu/Pt concentric bimetallic microtubes was performed using a modified electrochemical deposition procedure on a cyclopore polycarbonate template. Prior to the electrochemical deposition experiment, colloidal graphite ink was applied on one side of the polycarbonate template using commercial cotton swabs and immediately attached to a piece of flattened aluminium foil. The template was then assembled into a customised electrochemical deposition cell and acted as the working electrode. After rinsing the template thoroughly with ultrapure water (18.2 MΩ cm), 1 M CuSO₄ electrolyte was added to the cell to deposit an outer layer of Cu, along the pores of the template, galvanostatically at −4 mA for 450 s. Subsequently, the CuSO₄ electrolyte was removed, and the cell rinsed thoroughly with ultrapure water and replaced with a commercial platinum-plating solution. Following that, the electrodeposition of the platinum segment was carried out at −4 mA for 450 s. Upon completion of the deposition of microtubes, the electrochemical cell was disassembled and the template was washed with ultrapure water. The template was then ultrasonicated to remove the graphite layer before it was dissolved in methylene chloride. Ultrasonication was performed to aid the dissolution of the template and the electrochemically deposited microtubes were collected by centrifuging the solution at 6000 rpm for 3 min. The process of ultrasonication and centrifugation was repeated 3 times with methylene chloride to ensure complete dissolution of the template. Finally, impurities in the microtube solution were removed by washing with ethanol and water 2 times each, with
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3 min of centrifugation at 6000 rpm following each washing step. The microtubes were stored in water at room temperature.

13.3.4 Operation of micromotors

Fresh 25 mL mixtures containing 4% H$_2$O$_2$, 1% SDS, 500 μL BTB solution and varying numbers of micromotors dispersed in ultrapure water were prepared for the study of the micromotor gating effect on NaOH and dispersion in water. The BTB solution was made by dissolving 0.1 g of BTB powder in 100 mL of 50% (v/v) ethanol and served as a pH indicator. The total number of propelling micromotors present in the 25 mL mixture was calculated first by placing 20 μL of the mixtures on a glass slide and counting them under an optical microscope (n ≥ 5), before multiplying the average count by a factor of 1250. A Teflon maze made up of running pathways (width and depth = 1 cm) on a Teflon plate with dimensions of 42 cm by 26 cm by 2 cm was used for the experiments and a designated system of channels was constructed by blocking certain pathways of the maze using Teflon cubes (1 cm$^3$). The mixture was added to the system of channels before the start of each experiment and a NaOH pellet was subsequently placed at one end of the channel. The colour of the mixture changed as NaOH dissolved and spread towards the other end of the channel due to the presence of a pH indicator (BTB) in the experiment. Videos of the dissolution and dispersion process were recorded by placing a video-recorder over the system of channels to determine the time taken for the process to reach completion.
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References


Chapter 14

Conclusion and Epilogue

### 14.1 Conclusion

14.1.1 Development of voltammetric techniques for determination of engineered nanomaterials and study of their transformation in water

14.1.2 Toxicological studies on halogenated nanocarbons and two-dimensional transition metal dichalcogenide nanomaterials

14.1.3 Advancement towards real-world environmental remediation application of self-propelled micro-/nanomotors

### 14.2 Epilogue
Chapter 14: Conclusion

14.1 Conclusion

The increasing advent of ENMs into consumer products has raised concerns over the impact of their presence in the environment on the ecosystems and our health. The ENMs are likely to be released into environmental waters upon repeated use, washing, or disposal of the product. Once in the water, the ENMs might undergo transformation over time, resulting in alteration of its original physiochemical properties. In addition, several ENMs have been reported to be toxic to mammalian cells, and their toxicity is dependent on their final chemical state in the environment. As such, it is essential to develop efficient techniques that are able to detect and quantify the ENMs in a sample, as well as to study their fate in environmental waters systematically.

Several ENMs have shown potential in various applications owing to their superior physiochemical properties, and thus attracted immense interest from scientific communities and industries. Halogenated nanocarbons and two-dimensional transition metal dichalcogenide are two such nanomaterials. However, they are limited studies discussing the toxicological aspects of these emerging ENMs, which will eventually be introduced into several applications, including bioapplications. In view of this, it is necessary to establish the toxicity profiles of these nanomaterials and relate them to their physiochemical properties.

Besides ENMs, the progress in nanotechnology also led to the emergence of self-propelled micro-/nanomotors which are small scale devices that could convert energy into movement and force. Proof-of-concept studies have demonstrated that self-propelled micro-/nanomotors could be employed to improve the efficiency of pollutants removal or degradation, thus indicating their applicability in environmental remediation. However, there are several challenges pertaining to their usage for large-scale
remediation works and they need to be addressed in order to utilise these micro-/nanomotors for real-world environmental remediation.

Therefore, three aims were established in this project. The first aim is to introduce voltammetric technique for rapid and precise identification and quantification of electroactive ENMs and systematically investigate the transformations of ENMs in environmental waters using several analytical techniques, including cyclic voltammetry. The second aim is focused on the cytotoxicity assessments of halogenated nanocarbons and two-dimensional transition metal dichalcogenide nanomaterials to determine their toxicity profiles. The last aim is to tackle some of the issues that limit the real-world application of micro-/nanomotors for pollutant removal/degradation.
14.1.1 Development of voltammetric techniques for determination of engineered nanomaterials and study of their transformation in water

Electroactive ENMs could display unique voltammograms when subjected to voltammetry measurements and previous studies have shown that it is possible to utilise differential pulse voltammetry to quantify the amount of ENMs in a sample. As such, voltammetry could represent a facile and sensitive technique for rapid determination of ENMs and the study of its transformation in environmental waters.

The possibility of quantifying the amount of copper (II) oxide nanoparticles in alkaline media through cyclic voltammetry measurements was highlighted for the first time in Chapter 3. Subsequently, we studied the electrochemistry of iron (II,III) oxide nanoparticles using the same technique and demonstrated that the amount of iron-based nanoparticulate impurities in carbon nanotubes could be determined with cyclic voltammetry in Chapter 4.

Following that, it was found that the type of environmental water silver nanoparticles is released into could alter its morphology and extent of dissolution in Chapter 5. The amounts of dissolved oxygen and chloride in the water are likely to be the main factors that influenced silver nanoparticles dissolution. Then, comparison on the transformation of three graphene oxides (prepared using different oxidative treatments) in different types of water over a period of three months was highlighted in Chapter 6. Both studies showed that cyclic voltammetry could be employed to monitor the transformation of ENMs in water. Similar studies could be carried out on other commonly utilised ENMs (eg. ZnO, TiO₂, CNT) for commercial products in the future to investigate their transformations in different environmental waters.
14.1.2 Toxicological studies on halogenated nanocarbons and two-dimensional transition metal dichalcogenide nanomaterials

Recent advances in the research on halogenated nanocarbons and two-dimensional transition metal dichalcogenide nanomaterials could potentially lead to their incorporation in consumer products, including biomedical applications. Concerns over their toxicities to biological systems ought to be addressed before these nanomaterials can be introduced commercially. Initial evaluation of the nanomaterials’ biocompatibility was performed in this project by assessing their cytotoxicity in tandem with detailed characterisation of the nanomaterials to elucidate any relationship between the toxicological responses and the physiochemical properties.

It was revealed from cell viability measurements in Chapter 7 that size and morphology of the nanomaterial could influence the cytotoxicity of fluorinated nanocarbons. In addition, results from Chapter 8 suggested that fluorine content and in particular, the types of fluorine-containing group present in the nanomaterial, have an impact on the toxicity profiles of fluorinated graphenes. Subsequently, Chapter 9 demonstrated that the amount of halogen modified on graphene to produce halogenated graphenes would determine the level of cytotoxicity it exhibits. All three studies have also showed that thorough characterisations of the nanomaterials are a crucial aspect in any nanotoxicity assessments as it allowed us to understand the underlying factor that affected the nanomaterial’s toxicity.

Cytotoxicity assessment on exfoliated MoS$_2$, WS$_2$, and WSe$_2$ nanomaterials in Chapter 10 revealed that the disulphide nanomaterials were less toxic than organic analogues such as graphene oxides and halogenated graphenes, thus suggesting its potential for biomedical usage. The importance of performing control experiments to
determine possible nanomaterial-induced interference on cell viability assays was also highlighted in the study as it was found that the exfoliated transition metal dichalcogenides could reduce MTT reagent under cell-free conditions, resulting in false cell viability readings.

As we continue to assess the relationships between toxicity induced by nanomaterials and their physiochemical properties, it would be advantageous and more accurate to characterise these nanomaterials while they are incubated with the cells, so that we could eliminate possible error arising from differences in the physiochemical properties of the nanomaterials when they are dispersed in different medium (water versus cell culture).
14.1.3 Advancement towards real-world environmental remediation application of self-propelled micro-/nanomotors

As the research in self-propelled micro-/nanomotors gear towards their implementation in real-world scenarios, it is necessary to look into fabrication techniques that could lead to low cost and large-scale production of these small devices, as well as to observe their behaviour at the macroscale level.

Replacing platinum with an alternative catalyst to power the motion of micro-/nanomotors could potentially reduce the cost and increase the feasibility of producing the devices in huge quantities for real-world application. Chapter 11 illustrated the preparation of a silver-catalyst based micromotor which exhibited high mobility, high speed, and long lifetime. Subsequently, Fe(0) nanoparticles that could be easily produced in tons quantities were shown to propel autonomously in the presence of environmentally friendly citric acid in Chapter 12. In addition, the study demonstrated that the self-propelled Fe(0) nanomotors dramatically increased the degradation of azo-dyes pollutants. Therefore, the Fe(0) nanomotor represents a great replacement that could improve the efficiency of current decontamination works that already utilise static Fe(0) nanoparticles while maintaining the low cost and minimal damage to the environment.

Lastly, it was discovered in Chapter 13 that the presence of active bubble-propelled bimetallic Cu/Pt micromotors would result in a propelling micromotor quantity-dependent gating effect on the dissolution and dispersion of NaOH pellet in the macroscale level. We hypothesised that this phenomenon could affect the delivery of remediation agents to target pollutants located some distance away in the presence of active bubble-propelled micro-/nanomotors.
14.2 Epilogue

Perspective

The progression of nanotechnology has led to the incorporation of engineered nanomaterials in many consumer products today, with many others pending to be applied in various areas, such as drug delivery, sensing, optoelectronics, and catalysis. As the number of nanomaterial-containing consumer products increase, concerns about the safety aspect of using engineered nanomaterials and their long-term impact on the environment emerged. The scientific community begun to investigate the interactions between engineered nanomaterials and environmental waters, soil, and plants to determine the fate and transformation of these engineered nanomaterials upon release into the environment.

Since we are dealing with materials which are of nanometre dimensions and probably in minute concentrations in the environment, analytical techniques would require to be sensitive enough to detect and quantify them. It is also important that the techniques employed could characterise the engineered nanomaterials in their original environment as artifacts and alterations to the nanomaterial’s physiochemical properties will occur during sample preparation. Therefore, effort in the development of new analytical techniques will be crucial for facile and reliable determination of engineered nanomaterials in the environment and monitoring its transformation over time. In this way, we can better comprehend the acute and chronic toxicological impact of these nanomaterials on the ecosystems and our health if they are allowed to accumulate in the environment. As these engineered nanomaterials are estimated to be found in very low concentrations in environmental waters (in the ng L\(^{-1}\) range), present
voltammetric techniques might not be sensitive enough to detect and quantify these ENMs directly. Future works should focus on improving the sensitivity of these techniques or developing in situ pre-concentration techniques to allow these ENMs to be detected. In addition, present voltammetric studies would unlikely be able to differentiate ENMs from its bulk material, thus filtration of a real sample ought to be carried out first to accurately determine the amounts of the nanosized materials only.

The initial growth in nanotechnology was not met with a corresponding research in the toxicity of the engineered nanomaterials, especially when it came to carbon-based nanomaterials and inorganic analogues of graphene. Fortunately, this situation has improved in recent years with the increase in nanotoxicology studies of these nanomaterials. Current nanotoxicological research relied on the use of conventional in vitro testing methods which might not be suitable for nanomaterials due to possible interferences induced by the nanomaterials, resulting in false readings. In addition, the lack of standardised procedure to characterise the nanomaterials and determine their toxicity further impede the progress in this field. However, this is set to change with the use of novel electrochemical methods to assess both in vitro and in vivo toxicity of the nanomaterials and more efforts in place to implement standard protocols for the toxicological studies. These electrochemical methods have comparable sensitivity as the traditional in vitro toxicity assessment assays. In addition, some of these techniques could be utilised to measure single cell as opposed to collective signals from the whole cell culture.

Another product of nanotechnology advances is self-propelled micro-/nanomotors. They received immense attention since pioneering works started slightly more than a decade ago, and potential applications of these small devices such as
environmental remediation have been demonstrated over the past few years. However, the field of self-propelled micro-/nanomotors and their application as remediation agents are still in the proof-of-concept stage. These works lacked an in-depth discussion on the cost of employing these small devices as compared to current techniques used for environmental remediation, as well as investigations on possible adverse effects of employing these micro-/nanomotors on the ecosystem. As such, future works should focus on ensuring that these small devices will become the best system for real-world environmental applications.

Nanotechnology has improved our lives thus far and with due diligence, we will be able to overcome the current challenges and progress towards more beneficial applications without inflicting damages to the environment and our health.