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Biological cell–substrate interactions with few-layered nanomaterials

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Declaration

I hereby declare that this thesis has not been and will not be submitted in whole or in part to another University for the award of any other degree.

Signature:

Rhiannon W. Harries
Biology, fundamental to understanding life, remains a vitally important area of research. There is still much left for humankind to understand even after decades of research. This is clear now more than ever; as I write this, research has been disrupted for about 12 months due to various COVID-19 restrictions. Biology, therefore, is ripe for the fresh, new advances that result from interdisciplinary collaboration. Recent years have seen the exciting development of new few-layered materials, providing new possibilities in biology, as well as other areas. To this end, the work presented herein considers the interactions between different cell lines and various synthesised nanomaterial substrates.

To distinguish effects due to the inherent biology of the cell and effects due to the nanomaterial substrate, well-defined substrates are crucial to interdisciplinary research. Thin films created by the Langmuir–Schaefer (L–S) deposition technique are a good candidate. This technique provides an easily controllable method of producing single-layer substrates. Here, a method resulting in improved understanding of the physical and chemical influences on L–S film formation is described. Surface pressure-surface coverage data can be normalised to nanosheet size to account for edge effects. This new approach allows the L–S film density to be determined from standard dispersion properties alone. In addition, this work produced the first demonstration of the production of single layer hexagonal boron nitride films using this method.

To test nanomaterial–cellular interactions, various cell lines were seeded onto MoS$_2$ L–S substrates. To the best of our knowledge, this study resulted in the first demonstration of the internalisation of MoS$_2$ through mechanotransduction. The material showed localisation to the endoplasmic reticulum, which combined with the innate fluorescence or Raman signal of the MoS$_2$ nanosheet, could lead to a new theranostic tool. This study was expanded to consider cell interactions with other transition metal dichalcogenide materials, WS$_2$ and MoSe$_2$, to investigate the difference between structure and chemistry seen by the cell. This work provides a step change to studying nanomaterial–cellular interactions, opening the door to new therapies and diagnostics.
Publications

Elements of this thesis have contributed (in part or in their entirety) to the following publications:


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# List of Abbreviations

| # | A | B | C | D | E | F | G | I | L | M | N | O | P | Q | R | S | T | U |
| 0D | zero-dimensional. 7, 52 |
| 1D | one-dimensional. 7 |
| 2D | two-dimensional. 3, 4, 6, 7, 10, 14, 15, 20, 32, 44, 45, 52, 55, 58, 61, 67, 70, 71, 73, 76, 78, 79, 82, 83, 91, 100, 101, 102, 221 |
| 3D | three-dimensional. 4, 7, 9, 29, 33, 36, 102 |

**A**

AFM
   1) atomic force microscope. 1, 36, 38, 40, 62, 77, 80, 81, 215, 218
   2) atomic force microscopy. 36, 37, 38, 40, 62, 63, 73, 77, 215

AuNP gold nanoparticle. 14, 45, 46, 51

**B**

BN hexagonal boron nitride. 5, 7, 12, 13, 15, 22, 45, 46, 51, 52, 55, 58, 59, 61, 63, 64, 65, 67, 68, 69, 70, 73, 74, 100, 216, 218

**C**

CCD charge-coupled device. 32

CNT carbon nanotube. 7, 8, 15, 22, 45, 52

COVID-19 coronavirus disease 2019. 43, 49, 55, 104

CTAB cetyltrimethylammonium bromide. 46

CuNP copper nanoparticle. 14

CVD chemical vapour deposition. 11, 20, 77, 83, 91, 96

**D**

DI deionised. 20, 42, 43

DIC differential interference contrast. 29, 30, 40

DMEM Dulbecco’s modified Eagle’s medium. 42, 43

DNA deoxyribonucleic acid. 1, 45, 49

**E**

EDTA ethylenediaminetetraacetic acid. 42, 43

ER endoplasmic reticulum. 4, 75, 80, 82, 83, 84, 86, 87, 88, 89, 96, 102, 221
### ESF
European Science Foundation. 51

### F
**FCS** fetal calf serum. 42, 43

### G
**GO** graphene oxide. 9, 10, 44, 45, 53

### I
**IONP** iron oxide nanoparticle. 46

### L
**L-gra** larger-sized graphene flakes. 23, 59, 61, 63, 64, 65, 67, 68, 72, 215, 218

**LCC** liquid cascade centrifugation. 17, 18, 23

**LPE**
1) liquid-phase exfoliated. 11, 18, 33, 73, 96, 100
2) liquid-phase exfoliation by sonication. 14, 15, 16, 17, 19, 24, 57, 58, 59, 63, 77, 78, 79, 91, 92, 100

**L–S** Langmuir–Schaefer. 4, 5, 13, 22, 24, 59, 60, 67, 68, 73, 78, 79, 92, 101, 104

### M
**MEM** minimum essential medium. 42, 43

**MWCNT** multi-walled carbon nanotube. 45

**MXene** transition metal carbide, nitride, or carbonitride. 52

### N
**NP** nanoparticle. 14, 21, 22, 31, 43, 45, 46, 48, 49, 50, 51, 52, 54, 55, 56, 60, 66, 75, 89

### O
**OPL** optical path length. 29, 30, 35

### P
**PBS** phosphate-buffered saline. 41, 42

**PTT** photothermal therapy. 11, 12, 44, 47, 50, 51, 55, 88

### Q
**QD** quantum dot. 7, 45, 46, 47, 48, 49, 55

**QNM** quantitative nanoscale mechanical. 40

### R
**rGO** reduced graphene oxide. 10, 45
RNA ribonucleic acid. 49
RPM revolutions per minute. 17

S
SARS-CoV-2 severe acute respiratory syndrome coronavirus 2. 104
S-gra smaller-sized graphene flakes. 23, 59, 63, 64, 65, 67, 68, 71, 72, 216, 218
SWCNT single-walled carbon nanotube. 45

T
TMD transition metal dichalcogenide. 3, 4, 6, 7, 10, 11, 12, 15, 19, 20, 45, 52, 55, 57, 58, 68, 69, 70, 76, 88, 90, 91, 98, 99, 102, 103, 104

U
UPR unfolded protein response. 86, 88, 89, 102, 103
UV ultraviolet. 34, 35, 39, 62, 73
Chapter 1

Introduction

Nanotechnology is the study and application of matter at the nanoscale, which is about 1 to 100 nanometers. This encompasses a broad range of scientific disciplines, including surface science, organic chemistry, molecular biology, semiconductor physics, energy storage, and engineering. The field in its modern sense is still young, having been growing steadily over the past 50 years.

The term ‘nano-technology’ was first used by Norio Taniguchi at a conference in 1974 [1], but it was not until the invention of the scanning tunnelling microscope by Gerd Binnig and Heinrich Rohrer in 1981 [2,3] and the atomic force microscope by Gerd Binnig, Calvin Quate and Christoph Gerber in 1986 [4] that nanotechnology research began to take off in earnest. These instruments enabled researchers to image and to manipulate individual atoms for the first time. Without these inventions, and those that followed, the emergent properties of materials on the nanoscale would not be known and investigated for the wide array of applications researched today.

Nanobiotechnology refers to the application of nanotechnologies in biological fields, for example development of a “lab-on-a-chip”, real-time nano-sensors, drug delivery vectors, and tissue engineering [5, p. 1]. This is distinguished from bionanotechnology, which is the application of biological building blocks and tools for modern nanoscale technology, for example DNA nanotechnology (use of artificial nucleic acids for technology), or the use of peptide nanotubes or protein fibrils for the fabrication of physical elements at the nanoscale [5, p. 1]. According to the MedLine database, the
The term “nanobiotechnology” is first recorded in 2000 [5, p. 13]; this confirms that this is a young, interdisciplinary field which has gathered pace over the past few decades. Fresh perspectives from interdisciplinary collaboration could lead to new insights in several areas (Figure 1.1). These areas can be broadly categorised as being

- diagnostic,
- therapeutic, or
- “theranostic” (i.e. having combined therapeutic and diagnostic ability).

New inroads in these areas are in no small part attributable to the number of new nanomaterials and associated composites that have been synthesised and investigated since Geim and Novoselov’s Nobel Prize-winning work on the isolation of monolayer graphene in 2004 [6].

A whole host of few-layered and low-dimensional nanomaterials with interesting properties could introduce new ammunition to the arsenal of existing diagnostics, treatments, and theranostics. These materials typically have different properties when compared to the bulk form. Many exhibit enhanced physical, chemical, and
biological functionality due to their uniform shapes, high surface-to-volume ratios, and high specific surface areas [7] (some key materials and their properties are discussed in 2.1). This makes different nanomaterials ideal for applications such as biomedical nanocomposites (high surface-to-volume could improve scaffold or implant properties) and drug delivery (high specific surface area allows large numbers of drug molecules to be adsorbed), among others (see Figure 1.1). Furthermore, materials could exhibit different toxicities at the nanoscale. Ensuring biocompatibility is clearly vital if there is to be wide-scale adoption of biomedical applications based on nanomaterials. The burgeoning research on few-layer and low-dimensional materials means that, even for the most well established nanomaterials, their interactions with living cells and tissues is still poorly understood. One way to tackle this is to create well-defined substrates for cell and tissue studies; this would allow potential biological responses to be separated from any changes in the substrate properties.

1.1 Thesis outline

Reviewing the current state-of-the-art in nanobiotechnology, it is clear that well-defined substrates are of great importance to cell–substrate interactions. This thesis seeks to address two main themes:

- Is it possible to define a methodology to ensure a controlled and tuneable production of two-dimensional (2D) thin film substrates?

- Most nano–bio studies introduce the nanomaterial to the cell by dispersion in the cell medium — can nanomaterials be introduced to the cell from the substrate alone? Does this change the effect on the cell?

Chapter 2 covers the properties and common processing methods of layered nanomaterials, and techniques used to characterise these materials. Particular attention will be given to the transition metal dichalcogenides (TMDs), as these are investigated in more depth in later chapters. Additionally, various applications of low-dimensional materials in diagnostics and therapeutics will be discussed.
In Chapter 3, the Langmuir–Schaefer (L–S) deposition technique is discussed in more detail. Particular focus is given to the edge interactions of monolayer nanosheets. A framework to account for material interactions is developed by normalising the film surface coverage to nanosheet size, allowing greater understanding of thin film densities, and leading to a generic film formation methodology. This has wide implications, as control over thin film creation is crucial for various applications. One such application is also explored in this chapter, that of repeatable production of sample substrates for biological studies. The significant modification in cancer cell growth is demonstrated by the edge density of the material substrate used.

Chapter 4 details cell experiments on molybdenum disulfide (MoS\(_2\)) substrates. While most nano–bio experiments introduce the nanomaterial to the cell by dispersion in the cell medium, this work introduced MoS\(_2\) to the cell only via the thin film substrate onto which the cells were seeded. After a week of growth, bone carcinoma cells exhibited internalisation of the MoS\(_2\), with material clearly present within the cell, localised in the endoplasmic reticulum (ER). This is confirmed by optical microscopy and by 2D and three-dimensional (3D) Raman spectroscopy. The innate fluorescence and Raman signal of monolayer MoS\(_2\) lending itself to cell imaging, and its ability to target specific organelles, makes this an interesting phenomenon with potential theranostic applications.

This requires further investigation; Chapter 5 begins this work by considering the effect of other TMDs, tungsten disulfide (WS\(_2\)) and molybdenum diselenide (MoSe\(_2\)), on bone carcinoma cell growth. Cells were grown in the same conditions for one week to more easily compare with the work on MoS\(_2\). The work showed that internalisation of the nanomaterial is not unique to MoS\(_2\), but is also shown in WS\(_2\). However, MoSe\(_2\) was not visibly internalised within the cells. This suggests that the sulfur chemistry may play an important role in the internalisation process, although the reason for the lack of MoSe\(_2\) internalisation has not been confirmed. It is uncertain whether the material was never internalised, or whether there was a rapid internalisation and excretion of the material in the 7 day growth window before
trypsinisation and reseeding for imaging. The claim that cell–substrate interactions are an important feature for uptake was reinforced by visible cell extensions of U2OS cells on MoSe$_2$ substrates, which showed active sensing processes at work.

This thesis introduces a generic methodology for the controlled production of L–S films, applicable to a range of layered nanomaterials. This work describes the first demonstration of the deposition of L–S films of hexagonal boron nitride (BN) (Chapter 3). MoS$_2$ nanosheets are shown to be internalised and localised within the cell by mechanotransduction, leading to a potential new theranostic agent.
Chapter 2

Few-layered and low-dimensional materials

The last few decades have been a boom time for research into few-layered low-dimensional nanomaterials, with good reason. These materials, their properties long known and well characterised at the macro scale, can exhibit vastly different properties at the nanoscale. This has made possible many new applications, and research into potential medical and biological uses is ongoing, as described in 2.4.

Greater understanding of these nanomaterials and their properties is vital for the realisation of future applications. The ability to characterise them thoroughly is therefore necessary. This chapter discusses the properties, synthesis and processing of materials used during the course of this thesis, and the characterisation techniques used to investigate their properties. Experimental details for Chapters 3–5 are also included.

2.1 Properties of layered materials

Graphene is perhaps the most well known two-dimensional (2D) layered nanomaterial — many people globally will have come into contact with its bulk form, graphite (in the form of pencil lead), at an early age. However, there is increasing interest in other low-dimensional nanomaterials; in particular the transition metal dichalcogenides
(TMDs) are hailed as the new ‘wonder materials’ [8]. The following subsections will discuss carbon-based nanomaterials (2.1.1) and TMDs (2.1.2) in more detail, and other nanomaterials such as hexagonal boron nitride (BN) (2.1.3) will also be discussed.

### 2.1.1 Carbon-based nanomaterials

The carbon family of nanomaterials spans from the zero-dimensional (0D) to the three-dimensional (3D). In addition to the 2D graphene, these materials include the 0D fullerenes (first predicted by Osawa in 1970 [9] and discovered by Kroto et al. in 1985 [10]); the 1D carbon nanotube (CNT), whose first discovery is contentious* [15]; and the 3D graphite, a layered material formed of stacks of graphene monolayers, natural deposits of which have been known and used for centuries. Together, these can be considered the graphitic family; graphene can be used as an initial building block for the formation of these other materials (Figure 2.1). Additionally, there are a range of other carbon nanomaterials including carbon quantum dots (QDs) [16,17], carbon black [18], carbon nanofibres [19,20], and the more exotic carbon nanohorns [21,22] and carbon nano-onions [23,24].

There has been a surge of research involving graphene since 2004, when the work for which Novoselov and Geim won their Nobel Prize was first reported [6]. This described the isolation of graphene nanosheets large enough to measure its remarkable electronic properties for the first time [6], detailed further in [25]. These properties are produced as a result of the structure of graphene.

Carbon atoms have four valence electrons, two in the 2s orbital and the other two in the 2p$_x$ and 2p$_y$ orbitals. When bonding with other atoms, one of the electrons in the 2s orbital is promoted to the 2p$_z$ orbital, such that each orbital has one electron [27]. In graphene, only the 2s, 2p$_x$, and 2p$_y$ orbitals undergo hybridisation, forming three sp$^2$ orbitals [27]. These orbitals lie perpendicular to the remaining

---

*Single-walled CNTs were first discovered independently by Iijima and Ichihashi, and Bethune et al., in 1993 [11,12]. The discovery of multi-walled CNTs is also frequently ascribed to Iijima [13]; while this work certainly brought CNTs wider attention in the scientific community, they were perhaps first observed in 1952 by Radushkevich and Lukyanovich [14].
Figure 2.1: The iconic hexagonal structure of graphene is shown in blue (top). Graphene can be considered the initial building block for the graphitic family, comprising fullerenes (left), CNTs (centre), and graphite (right). Image from [26].

2p\(_z\) orbital, forming covalent σ bonds in a trigonal planar geometry, resulting in graphene’s familiar layered hexagonal lattice [25,27]. This is shown in Figure 2.1. The 2p\(_z\) orbitals form π bonds between graphene sheets, and are the cause of the weak bonding which allows the easy exfoliation of graphite.

It is these σ and π bonds that give graphene its remarkable mechanical and electronic properties. The delocalised electron density associated with σ bonds ranks these among the strongest covalent bonds, giving graphene its extreme strength (intrinsic strength of 42 N/m in a defect-free sheet) [28]. This delocalisation also improves the electronic transport properties [27]. While the π bonds explain the weak, easily-broken bonding between sheets of graphene, they are also the cause of graphene’s unique electronic properties [29].

A tight-binding model can be used to derive the energy dispersion relation of graphene [25,30]. The band structure can therefore be visualised by plotting this relation, shown in Figure 2.2. The dispersion relation is linear at the \(K\) points, called the Dirac points, which correspond to the corners of the first Brillouin
zone [25,30]. This makes graphene a zero-gap semiconductor, or semi-metal; its electrons have no effective mass and are scattering-free over distances on the order of micrometers [25]. Further, the conductivity of graphene can be tuned with an external gate potential [6,25]. Graphene has also been investigated for biological applications, although its inert nature doesn’t lend itself easily to ensuring biocompatibility. However, while modification of graphene can enhance its compatibility in biological systems [31], graphene oxide (GO) is a more obvious choice for use in nano–bio research.

GO has received more attention in this sphere due to its excellent surface functionality, amphiphilicity, aqueous dispersibility, fluorescence quenching ability and surface-enhanced Raman scattering property [31]. Unlike graphene, the oxygenated sheets of GO allow for easy functionalisation or loading of biological compounds, leading to improved biocompatibility, solubility and selectivity [32]. However, this benefit also limits its use as a substrate or scaffold, as these functional groups increase
the hydrophilicity compared to graphene, meaning that GO readily disperses in water and cell media [27, 33]. For this reason, reduced graphene oxide (rGO) could offer ‘the best of both worlds’ — rGO maintains enough functional groups to be easily functionalised [34, 35], while also becoming less soluble [36].

2.1.2 Transition metal dichalcogenides

Transition metal dichalcogenides (TMDs) are compounds described by the chemical formula MX$_2$, where M is a transition metal (e.g. Mo, W) and X is a chalcogen (e.g. S, Se) [8, 38]. The monolayer, formed of a hexagonal plane of transition metal ions sandwiched between planes of chalcogen atoms [39], typically has a thickness of $\sim$6–7 Å (Figure 2.3) [38]. Similar to graphene, the in-plane covalent bonding is strong and the bulk TMD is formed by the stacking of weakly bonded monolayers; this means that the bulk crystal is similarly easy to exfoliate [8]. While not strictly 2D materials, as the monolayer is formed of three planes of atoms, TMDs are often classed as such due to their extremely thin monolayers [38–40].

Figure 2.3: Crystal structure of MoS$_2$. Image from [37].
Some TMDs occur naturally as crystalline minerals such as molybdenite (MoS$_2$) or the rarer tungstenite (WS$_2$) [8]. TMDs are also produced synthetically by chemical vapour deposition (CVD), hydrothermal, and solid phase pyrolysis processes [39,40]. Chemical exfoliation by intercalation was the predominant method of few-layered TMD production for study in the decades before graphene was first isolated [41–48]. However, the work in this thesis uses liquid-phase exfoliated (LPE) materials; this technique is discussed further in 2.2.1.

Naturally occurring MoS$_2$ commonly has the 2H-MoS$_2$ crystal phase, while synthetic MoS$_2$ often has the 3R-MoS$_2$ phase [8]. Both of these polytypes, with respective hexagonal and rhomboedral symmetries, have a trigonal prismatic geometry and are semiconducting [8,49,50]. MoS$_2$ is probably the most well studied TMD. It has been investigated for several decades; few-layer MoS$_2$ has been reported since the 1960s [51], and the monolayer was first reported 20 years later [45]. In this thesis few- or monolayered MoS$_2$, WS$_2$, and MoSe$_2$ are studied, although these three materials are only some of the about 40 TMDs known to have a layered structure [8].

Bulk MoS$_2$ is an indirect band gap semiconductor with a band gap of 1.29 eV [53]. As the layer number decreases towards the monolayer, the indirect band gap increases to over 1.90 eV [53]. This shifts the lowest energy transition to the direct band gap (Figure 2.4), resulting in a strong photoluminescence in monolayer MoS$_2$ [52,53].

MoS$_2$ has been used for applications such as lubrication (its weak interplanar bonding gives it a low coefficient of friction) [54–56] and catalysis [57–61] for the best part of a century. More recent research has focussed on the potential of few-layered MoS$_2$ for new applications, ranging from microelectronics [62–68] to superconductivity [69–76] to photovoltaics [77–84]. Of particular relevance to this thesis are possible biological applications. MoS$_2$ has been considered for bioimaging because of the photoluminescence of the monolayer form [85–88]. Due to its high fluorescence quenching, recent years have also seen increasing interest in the use of MoS$_2$ for biosensors [89–96]. Additionally, MoS$_2$ has high absorbance of near infrared light, leading to its investigation for potential photothermal therapies (PTTs) [97–102].
Drug delivery vehicles based on MoS$_2$ have been described in the literature [103–106], as well as combination PTT/drug delivery applications [107–110]. A new potential theranostic application for MoS$_2$, utilising its inherent photoluminescence and local heating, is described in detail in Chapter 4.

### 2.1.3 Other nanomaterials

Like the TMDs, **hexagonal boron nitride (BN)** is another graphene analogue. Its crystal structure is shown in Figure 2.5; the in-plane covalent bonding is very strong, and the layered structure of the bulk is formed by van der Waals bonding between these planes [111]. This differs slightly from graphene because of the two elements involved — when stacked, the boron atoms lie above and below the nitrogen atoms [111]. Although BN also exists in other forms (e.g. cubic, wurtzite, rhombohedral), the hexagonal form is the most common as it is the most stable [112].
Despite the similarity in structure, BN exhibits different electronic properties to those of graphene. This change is caused by the narrowing of the sp\(^2\) \(\pi\) bands (due to the difference in electronegativity) compared to graphene [113]. As a result there is a loss of conductivity, yielding an insulator (or a wide-band semiconductor) [113]. A range of wide band gaps (5.5–5.9 eV) have been reported recently [114,115], though depending on the method used, this range increases to 3.6–7.1 eV [116].

Because it acts as an insulator, BN is a promising material for use as a dielectric in electronic devices [117]. However, to scale up, this requires the production of large areas of flat BN. Dean et al. used a mechanical transfer process to produce graphene-on-BN devices [117]. Another method which could produce thin films of BN suitable for such devices is Langmuir deposition (technique described in more detail in 2.2.3). Recent work has produced the first demonstrations of Langmuir–Schaefer [118] (discussed in Chapter 3) and Langmuir–Blodgett [119] films of BN. Although this research is in its infancy, utilising such processing techniques could give new approaches to transparent and flexible devices [119], or to controllable substrates for biological studies [118].
Potential applications of other nanomaterials including

- **metal nanoparticles (NPs)**
  (e.g. AuNPs, CuNPs, metallofullerenes, etc.),

- **transition metal oxides and hydroxides**
  (e.g. TiO$_2$, ZnO NPs, layered Zn(OH)$_2$), and

- **silica NPs**

are discussed in the review of low-dimensional nanomaterials with intercellular function for diagnostics, therapeutics and theranostics in 2.4.

### 2.2 Synthesis of layered materials and substrate production

To achieve the properties associated with few-layered nanomaterials, the initial material needs to be well exfoliated. The reasons that exfoliated materials are desirable are numerous. As discussed at the beginning of this chapter, it is as the 2D landscape is approached that the unique and special properties of nanomaterials are enabled. Moreover, delaminating the bulk material increases the accessible surface area of the material, leading to enhanced reactivity in catalytic applications [120].

Synthesis of few- or monolayered nanomaterials can be separated into two broad categories: top-down, where bulk material is exfoliated to produce thin sheets; and bottom-up, where the material is created from scratch from the relevant building blocks. **Liquid-phase exfoliation by sonication (LPE)** is one example of a top-down approach. The work presented in this thesis uses nanomaterials produced by this method, and LPE is discussed in more detail in 2.2.1. Some other synthesis techniques are discussed briefly in 2.2.2.

Once a nanomaterial is produced, the resultant dispersion is available for processing. Nanomaterial dispersions provide a convenient basis for the creation of a number of useful substrates for further experimentation. The work in this thesis
focuses on 2D thin films produced by Langmuir film deposition, and this method is described further in 2.2.3. Additionally, the experimental details of the synthesis and processing techniques used for work in this thesis are provided in 2.2.4.

### 2.2.1 Liquid-phase exfoliation by sonication

Liquid-phase exfoliation by sonication (LPE) to produce monolayer graphene was first described by Hernandez et al. in 2008 [121]. This paper describes the production of graphene, though the same sonication process was quickly shown to debundle CNTs [122,123], and thereafter shown to exfoliate a wide range of layered materials including TMDs [49,124], BN [124,125], and black phosphorus [126,127].

LPE is easily scaleable compared to other methods such as mechanical exfoliation. This scalability means that it is possible to achieve higher production rates, important if nanomaterials are to become key materials in industry [120]. LPE also produces defect-free nanosheets [128], which could be important in applications where the material quality is paramount. However, it is important to note that this method isn’t without problems. Although defect-free, the size of the nanosheets produced are often small compared to those produced by mechanical exfoliation [129]. Despite this drawback, nanosheets produced by LPE can still be harnessed for their enhanced properties [129].

The LPE process begins with a mixture of the bulk material in the chosen dispersant. This is introduced to either bath or probe sonication, which produces ultrasonic waves [120]. These waves cause cavitation bubbles to form in the dispersant;

![Figure 2.6: Schematic description of sonication-assisted liquid-phase exfoliation (LPE). Image adapted from [120].](image-url)
when these bubbles burst, high-energy jets are released [120]. This energy is enough to break the inter-layer bonding of the bulk material, producing exfoliated few-layer nanosheets [120]. This is shown in Figure 2.6. The success of the sonication depends in no small part on the dispersant used. Solvents [130–132], surfactants [133–137], polymers [138,139], and biomolecules [140–142] have all been demonstrated as dispersants. Du et al. have reviewed many different dispersants used for LPE of various layered materials [143]. Water is available in abundance and is nontoxic, so would make an ideal dispersant for nanomaterials. However, nanomaterials are not soluble in water, so the addition of surfactants or polymers is necessary. The work in this thesis uses nanomaterial dispersions in organic solvents, as surfactants are toxic to biological systems. As such, only solvent dispersants are discussed in detail.

The success in producing a stable nanomaterial dispersion, i.e. a dispersion which doesn’t reaggregate and precipitate quickly, has been shown to depend on surface energy matching between material and solvent [121]. Solvents with surface energies close to that of the layered material produce a more stable dispersion; reaggregation is less likely because the energy difference between the exfoliated and reaggregated states is small [120,128]. Good solvents for the exfoliation of graphene therefore require surface tensions of ~40–50 mJ/m² [121]; this explains the inability of pure water to satisfactorily exfoliate graphite, as it has a surface tension of 72 mJ/m² [120].

Which solvents make ‘good’ dispersions is a subject which has received much investigation. As discussed above, the energy cost of exfoliation is a key factor. Hernandez et al. identified the use of Hansen solubility parameters to estimate the energy cost of nanomaterial exfoliation [144]. Originally developed to explain the solubility of polymers, this framework is also able to describe the dispersibility of nanomaterials satisfactorily. The intermolecular interactions between solvent and solute can be broadly classified into dispersive (D), polar (P), and hydrogen-bonding (H) components [144]. The distance in Hansen parameter space from solvent (B) to solute (A) can be used as a simple parameter to represent the similarity of the
materials’ surface energies. This is given as

\[ R = \sqrt{4(\delta_{D,A} - \delta_{D,B})^2 + (\delta_{P,A} - \delta_{P,B})^2 + (\delta_{H,A} - \delta_{H,B})^2} \]  

(2.1)

where \( \delta_{D,B}, \delta_{P,B}, \delta_{H,B} \) are the solvent Hansen parameters and \( \delta_{D,A}, \delta_{P,A}, \delta_{H,A} \) are the solute Hansen parameters [145, p. 29]. The constant “4” is included in front of the first term having been found empirically to ensure that \( R \) is spherical in Hansen parameter space [145, p. 29]. When \( R \) is minimised, so is the energy cost of graphene exfoliation. Although shown to work for graphene, this framework has also successfully described other materials [124,146].

Hernandez et al. showed that dispersion concentration increases as \( R \) decreases [144], and dispersions of MoS\(_2\) have been produced with concentrations as high as 40 mg/mL [147]. Graphene dispersions have been shown to reach concentrations of at least 63 mg/mL, though this method included a centrifugation step before redispersion of the material in fresh solvent [148].

Centrifugation is therefore an important step in obtaining high quality nanomaterial dispersions. This method is well established, having been used in laboratory practice for well over 100 years [149]. In short, the dispersion is rotated at a high number of revolutions per minute (RPM); this artificially increases the gravitational force felt by nanosheets in the dispersion. The largest sheets feel the largest gravitational force, so separate quickest from the rest of the dispersion. Hence, separation occurs at different rates depending on sheet size, allowing the isolation of few-layered nanosheets in minutes, rather than days.

Not only does centrifugation remove unexfoliated bulk material and increase the concentration of the final dispersion, but it can also be used to obtain specific sizes of nanosheets. LPE produces nanomaterials with a broad range of sheet sizes and thicknesses [150]. If nanosheets within a specific, narrow range are required then additional processing is needed. One size selection technique is known as liquid cascade centrifugation (LCC) [151]. This process covers a series of successively stronger centrifugation steps. Each step has a gradually increased RPM, such that
Figure 2.7: Diagram illustrating the liquid cascade centrifugation (LCC) process, beginning with a graphene dispersion in cyclohexanone. Details in figure relate to 2.2.4.1.

the dispersion is subjected to steadily increasing gravitational forces. These fractions, each with smaller and thinner nanosheets, can be redispersed to produce samples. The LCC technique is shown in Figure 2.7.

### 2.2.1.1 High-pressure homogenisation

While sonication-assisted LPE materials are mainly used in this thesis, some material was produced by high-pressure homogenisation. The shear forces required to exfoliate the material are provided by forcing the dispersion through a narrow nozzle at high pressure [152]. The specific system used in this thesis is described by Large et al. and is shown in Figure 2.8 [137]. The initial dispersion is poured into the inlet reservoir, and from here it is drawn into the intensifier pump. The system can generate pressures of up to 3000 atm, and the compressed dispersion is decompressed through a diamond nozzle within the process cell [137]. The resultant high-speed jet of dispersion collides with fluid flowing in the opposite direction, generating the shear forces which exfoliate the bulk material. From here the dispersion is passed into a heat exchanger; the turbulent forces necessary for exfoliation produce a lot of
heat, so the dispersion is cooled to a specified temperature controlled by an external chiller. At the end of the cycle, the dispersion can either be collected or recirculated, depending on the system configuration [137].

Figure 2.8: Schematic of the high-pressure homogenisation equipment used, overlayed on a photograph of the system. Image from [137].

2.2.2 Other synthesis techniques

While liquid-phase exfoliation by sonication and high-pressure homogenisation were the only synthesis techniques used in this thesis, other methods are commonly used in the literature. Some other techniques are discussed briefly in this section.

One method mentioned in passing in 2.1.2 is ion intercalation. Whereas LPE utilises sonication to break the weak bonding between the layers in layered nanomaterials, ion intercalation involves the introduction of ion guests between these layers [153]. The ions increase the size of the gaps between layers [154], leading to exfoliation if enough energy is supplied [120]. In 2.1.2 the long history of TMD exfoliation by ion intercalation was mentioned, but it is important here to note that graphite has also been subject to ion intercalation studies for the past several decades [155].
No discussion of nanomaterial exfoliation would be complete without mentioning **mechanical exfoliation**. This method is well known as the technique first used to isolate monolayer graphene by Novoselov and Geim in 2004 [6]. This simple yet powerful technique works by physically peeling individual layers from the bulk crystal using scotch tape, allowing each layer to be deposited on a substrate of choice. This synthesis technique has also been used to obtain other few-layered materials, including but not limited to TMDs [156] and group IV metal chalcogenides [157].

Possibly the most common bottom-up synthesis method is **chemical vapour deposition (CVD)**. The process involves the introduction of the precursor material as a vapour to a vacuum chamber which contains the substrate; the vapour then reacts with the substrate to produce a monolayer of the desired material [158]. This can create monolayers with large areas, but is an expensive process that currently lends itself mainly to high-performance applications [159]. Moreover, the nanosheets produced can be quite defective, and cannot be transferred from the initial substrate used. Regardless, CVD is the one of the most common bottom-up synthesis techniques in industry, used for microelectronics (mobile phones, laptops, etc.), data storage, and medical equipment applications [160].

### 2.2.3 Langmuir deposition

The Langmuir deposition technique functions by utilising the air–liquid interface as a **2D** plane for the assembly of exfoliated nanosheets. Nanomaterial dispersion is dropped onto the liquid subphase (frequently deionised water is used, though some materials may require a modified subphase); the droplets spread on the subphase surface until the solvent evaporates and a thin, monolayer film is left at the air–liquid interface.

Therefore, solvent choice is an important aspect of the process. The chosen solvent must fulfil three main requirements. It must:

- have a high vapour pressure, so that it evaporates and leaves a film of particulates at the interface [161];
• spread on the water surface, to maximize the area over which nanoparticles are spread to minimize reaggregation during solvent evaporation \[162\]; and

• be water-immiscible, to avoid mixing with the subphase.

The density of the nanomaterial monolayer can be modified before deposition by controlling the film area with barriers. This was first described by Agnes Pockels in 1891 \[163\]; she had created a trough with moveable barriers from a tin pan and inserts. The trough was further developed by Irving Langmuir and Katherine Blodgett \[164\].

Control over the film density is important, as this is related to the surface pressure of the film and can affect its behaviour. When trough area is large and the film density is low, the material is sparsely distributed in the trough; these ‘films’ are not continuous, and may consist of islands of material across the trough area. As the trough area decreases, the film density increases until nanosheets come into contact with each other. If this continues, the film will eventually cease to be monolayer as the density reaches a maximum and the nanosheets begin to overlap. This process describes the change of surface pressure from low to high, and can be described as the ‘phase transitions’ \[165\] of the monolayer film (Figure 2.9).

![Figure 2.9: Typical pressure–area isotherm indicating the different phases in a Langmuir monolayer. As the trough area, and hence area per molecule, decreases, the surface pressure increases, causing the monolayer to undergo ‘phase transitions’. The molecular arrangement in the different phases is indicated. Image from \[166\].](image-url)
Once the nanomaterial film is at the desired density it is deposited onto a suitable substrate. The thin films studied throughout this thesis are deposited onto glass cover slips as these are standard substrates in the biological sciences, so compatibility with cell study methods was ensured. There are two main techniques to deposit the nanomaterial film from the air–water interface onto a substrate: Langmuir–Blodgett deposition, which involves the vertical dipping of a substrate through the monolayer [164], and Langmuir–Schaefer (L–S) deposition, which involves the horizontal dipping of a substrate [167].

The Langmuir deposition technique was developed with amphiphilic molecules, as the polar nature of these molecules meant that they would align in the same orientation on the subphase and transfer easily to the substrate [168]. The technique has since been shown to work for a range of nanoparticles [166] including but not limited to pure and functionalised fullerenes [169–171]; capped metallic NPs [172–174]; functionalised CNTs [175–177]; silver nanowires [178]; graphene [179–181]; MoS$_2$ [182–184]; and phosphorene [185].

### 2.2.4 Synthesis and processing of materials for Chapters 3–5

This section contains experimental details of the material synthesis and substrate production for the work presented in Chapters 3–5.

#### 2.2.4.1 Synthesis and processing of materials for Chapter 3

**Synthesis of materials**

BN powder (0.5 g, Aldrich Chemistry) was added to 20 mL of cyclohexanone (VWR Chemicals) and probe sonicated using a Sonics Vibracell VCX750 and ½-inch (13 mm) tip at 60% amplitude for 3 hours. The resulting dispersion was centrifuged for 25,000 g min using a Thermo Scientific Sorvall Legend X1. The supernatant was collected for further characterisation.
MoS$_2$ powder (0.4 g, Aldrich Chemistry) was added to 20 mL of cyclopentanone (VWR Chemicals) and probe sonicated using a Sonics Vibracell VCX750 and $\frac{1}{2}$-inch (13 mm) tip at 60% amplitude for 1 hour. The resulting dispersion was centrifuged for 150,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was discarded, and 20 mL of fresh cyclopentanone added. This was probe sonicated using a Sonics Vibracell VCX750 and $\frac{1}{2}$-inch (13 mm) tip at 60% amplitude for 3 hours. The resulting dispersion was centrifuged for 25,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was collected for further characterisation.

WS$_2$ powder (0.75 g, Aldrich Chemistry) was added to 30 mL of acetone (VWR Chemicals) and probe sonicated using a Sonics Vibracell VCX750 and $\frac{1}{2}$-inch (13 mm) tip at 60% amplitude for 1 hour. The resulting dispersion was centrifuged for 150,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was discarded, and 20 mL of fresh cyclopentanone added. This was probe sonicated using a Sonics Vibracell VCX750 and $\frac{1}{2}$-inch (13 mm) tip at 60% amplitude for 3 hours. The resulting dispersion was centrifuged for 25,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The resultant WS$_2$/acetone dispersion was centrifuged for 5,400,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was discarded, and the solute redispersed in 5 mL of fresh cyclohexanone added to each centrifuge tube.

Graphene dispersions in cyclohexanone were processed as described in Large et al. [137]. Size-selected graphene dispersions were required. The dispersion was diluted 1:1 with cyclopentanone (Aldrich Chemistry) for the largest flake size (referred to as L-gra). LCC [151] was used to obtain fractions of the original dispersion with different nanosheet size by extraction and re-centrifugation of the supernatant after each centrifugation step (Figure 2.7). Each time, this process leaves behind a solute that has an increasingly smaller average flake size. The dispersion to be size-selected for small flake size (referred to as S-gra) was further diluted 1:10 in cyclopentanone before centrifugation for 4,800,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was collected for further characterisation.
Langmuir–Schaefer deposition of films

A NIMA 102A Langmuir trough and NIMA surface pressure sensor (type PS4, serial no. 045) equipped with platinum Wilhelmy plate was used. Material dispersions were used to create thin films with varying surface coverages. The dispersion concentrations used ranged between 0.018–2.558 g/L (i.e. within the range typically produced by LPE), and the amounts deposited ranged between 100–900 µL. For film fabrication calibration, a range of surface pressures has been used (ranging from approximately 0.5–40 mN/m). To investigate quantitatively, a series of thin films of various materials were deposited by L–S technique at varying pressures.

2.2.4.2 Synthesis and processing of materials for Chapter 4

Synthesis of materials

MoS$_2$ powder (0.4 g, Aldrich Chemistry) was added to cyclopentanone (20 mL, VWR Chemicals) and probe sonicated using a Sonics Vibracell VCX750 and $\frac{1}{2}$-inch (13 mm) tip at 60% amplitude for 1 hour. The resulting dispersion was centrifuged for 150,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was discarded, and fresh cyclopentanone (20 mL) added. This was probe sonicated using a Sonics Vibracell VCX750 and $\frac{1}{2}$-inch (13 mm) tip at 60% amplitude for 3 hours. The resulting dispersion was centrifuged for 25,000 $g$ min using a Thermo Scientific Sorvall Legend X1 to remove unexfoliated nanosheets. The supernatant was collected for further characterisation.

Langmuir–Schaefer deposition of films

A MoS$_2$ dispersion was used to create thin films by the Langmuir deposition technique. Films were created using both a NIMA 102A Langmuir trough and NIMA surface pressure sensor (type PS4, serial no. 045) equipped with platinum Wilhelmy plate, and by using a Petri dish without a surface pressure sensor. Material was deposited onto pristine glass cover slips once the films appeared dense to the naked eye.
2.2.4.3 Synthesis and processing of materials for Chapter 5

Synthesis of materials

WS$_2$ powder (0.75 g, Aldrich Chemistry) was added to 30 mL of acetone (VWR Chemicals) and probe sonicated using a Sonics Vibracell VCX750 and ½-inch (13 mm) tip at 60% amplitude for 1 hour. The resulting dispersion was centrifuged for 150,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was discarded, and 20 mL of fresh cyclopentanone added. This was probe sonicated using a Sonics Vibracell VCX750 and ½-inch (13 mm) tip at 60% amplitude for 3 hours. The resulting dispersion was centrifuged for 25,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was collected for further characterisation.

The WS$_2$/acetone dispersion was centrifuged for 5,400,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was discarded, and the solute redispersed in 5 mL of fresh cyclohexanone added to each centrifuge tube.

MoSe$_2$ powder (0.5 g, Aldrich Chemistry) was added to 20 mL of cyclopentanone (VWR Chemicals; Sigma-Aldrich) and probe sonicated using a Sonics Vibracell VCX750 and ½-inch (13 mm) tip at 60% amplitude for 1 hour. The resulting dispersion was centrifuged for 150,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was discarded, and 20 mL of fresh cyclopentanone added. This was probe sonicated using a Sonics Vibracell VCX750 and ½-inch (13 mm) tip at 60% amplitude for 3 hours. The resulting dispersion was centrifuged for 25,000 $g$ min using a Thermo Scientific Sorvall Legend X1. The supernatant was collected for further characterisation.

Langmuir–Schaefer deposition of films

WS$_2$ and MoSe$_2$ dispersions were used to create thin films by the Langmuir deposition technique. Films were created using both a NIMA 102A Langmuir trough and NIMA surface pressure sensor (type PS4, serial no. 045) equipped with platinum
Wilhelmy plate, and by using a Petri dish without a surface pressure sensor. Pristine glass cover slips were masked with scotch tape approximately halfway; material was deposited onto these substrates once the films appeared dense to the naked eye.

2.3 Characterisation techniques

This section describes the physics behind the characterisation techniques used in this thesis. Broadly, these encompass microscopy (2.3.1, 2.3.4) and spectroscopy (2.3.2, 2.3.3).

2.3.1 Optical microscopy

While this technique is well known, it is worth mentioning here as it is used extensively throughout the work in this thesis. Light microscopes use visible light to produce a magnified image of an object that is projected onto a sensor (e.g. the retina of the eye or the photosensitive surface of an imaging device) [186]. Compound light microscopes use two lenses together to produce the final image magnification [187].

The optical components of a typical upright optical microscope are shown in Figure 2.10a. Light is provided to the microscope stage by a mirror or an integrated light source. The light passes through the condenser, which focuses the light onto a small area of the specimen, before illuminating it [186]. From the specimen, the light next reaches the objective, which collects the light diffracted by the specimen and forms a magnified real image near the eyepieces (depicted in Figure 2.10b) [186]. The eyepiece together with the detector (eye, camera, etc.) forms a final real image which is interpreted as having been magnified [186].

Bright-field microscopy occurs when light is transmitted through the sample to the objective; the image contrast is generated by the absorption of light in dense areas of the sample [188]. Until the late 1980s, other imaging modes were laborious to implement because most microscopes had a fixed tube length (i.e. the distance between the objective and the eyepiece is specified) [186]. This ‘finite’ design,
Figure 2.10: (a) Diagram showing optical components of an upright optical microscope. (b) Perception of a magnified virtual image of a specimen in the microscope. The objective forms a magnified real image of the specimen (called the real intermediate image) in the eyepiece. The intermediate image is examined by both the eyepiece and eye, which together form a real image on the retina. Because of the perspective, the retina and brain interpret the scene as a magnified virtual image about 25 cm in front of the eye. Image and caption adapted from [186].

shown in Figure 2.11a, doesn’t easily allow for additional optical accessories such as polarisers to be added to the light path [186]. These additions increase the effective tube length, resulting either in image artefacts or in the placement of additional optical elements to counteract the effect of the accessories [186].

To combat the problems associated with fixed tube length microscopes, an alternative approach dubbed ‘infinity optics’ was developed (shown in Figure 2.11b) [186]. Instead of focusing light rays from the objective at the intermediate image plane, the design is modified such that the objective produces parallel light rays that image at infinity [186]. These rays are brought into focus by an additional component, the tube lens [186]. The area between the objective and the tube lens is designated the ‘infinity space’, and it is possible to introduce additional optical accessories into the light path here without producing artefacts [186].
Although bright-field microscopy can be used to image living cells, many biological samples appear with low contrast. In particular, specimens which are colourless and transparent, such as many types of cells, are not easily seen. To improve visibility of these samples, other imaging modes must also be utilised.

Dark-field microscopy excludes the unscattered light ray from the final image, which appears as a very dark background with a bright sample on it [189]. The technique is simple, and requires only the addition of a dark-field condenser lens. Artefacts which are common in phase-contrast microscopy, such as halos or shadows, do not usually appear in dark-field images. However, due to the low light in the final image the sample must be strongly illuminated [189], which can damage biological samples.
Another method that improves the contrast in transparent samples is differential interference contrast (DIC) microscopy. As its name suggests, DIC microscopy uses principles of interferometry to resolve features that would otherwise be invisible. This is because most detectors are sensitive only to the intensity of light, only showing contrast based on how much light is absorbed; DIC microscopy instead utilises the phase changes caused by transparent specimens to produce an image of the specimen [190].

In microscopes adapted for this technique, the initial light is polarised first by a polariser and then by the first Nomarski-modified Wollarston prism [190]. This produces two laterally-separated light rays which are orthogonally polarised and coherent [191]. These rays are focused by the condenser such that they pass through the sample a small (sub-resolution) distance apart from each other [190]. Because the adjacent areas of the sample will have different thicknesses or refractive indices, the light rays will experience different optical path lengths (OPLs), causing a relative change in phase between the rays. The light rays travel through the objective and are focused onto the second Nomarski-modified Wollarston prism, which recombines the light rays into one polarised beam and gives rise to interference of the rays [188].

Figure 2.12: Gradients in optical path length (OPL) yield differences in amplitude. (a) DIC image of a mammalian erythrocyte (its diameter indicated by the white line). (b) Plot and (c) first derivative of the OPL across the diameter of the cell. Positive and negative slopes in panel (a) correspond to regions of higher and lower amplitude in panel (c). Regions of the object exhibiting no change in slope (e.g. centre of the cell) have the same amplitude as the background. This gives DIC images their misleading “3D” appearance. Image from [186].
Figure 2.13: Comparison of images of the same Madin Darby canine kidney cell obtained from bright-field (left), dark-field (centre) and DIC (right) microscopy. Scale bar = 10 µm. Image from [192].

The final DIC image depends on the gradient of the OPL (Figure 2.12). The advantage of DIC microscopy is apparent when compared with bright-field and dark-field microscopy; this technique produces images with higher contrast and better z-resolution [192] (Figure 2.13).

Fluorescence microscopy is a technique commonly used for biological imaging. The term ‘fluorescence’ describes the process where specimens absorb light of one wavelength and re-emit it at a longer wavelength [187]. It is an example of the wave–particle duality of light. When a photon of light is incident upon fluorescent material an electron is excited from the ground state to a higher energy state. As the electron relaxes within the excited state, energy is dissipated to the surroundings until the electron reaches the lowest level of the excited state [187]. At this point it returns to the ground state, and a photon of corresponding energy is emitted. Because some of the initial energy has dissipated, the emitted photon has less energy than the incident photon; this is why light emitted by fluorescence always has a longer wavelength than the incident light (Figure 2.14) [187].

The fluorescence microscopy technique has the ability to localise enzymes, substrates, and genes, and to characterise physicochemical properties of the cell, including membrane potentials, viscosity, and pH with high resolution and contrast [187]. Usually nonfluorescent molecules or antibodies are tagged with a fluorescent dye or material to make these structures visible [186]. However, recent research increas-
ingly utilises fluorescent nanoparticles for imaging purposes; unlike more traditional fluorescence microscopy, these materials don’t suffer from photobleaching, and don’t require tagging. Some nanomaterials used for fluorescence imaging are discussed in 2.4.

Figure 2.14: Energy diagram of a fluorescent molecule. The absorption and fluorescence emission spectra of the molecule are shown on the right. Image from [187].

2.3.2 Raman spectroscopy

Light is not only absorbed and emitted by atoms and molecules; photons can also be scattered by a sample. Scattering is either elastic, where molecules are left in the same state, or inelastic, where molecules are left in a different state. These two types of scattering are known as Rayleigh scattering and Raman scattering, respectively.

Rayleigh scattering is much more common than Raman scattering, the latter comprising only $\sim 10^{-5}$ of the incident beam [193, p. 15]. It is responsible for the blueness of the sky due to its strong ($I \propto \lambda^{-4}$) wavelength dependence [194,195]. Rayleigh scattering is shown in Figure 2.15.

Raman scattering causes the wavelength (and hence energy) of the scattered light to change compared to the incident light. This can be split into two categories depending on whether the scattered light has an increased or decreased wavelength compared to the incident light. Stokes scattering occurs when the emitted light has a longer wavelength; this is caused by the excitation of a ground state molecule to
a virtual state, before relaxation back down to a higher energy vibrational state. Anti-Stokes scattering occurs when the emitted light has a shorter wavelength; this is caused by the excitation of a molecule in a vibrational state to a virtual state, before relaxation back down to the ground state. Stokes scattering is more common than anti-Stokes scattering because it is more likely for the molecule to be in the ground state when light is incident upon it. However, the ratio of anti-Stokes to Stokes scattering increases with temperature following the Boltzmann distribution, \( N \propto \exp\left(-\frac{\epsilon}{k_B T}\right) \), because more molecules will be in the first vibrationally excited state under these circumstances. Both of these are shown in Figure 2.15.

![Energy diagram of Rayleigh, Stokes Raman, and anti-Stokes Raman scattering.](image)

Figure 2.15: Energy diagram of Rayleigh, Stokes Raman, and anti-Stokes Raman scattering.

A Raman spectrometer is used to measure the Raman scattering of a material (a basic schematic is shown in Figure 2.16). In simple terms, this comprises a laser to illuminate the sample, followed by filters to collect either Stokes or anti-Stokes Raman scattered light (filters out other light components) and diffraction gratings to diffract the scattered light into a spectrum, before finally being detected by a CCD detector. This gives a Raman spectrum at a single point of the sample. Some Raman spectrometers have the ability to obtain a 2D map by scanning in \( x \) and \( y \) directions across a specified area, giving a picture of the material across a wider
area. With a confocal system, which is additionally capable of incrementing in the $z$ direction automatically, this process can be extended into three dimensions to create a 3D volumetric map.

For a material to be Raman-active, a molecular rotation or vibration must cause a change in the molecular polarisability, $\alpha$ [197, p. 63]. If Raman-active, then Raman spectroscopy measures the intensity of the scattered light as a function of Raman shift (the difference in wavenumber between the excitation and scattering). These spectra exhibit bands at various frequencies that correspond to vibrations of specific bonds in the material; these are known as Raman modes. The vibrational frequencies of the Raman modes depend on the masses of the atoms involved and the strength of the bonds between them; low vibrational frequency indicates heavy atoms and weak bonds, while high vibrational frequency indicates light atoms and strong bonds [193, p. 18]. An overall idea of the composition of the material is gained by observing which modes are present, and their ratios to one another.

It is well established that the ratio between Raman mode intensities, e.g. between the $I_D$ and $I_G$ modes for graphene, changes with nanosheet size [198–201]. Metrics based on this concept were developed for LPE graphene samples by Backes et al. [202].
Recently, improved Raman metrics utilising pixel-wise metricised Raman mapping have been developed by Amorim Graf et al., and are shown to apply to both graphene and MoS$_2$ [203]. Furthermore, a new metric for MoS$_2$ layer number was developed based on an intensity ratio, $I_{465}/I_{453}$, of resonant Raman modes [203].

Raman spectroscopy is a non-destructive, non-invasive technique, which lends itself well to the study of sensitive samples, such as biological cells or tissues. Spectra are acquired rapidly, without need for sample preparation. However, the Raman signal can be swamped by fluorescence from some materials; this characterisation technique can be used in combination with other techniques that may be less affected by, or that actively utilise, fluorescence signals.

\subsection{2.3.3 UV–visible spectroscopy}

UV–visible spectroscopy is a technique used to measure the optical extinction (comprising absorbance and scattering components) or transmittance of a solid or liquid sample as a function of wavelength.

![Figure 2.17: Schematic of a UV–visible spectrometer. Image from [204].](image-url)
The internal components of a UV–visible spectrometer are shown in Figure 2.17. Briefly, a broadband light source is filtered and monochromated such that only light of a single wavelength is used for illumination. This light is passed through a beam splitter, forming two paths so that both the sample and reference can be measured simultaneously. Some of this light is absorbed by the sample, some is scattered, and some is transmitted; the transmitted light is measured by photodiodes. Any difference between the sample and reference transmittance measurements is assumed to be caused by the material in the sample.

Often, knowing the extinction (Ext) may be more useful than knowing the transmittance \( T \) of a sample. The two are related by the equation

\[
\text{Ext} = -\log_{10}(T).
\]  

(2.2)

For many samples, extinction can be directly related to concentration \( c \) by the Beer–Lambert law. This can be written as

\[
\text{Ext} = \varepsilon \ell c,
\]  

(2.3)

where \( \varepsilon \) is the extinction coefficient and \( \ell \) is the OPL through the sample.

UV–visible spectroscopy can reveal exciton peaks in the spectra which are caused by electronic transitions in the material. Electronic structure is affected by the exfoliation of the material (as shown for MoS\(_2\) in Figure 2.4), so there is a correlation between the measured exciton peaks and the thickness of the material. This has led to the development of spectroscopic metrics based on UV–visible spectroscopy measurements, which can provide information about the lateral size and the layer number of the material in question [202,205–208].
2.3.4 Atomic force microscopy

Scanning probe microscopy encompasses several techniques that obtain images of sample surfaces by using a physical probe to scan the sample. Atomic force microscopy (AFM) is one of these techniques. Compared to the traditional optical microscope it has a very high resolution. AFM can overcome the optical diffraction limit, so has a resolution on the nanometer scale [209]; however, a slow imaging speed is required to achieve the best resolution. Additionally, it provides a 3D image of the sample surface [209]; to use reading a book as an analogy, AFM is to optical microscopy what Braille is to standard print on a page.

A diagram of a typical atomic force microscope (AFM) setup is shown in Figure 2.18. The force sensor is based on a cantilever with a sharp tip (tip radius of a few nanometers). As the cantilever rasters across the sample surface, the tip interacts with the sample, sensing attractive or repulsive forces between the molecules of the tip and sample [209]. Even small changes in the forces cause the cantilever to deflect, altering the reflection angle of the laser light incident upon the cantilever. This shifts the position of the reflected light on the photodiode array, and hence influences the photocurrent produced. Combining feedback from the photodiode array with the piezoelectric sensor controlling the motion of the cantilever allows precise measurements of the surface structure to be obtained.

There are three main scanning modes used for AFM measurements: contact mode, noncontact mode, and tapping mode [209]. Contact mode can be used to achieve high resolution images in both air and fluid environments; however, it can only be used to image samples with hard surfaces which cannot be deformed, because more fragile surfaces can be damaged. In noncontact mode the tip never makes contact with the sample surface. While this avoids issues associated with contact mode, such as damaging soft samples (e.g. some biological tissues), the resolution is lower due to the long-range forces used, and the image can be inaccurate due to the contamination layer present on all samples in ambient conditions.
Figure 2.18: Schematic of AFM. The laser path goes from the laser source to the photodiode via reflection on the cantilever. The cantilever is attached to the tip, which interacts with the sample.

Tapping mode is a good alternative, combining features from both contact and noncontact modes. In this mode, the cantilever is oscillated at or near its resonant frequency, and the phase shift between the driving oscillation and the feedback is measured. This mode is suited to most biological samples, as it can provide high resolution images of soft samples which may be damaged easily. Figure 2.19a shows the force response curve of the tip. When close to the surface repulsive forces are dominant; moving further from the surface attractive forces are dominant, until the tip is far enough away that any forces from the surface are negligible [210]. The domains for contact, noncontact, and tapping mode can be mapped onto this graph, and are shown in Figure 2.19b.

Artefacts are features that appear in images but which are not naturally present. They occur as a result of the imaging procedure. Some of the main sources of artefacts when using AFM are the quality of the tip used, vibrations during imaging, and issues arising from image processing. This underlines the importance of ensuring that the tip is undamaged, ensuring that there are as few environmental vibrations as possible (e.g. by mounting the stage on a bed of gas, etc.).
Figure 2.19: Force–distance graph for tip–sample interactions during AFM. (a) Plot shows the force response curve resulting from the repulsive and attractive forces felt by the tip. (b) The domains for contact, noncontact, and tapping mode are overlaid onto this plot. Image adapted from [211].

AFM has been used in this thesis to obtain statistics related to nanosheet lateral size and layer number. The nanosheet layer number can be obtained from the measured AFM thicknesses using established metrics based on step height analysis [151, 205, 206, 212]. It is well established that direct conversion of AFM-measured height to layer number is complicated by adsorbed surfactant or solvent on nanosheets and substrate. Step height analysis accounts for this by considering the heights of several nanosheets simultaneously. By plotting each nanosheet height in ascending order, a series of steps appear in the data; the first step in the data gives the thickness of the monolayer.

2.3.5 Characterisation techniques for Chapters 3–5

This section contains experimental details of the characterisation techniques used for the work presented in Chapters 3–5.

2.3.5.1 Characterisation techniques for Chapter 3

Optical microscopy

Optical microscopy was performed using an Olympus BX53M microscope with a 5× magnification in bright-field mode. The optical micrographs were pre-processed to crop the image down to the area of the cover slip only.
Optical images of the samples were required for calculation of surface coverage. Images were taken of large sample areas (at least half of each 18 mm × 18 mm sample) to allow a more accurate approximation of the film distribution over the whole sample.

**Binary threshold method to determine film surface coverage**

A simple, two-step post-processing method was used to determine the film surface coverage from the optical micrographs. Firstly, the open-source ImageJ software [213] was used to run a binary threshold on the micrographs to separate the nanomaterial film from the substrate (a small section of a typical optical micrograph and its corresponding threshold is shown in Figure 2.20 as an example). Secondly, a custom Python script was written to automate the calculation for determining the fraction of nanomaterial present.

![Figure 2.20: (a) Optical micrograph. (b) Binary threshold of the optical micrograph.](image)

**UV–visible spectroscopy**

UV–visible spectroscopy measurements were performed using a Shimadzu UV-3600 Plus spectrophotometer. Liquid characterisation was performed using quartz cuvettes (Starna Scientific); solid characterisation was performed using a microscope slide holder.
**Atomic force microscopy**

AFM measurements were performed using a Bruker Dimension Icon AFM system in PeakForce QNM® mode. AFM was performed on sparse Langmuir films of each material to obtain statistics on flake length, width, and layer number using NanoScope Analysis software. This allows flake area to be calculated. At least 100 flakes of each material were used to obtain reliable statistics.

**2.3.5.2 Characterisation techniques for Chapter 4**

**Atomic force microscopy**

AFM measurements were performed using a Bruker Dimension Icon AFM system in PeakForce QNM® mode. AFM was performed on sparse Langmuir films to obtain statistics on flake length, width, and layer number using NanoScope Analysis software.

**Optical microscopy**

Optical microscopy was performed using an Olympus BX53M microscope with a range of objectives (5×–100×) in both bright-field and DIC modes.

**Raman spectroscopy**

Raman spectra were taken using an upright Renishaw InVia confocal Raman microscope. A 660 nm laser was used for resonant measurements of MoS$_2$. Volumetric maps were taken using resonant laser (660 nm) and 100× objective and a grating of 1800 l mm$^{-1}$. The maximum depth uncertainty is $\sim 3 \mu$m, but using small step sizes and overlapped data provides a much greater accuracy (closer to 1 $\mu$m). The step size and area were varied depending on the sample size (step size ranged from 0.1 to 0.5 $\mu$m). Very low power (0.001 mW) and short exposure times (< 0.5 s) were used.
2.3.5.3 Characterisation techniques for Chapter 5

Optical microscopy

Optical microscopy was performed using an Olympus BX53M microscope with a range of objectives ($5\times$–$100\times$) in both bright-field and dark-field modes.

Raman spectroscopy

Raman spectra were taken using an upright Renishaw InVia confocal Raman microscope. A 532 nm laser was used with a grating of 2400 l mm$^{-1}$. A power of 5 mW was used for exposure times of 10 s.

2.3.6 Cell experiments for Chapters 3–5

This section contains experimental details of the cell experiments conducted for the work presented in Chapters 3–5.

2.3.6.1 Cell experiment for Chapter 3

The cell line used for the cell study was U87 glioblastoma from the American Type Culture Collection. The study ran for 14 days, with observations made at day 3 and day 13, and images taken at day 4 and day 12 using an EVOS FLC imaging system (Life Technologies) at $20\times$ magnification.

2.3.6.2 Cell experiments for Chapter 4

The cell lines used for the cell studies were U2OS (obtained from the American Type Culture Collection) and 1BR primary fibroblasts (obtained from the Genome Damage and Stability Centre Research Tissue Bank, University of Sussex). Cell lines were grown on MoS$_2$ thin films (deposited onto pristine glass cover slips as described in 2.2.4.2), and grown on pristine glass cover slips used for the control. The 1BR primary fibroblasts were seeded with $0.4\times10^3$ #/mL and the U2OS with $0.6\times10^4$ #/mL cell seeding density. Cells were fixed on cover slips using 3% paraformaldehyde and 2% sucrose PBS, for 10 minutes at room temperature. Samples
were washed with DI water before imaging. Study lengths ranged from 3 to 14 days. Cells were grown in DMEM and MEM, respectively, supplemented with 15% FCS, L-glutamine, and pen-strep at 37 °C and 5% CO₂. Cell counting was performed using hemocytometry after trypsinisation using trypsin EDTA (0.25%). For the nystatin studies, cells were grown with a 20% nystatin solution in the complete media. Error bars in cell counts are calculated as the standard error in the mean of three repeated experiments (one experiment has two repeats per sample, four counts per sample) for the U2OS cells. Error bars for the fibroblasts come from experimental error in single experiment (two repeats per sample, four counts per sample).

In one experiment, U2OS cells were grown on MoS₂ substrates and pristine glass controls. Once the cells reached confluence after 7 days in culture, they were trypsinised, split, and reseeded with fresh media and onto fresh substrates at the initial (day 0) density to allow room for further proliferation on the new substrates. The new substrates used were either further fresh MoS₂ substrates or pristine glass controls. The cells were reseeded such that cells initially from MoS₂ substrates (M) were grown on further fresh MoS₂ substrates (MM) or glass controls (MC), and cells from control substrates (C) were seeded onto MoS₂ substrates (CM) or further control substrates (CC). Error bars are calculated from standard error in the mean from two repeat experiments (two samples per type in each experiment).

2.3.6.3 Cell experiments for Chapter 5

The cell lines used for the cell studies were U2OS (obtained from the American Type Culture Collection) and 1BR primary fibroblasts (obtained from the Genome Damage and Stability Centre Research Tissue Bank, University of Sussex). Cell lines were grown on WS₂ and MoSe₂ thin films (deposited onto pristine glass cover slips as described in 2.2.4.3), and grown on pristine glass cover slips used for the control. The 1BR primary fibroblasts were seeded with 0.4×10³ #/mL and the U2OS with 0.6×10⁴ #/mL cell seeding density. Cells were fixed on cover slips using 3% paraformaldehyde and 2% sucrose PBS, for 10 minutes at room temperature.
Cells were grown in DMEM and MEM, respectively, supplemented with 15% FCS, L-glutamine, and pen–strept at 370 °C and 5% CO₂. The study ran for 7 days on the original substrates. At this point, cell counting was performed using hemocytometry after trypsinisation using trypsin EDTA (0.25%). The trypsinised cells from control, WS₂, and MoSe₂ substrates were all reseeded onto pristine glass cover slips for a further 2 days before fixation. Samples were washed with DI water before imaging.

2.4 Applications of low-dimensional nanomaterials in diagnostics, therapeutics and theranostics in life sciences

The rise of nanobiotechnology research has led to new nano–bio interfaces aiming to achieve controlled manipulation of cellular functions and features. Herein, recent developments in the use of a range of nanomaterials (e.g. carbon-based materials, transition metal dichalcogenides, layered metal hydroxides, etc.) for diagnostic, therapeutic and theranostic applications is reported. Imaging is the area of diagnostics considered, encompassing fluorescence imaging, live cell imaging, and biosensing. Photothermal therapy, drug delivery, and stem cell control are the discussed therapeutic applications. Toxicity studies for various materials are also included. Finally, theranostic applications are considered. The recent COVID-19 pandemic has exposed the necessity of innovation in many of these areas, particularly for improved biosensing systems. The vast array of well characterised nanoparticles now available offers a promising route to develop many of these technologies, with optionality through control of chemistry, structure and optoelectronic properties.
2.4.1 Introduction

Nanobiotechnology is a rapidly developing field of research. The use of nanomaterials in cellular biology is enabling growth of new imaging techniques, drug delivery and therapies. In particular, the more recent acceleration of research into nanomaterials provides a new opportunity to develop nano–bio interfaces that have not been possible previously. Cells and their organelles, transport vesicles, etc. operate on the nanoscale, so interdisciplinary research provides a new way to address these nanoscale features and functions in a controlled way.

Exhibiting properties vastly different to those of bulk material, nanomaterials have become a promising new tool in this field. Their small size allows for nanomaterials to be internalised by the body’s own mechanisms, while researchers’ high degree of control over shape and functionalisation gives the ability to target these materials to particular areas of the body.

There remains debate over the role of material morphology and the chemistry of the material. While a resolution to this question is not yet apparent, simplifying the parameter space can help address this problem. The use of synthetic analogues is one way to achieve this, as is to focus on lower-dimensional cases as a starting point.

While recent decades have seen the isolation of myriad new two-dimensional (2D) materials with wide-ranging properties and possibilities, carbon remains an obvious first choice for research into diagnostics and therapeutics as it provides a starting point for biocompatibility in carbon-based life.

At the nanoscale, therefore, carbon provides a plethora of potentially useful properties. The strength and chemical stability of carbon nanomaterials lends them well to production of tissue scaffolds and implant production, while the easy manipulation of the material is a recurring theme for intercellular processes such as fluorescent tagging, drug delivery, and photothermal therapy. Carbon-based materials considered in this section include graphene, graphene oxide (GO), and
reduced graphene oxide (rGO); carbon and graphene nanodots and quantum dots (QDs); carbon black; nanodiamonds; carbon nanotubes (CNTs), carbon nanofibers; carbon fullerenes; and other more exotic carbon nanomaterials.

Although carbon-based materials are clearly well researched for biological applications, other low-dimensional materials also receive increasing attention in this sphere. In particular, nanohybrids or nanocomposites combining materials seem to be a popular choice to enable precise control over chemical properties, and hence over nano–bio interactions. Other materials included in this review are transition metal dichalcogenides (TMDs); gold nanoparticles (AuNPs); silica NPs; hexagonal boron nitride (BN); copper nanoclusters; metal oxide NPs; layered zinc hydroxide; TiO$_2$; metallofullerenes; metallo-QDs; among others.

There are good and bad aspects to each of these materials. Some common aspects are discussed by Anık, Timur, and Dursun [214] and have been summarised in Table 2.1.

Table 2.1: Pros and cons for a range of materials commonly researched for nanobio-technological applications.

<table>
<thead>
<tr>
<th>Material</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D graphene-based nanomaterials (inc. GO, rGO)</td>
<td>• Enters the cell membrane easily • Can merge with DNA and protect it from enzymatic degradation • Can be utilised as nanocarriers in <em>in vivo</em> structures • Higher drug loading because of their large surface areas</td>
<td>• Aggregation of graphene-based nanomaterials in lungs and liver • Cause oxidative stress • Damage to cell membranes • Bonding or adhesion with cell receptors can activate stress and apoptotic mechanisms • Toxicity depends on time, concentration, aspect, and surface chemistry</td>
</tr>
<tr>
<td>Carbon nanotubes (CNTs)</td>
<td>• Functionalised, water-soluble CNTs can enter into cells easily (depending on size and surface chemistry) • Drugs can be loaded within CNTs’ hollow inner channel, or on the wall surface with the help of hydrophobic interaction</td>
<td>• Some MWCNTs and SWCNTs can accumulate in the lungs causing pulmonary toxicity and inflammation (based on CNT size and uniformity) • Large MWCNTs, and SWCNTs injected intraperitoneally, can cause inflammation and granuloma formation • Raw CNTs show worse <em>in vivo</em> toxicity than functionalised CNTs</td>
</tr>
</tbody>
</table>
Table 2.1: Pros and cons for a range of materials commonly researched for nanobio-technological applications (continued).

<table>
<thead>
<tr>
<th>Material</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
</table>
| Fullerene         | • Fullerene C60 has a spherical structure and is apolar, allowing it to pass the cell membranes  
                    • Can easily be modified  
                    • Convenient structure leads to use as drug delivery vehicles and sometimes as nano drugs | • Fullerene exposition resulted in lipid peroxidation and glucose depletion in the brain  
                    • Fullerenes that reach the brain through circulation or axonal translocation dissolve into the lipid-rich brain tissue, and because of their direct redox activity, brain damage might occur |
| Quantum dots      | • Fluorescent properties in broad-spectrum  
                    • High quantum yield  
                    • Low photobleaching  
                    • Can be used in photodynamic therapy, in vivo imaging, and drug biodistribution | • Based on the usage of heavy metals in their composition, and also their discharge from the body, in vitro studies have shown that QDs may be toxic  
                    • Degree of toxicity sometimes depends on the surface coating  
                    • Plain QDs can be cytotoxic by induction of reactive oxygen species which leads to damage to plasma membranes, mitochondria, and nucleus |
| Gold nanoparticles | • Biocompatible nature  
                    • Controllable size distribution  
                    • Possible to vary NP shape  
                    • Easily conjugated  
                    • Ease of preparation | • The stabiliser CTAB which has been used in the production of gold nanorods causes considerable cytotoxicity  
                    • Conformational change in protein structure, e.g. serum albumin, has also been reported upon exposure of the protein to AuNPs  
                    • Surface interaction of proteins at the nanoscale affects protein fibrillation, which could lead to neurodegenerative diseases such as Parkinson’s and Alzheimer’s |
| Iron oxide nanoparticles | • Less cytotoxic  
                    • Magnetic properties  
                    • Controlled size and surface modification | • Superparamagnetic IONPs tend to accumulate in the liver without showing any significant biological degradation |

This review will consider three overarching themes: diagnostic (with a particular focus on imaging), therapeutic, and theranostic applications of low-dimensional materials. Within these themes, narrower topics will be considered. Discussion of
imaging applications includes fluorescence imaging, live cell imaging, and cell sensing, while discussion of therapeutic applications will include photothermal therapy, drug delivery, stem cell control, and toxicity studies.

2.4.2 Imaging

The ability to diagnose illnesses quickly, correctly, and efficiently is an important first step to combating diseases. Without an accurate understanding of the disease in question, treatment may be ineffective, or worse, actively detrimental to the patient. However, diagnosis is important not only at the macro scale, but also at the cellular level. Some of the most promising nanoscale approaches to this small-scale diagnosis include fluorescence probes, live cell imaging, and cell sensing. Other imaging techniques are also being investigated. In particular, non-fluorescence Raman imaging could provide a new avenue of exploration, as a non-invasive technique that has the potential to be used with live cells [215, 216]. However, studies dedicated to these other techniques are still relatively few in number, so the focus of the following sections will be on the three methods mentioned above.

2.4.2.1 Fluorescence imaging

Fluorescence imaging allows for certain molecular processes or structures to be observed. This is achieved by utilising fluorescent probes that are able to target specific locations within the cell. Targeting is traditionally best achieved with carbon materials that are easily functionalised to attach to particular probes, often fluorescent dyes or proteins, however these carbon materials are not innately fluorescent. Advances in recent years have given rise to studies using different targeting materials that are inherently fluorescent and so do not necessarily require functionalisation with a separate fluorescent probe.

Of particular interest in this area are nanodots. Carbon nanodots, or carbon QDs, have properties that make them well suited for use as fluorescence probes. Not only does their small size (< 10 nm) and low cytotoxicity make them an obvious choice
for such biological applications, carbon nanodots also show low or no photobleaching, a common problem associated with the fluorescent dyes and proteins which have been in use for some time [217].

There is a good range of analyte specification across the various available NPs in serum and blood, but for cell imaging the fluorescent NPs are mostly limited to the cytoplasm, indicating that work on improving localisation and perhaps adding targeting functional groups would develop these materials further.

Although carbon or graphene nanodots in various forms constitute a large proportion of the materials included, others are also referenced. Research is grouped by material in Table A.1, beginning with carbon and graphene nanodots/QDs before moving on to various other materials investigated for use in fluorescence imaging. Where known, the table gives the proposed material, probe (if different to material), specific target, and cell lines and/or animal models tested.

### 2.4.2.2 Live cell imaging

Live cell imaging expands further on fluorescence imaging by tracking molecular processes or structures in real time. This is predominantly mediated through fluorescence imaging, and so similar fluorescent nanoparticles are used for this specific case in addition to the more general case discussed in 2.4.2.1. However, some other detection methods have been considered, including photoluminescence, stimulated emission depletion (STED) nanoscopy, and amperometric experiments (these are all included in Table A.2).

Perhaps unsurprisingly given the strong link between fluorescence imaging and live cell imaging, carbon nanodots and QDs are also popularly investigated for this application. These are discussed by Kim et al. [218] in addition to a host of other graphene or graphene-based nanomaterials used for live cell biosensing platforms.
Again, we see that the current targets are somewhat limited in this field. Only ions are so far reported, with no organelle staining for example, so there is scope to further improve the localisation and specificity of these NPs. Interestingly, a combination of imaging techniques may be relevant here; combining Raman with fluorescence could improve live cell imaging.

As in 2.4.2.1, although carbon or graphene nanodots in various forms constitute a large proportion of the materials included in Table A.2, other materials are also referenced. Research is grouped by material, beginning with carbon and graphene nanodots/QDs before moving on to other carbon and non-carbon nanomaterials and composites. Where known, the table includes the proposed material, probe (if different to material), detection method, specific target, and cell lines and/or animal models tested.

2.4.2.3 Biosensing

Biosensing refers to the detection of biomolecules using an analytical device (i.e., biosensor) that combines a biological component with a physicochemical detector [219, 220]. Conventionally, a biosensor consists of a bioreceptor (often a biological component such as cells, enzymes, antibodies, peptides, oligonucleotides, etc.) which interacts with the target and yields a physical or chemical change that is amplified into a readable signal with the help of a transducer (detector) component [221]. More recent developments have led to the aptasensor, a type of biosensor that uses DNA or RNA aptamers as the biological recognition element [222]. In aptasensors, the recognition reaction is independent of the transducer used [222].

The past ∼15–20 years have seen a rapid increase in the influence of nanomaterials on biosensor development [223]. Moreover, the COVID-19 pandemic (currently ongoing) has emphasised the need for “easy-to-use, quick, cheap, sensitive and selective detection of virus pathogens” [224]. Comprehensive reviews focusing on biosensors for COVID-19 detection can be found at [225, 226].
Graphene is commonly investigated for biosensors and aptasensors, helped by the fact that it is abundant, chemically stable, and conductive [224]. Vermisoglou et al. summarise state-of-the-art graphene-based sensing systems for a variety of viruses [224]. However, the biosensing field is broadly open for a huge variety of NPs. The range of possible analytes is enormous, and with such a wide palette of nanomaterials to choose from, it should be possible to select for end application. There is likely to be huge growth in the area of nanomaterial biosensors over the coming years as affinities and targets are identified for the various NPs and the technology to incorporate them into sensing platforms becomes more standardised.

Research reviewed here is grouped by material in Table A.3, beginning with graphene-based systems, then covering other carbon-based systems and systems based on other materials. The proposed material, probe (if different to material), detection method, target, and cell lines and/or animal models tested are listed in the table.

2.4.3 Therapeutics

Once disease has been successfully diagnosed, the aim is to treat it as efficiently as possible. Without effective therapy, a diagnosis is useful only to curb curiosity. Some of the most cutting edge nanoscale approaches include photothermal therapy, drug delivery, and stem cell control. In addition, nanomaterials are not native to the human body, so a wide range of toxicity studies have been conducted on various materials considered for these and other treatments. These areas of research are discussed further in this review.

2.4.3.1 Photothermal therapy

One of the most developed technologies discussed in this review, photothermal therapy (PTT) is a minimally invasive technique which uses laser light to heat contrast agents or nanomaterials presented to the tissue. This causes thermal damage in this region.
Lower laser wavelengths are desirable for PTT, as they are less energetic and so are less likely to cause damage to surrounding healthy tissues. Recent developments focus on nanomaterials tailored to target specific tissues.

Nanostructures based on gold are heavily researched due to its good biocompatibility and localised plasmon surface resonance (meaning that AuNPs can absorb light at specific wavelengths) [227]. In fact, AuNPs are used in clinical settings already. Gold-based therapies feature several times in Table A.4, and the use of AuNPs for photothermal cancer therapies is reviewed by Vines et al. in [227]. However, many of the new layered and carbon-based NPs are also being investigated as the use of infrared excitation is appealing in biological applications.

Research is grouped by material in Table A.4, beginning with carbon-based materials before moving on to other materials investigated for use in PTT. The table gives the proposed material, probe (if different to material), irradiation method, specific target, and cell lines and/or animal models tested.

### 2.4.3.2 Drug delivery

Drug delivery is a broad term that encompasses several aspects important for the therapeutic delivery of drugs, including development of new materials or carrier systems, research into the administration route to the disease, etc. [228]. In 2004, nanomedicine was defined by the European Science Foundation (ESF) [229] as

The science and technology of diagnosing, treating and preventing disease and traumatic injury, of relieving pain, and of preserving and improving human health, using molecular tools and molecular knowledge of the human body.

‘Novel therapeutics and drug delivery systems’ was chosen by the ESF as one of five key areas of focus [229]. Drug delivery systems could offer site-specific, time-controlled, noninvasive delivery of different molecular weight drugs and other bioactive molecules for the treatment of medical infections and diseases [230].
Drug delivery is perhaps the area which is most suited to the new nanomaterials available. The vast array of chemistries and morphologies now available in NPs should make drug attachment and targeting more specific, providing a whole host of new vector systems. Research is ongoing to identify the suitable NP–drug partnerships to optimise these systems. Perhaps unsurprisingly given the emphasis placed on developing new drug delivery systems, there are several reviews concerning suitability of a range of materials for this purpose. Plachá and Jampilek [231] review graphene-based nanomaterial drug delivery systems research in 2017–2019; Guo et al. [232] review research into \textit{in vitro} and \textit{in vivo} CNTs-based drug delivery to cancer and the brain (as of 2017); and Wang et al. [233] provide a review of nanocarriers based on 2D materials beyond graphene, such as TMDs, MXenes, BN and others (as of 2019).

The materials included in Table A.5 are mostly carbon-based, with several small 0D materials such as carbon dots, nanodiamonds, and C_{60} fullerenes, possibly because these smaller particles can follow internalisation pathways more easily or be used as cages. Research is grouped by material in Table A.5, beginning with carbon-based materials before moving on to various other materials proposed. Where known, the table gives the proposed material, probe (if different to material), drug, specific target, and cell lines and/or animal models tested.

\subsection*{2.4.3.3 Stem cell control}

In this review, stem cell control is used as a term to describe the effect of various materials on stem cell behaviour (e.g. differentiation, proliferation, etc.). Stem cells exist at the origin of a cell lineage, can differentiate into various types of cells, and proliferate indefinitely to produce more of the same stem cell [234]. The ability to stimulate stem cell differentiation is a topic of interest, because this control could allow for better medical implant biocompatibility, improved wound healing, and tailored tissue engineering scaffolds, among other possible applications for a range of diseases.
Stem cell control is one of the newest research areas. Since our understanding of how much cell function is dependent upon nanoscale cues has increased, the possibility to use nanomaterials to manipulate and stimulate at the nanoscale has become apparent. Stem cell fate is however a hugely complex process and isolation of individual components or response is difficult. Akin to drugs requiring delivery to specific areas in the body, discussed in 2.4.3.2, often various molecules (e.g. proteins) need to be transported to the stem cells to induce specific behaviours. GO has been investigated for various stem cell control studies because it can be easily functionalised with such molecules. Various metals have also been researched, possibly due to their common use in medical implants; as mentioned above, better stem cell control could lead to medical implants that adapt to the body more quickly and that take longer to be rejected by the body. Research is grouped by material in Table A.6, beginning with graphene and GO materials before moving on to other carbon-based materials and then metal nanomaterials. In general, complexes involving GO seem to result in enhanced stem cell differentiation and proliferation, those involving nanodiamonds seem to show improved stem cell adhesion, and Ti surfaces with a rougher topography seem to improve stem cell differentiation and proliferation. Key conclusions for each paper are included in the table, along with the proposed material, probe (if different to material), and cell lines and/or animal models tested.

2.4.3.4 Toxicity studies

For any of the applications discussed throughout this review to be put into practice, it is vital that the proposed materials themselves, alone or in composites, are not toxic to humans. Toxicity studies aim to address this by systematically testing different materials with standard tests (e.g. cytotoxicity, effect of dosage or repeat dosage, duration of treatment, etc.). Testing can be categorised into three main sections [235]:
• acute toxicity studies (adverse effects occurring within a short time, up to 14 days, after administration of a single dose or after multiple doses administered in a short time period [236]);

• sub-acute toxicity studies (adverse effects occurring after administration for 14–28 days of a single dose or after multiple doses administered in a short time period [237]); and

• chronic toxicity studies (adverse effects occurring after repeated or continuous long-term exposure [238]).

However, as new materials begin to be used in biological applications, their toxicity is also becoming assessed. It is clear that much more plays into toxicity than just the chemistry of the material, with size and shape also a key factor. This makes understanding of NP toxicity particularly challenging, as a blanket recommendation for a material will not catch such nuances. As shown in Tables A.1–A.6, carbon-based nanomaterials are commonly explored for new nanodiagnostics and nanotherapies. As such, the toxicity of these materials is particularly important to study, and carbon-based nanomaterials feature heavily in Table A.7. Research is grouped by material in Table A.7, beginning with carbon-based materials before moving on to various other materials proposed. Where known, the table gives the proposed material, tests, cell lines and/or animal models tested, and key conclusions for each paper.

2.4.4 Theranostics

Theranostics, a term coined by combining the words therapeutics and diagnostics, is exactly that — NPs or nanocomposites that provide simultaneous diagnostic and therapeutic effects [239]. Materials capable of theranostic applications could be the key to unlock the door to exciting new avenues of research into areas such
as personalised medicine. This combination therapy could improve early disease detection and optimise treatment for individuals [240]. Other benefits could include enhanced drug efficiency, better disease management, and improved healthcare [240].

That theranostics is a subject of burgeoning interest is therefore unsurprising, and this is highlighted by the range of reviews on different aspects of theranostics. Panwar et al. [241] review a range of nanocarbons for theranostic applications including sensing, imaging, and drug delivery, and also provide an outlook on current challenges (as of 2019). Also considering carbon materials, Hassan et al. [242] review specifically carbon QDs for photomediated theranostics, with emphasis on the importance of the integration of light with nanotechnology (as of 2017). Hu et al. [243] discuss QDs, NPs, and 2D materials decorated with various biomolecules for targeted diagnosis and treatment (as of 2020). Gong et al. [244] provide a review of photothermal combination cancer therapy based on 2D TMDs, and consider future prospects and challenges of using 2D TMD-based nanomaterials for theranostics (as of 2017). Yang et al. [245], Raja et al. [246], and Mohammandpour and Majidzadeh-A [247] all present reviews on 2D nanomaterials for cancer theranostics.

The present work also considers a range of materials, mostly carbon- and metal-based. Research is grouped by material in Table A.8, beginning with carbon-based materials before moving on to various other materials. The proposed material, probe (if different to material), detection method, therapy, specific target, and cell lines and/or animal models tested are given in the table.

2.4.5 Conclusions

While some technologies are already well developed, such as fluorescence imaging and PTT, there remains more scope for development in areas such as biosensing and drug delivery. This has been shown especially by the push for improved sensing systems spurred by the COVID-19 pandemic. Carbon-based nanomaterials still
hold much promise in all these areas, and are shown here to be the focus of much research. However, the new layered NPs are beginning to shine through, especially for biosensing and imaging applications with their tuneable optoelectronic properties.
Chapter 3

Langmuir films of layered nanomaterials: edge interactions and cell culture applications

The application of nanomaterials in technology is limited by challenges in their processing into macroscopic structures with reliable and scaleable methods. Herein, it is demonstrated that using scaleable fabrication methods such as liquid-phase exfoliation by sonication (LPE) it is possible to produce dispersions of a wide variety of layered nanomaterials with controllable and standardised size and thickness scaling. These can be used as-produced for Langmuir deposition, to create single layer films with tuneable density, including the first demonstration of hexagonal boron nitride (BN). Of particular importance, we show that the difference in edge chemistry of these materials dictates the film formation process, and therefore can be used to provide a generic fabrication methodology that is demonstrated for various layered nanomaterials, including graphene, BN and transition metal dichalcogenides (TMDs). We show that this leads to controllable cancer cell growth on graphene substrates with different edge densities but comparable surface coverage, which can be produced
on a statistically relevant cell study amount. This opens up pathways for the generic fabrication of a range of layered nanomaterial films for various applications, towards a commercially viable film fabrication technology.

3.1 Introduction

LPE is a process which has been shown to be the most effective way to produce large-scale yields of various layered nanomaterials, making it the most practical production technique available [120, 248]. Layered nanomaterials incorporate a range of two-dimensional (2D) materials with a variety of different properties associated with them. Graphene is possibly the most well known of these, having received much attention both in the academic sphere and also in public discourse. Since it was first isolated [6], its interesting mechanical, electronic, optical and thermal properties have been studied extensively [25, 26, 249, 250]. Further research has looked into these properties in other layered nanomaterials such as TMDs [49, 251, 252], BN [112, 253], and other exotic layered crystals [254–256].

These new materials could lead to many innovations. 2D materials can be utilised for such wide-ranging applications as electronics and optoelectronics [117, 249, 252], biomarker detection [257, 258], and energy-related areas [259]. Liquid processing of 2D layered nanomaterials is necessary to obtain dispersions suitable for Langmuir deposition; this is a well known method of creating thin films, whereby a nanomaterial dispersion is spread on a liquid subphase [163, 260].

The thin films produced by this method are 2D, in the sense that they comprise a single layer of particles, i.e. they are only as thick as the thickness of the exfoliated layered nanomaterial used, and depending upon the parameters used varying from monolayer to multilayer. By use of a moveable barrier it is possible to vary the surface area of a Langmuir trough, and hence to compress a nanomaterial film. This technique gives simple control of film creation, with small quantities (mg m$^{-2}$) of material resulting in high material efficiency. This allows production of single layer particulate films with high surface coverages.
Control over thin film creation is therefore crucial as different applications may require different finely-tuned film properties \[183\]. Control over Langmuir film assembly would allow for generic scaleable procedures for a range of materials dependent upon application requirements. By providing a framework for normalising for material interactions, it is possible to understand film densities for different layered nanomaterials, including BN (the first time its Langmuir behaviour and deposition has been shown), graphene, MoS\(_2\), and WS\(_2\). This will be useful for various applications, including in biomaterials, where edge interactions play a critical role in cellular growth and adhesion as well as the optimisation of the nanomaterial surface for functionalisation or surface interaction \[182\]. Moreover, sample substrates for biological studies must be able to be produced repeatably to allow for statistically significant trials. We demonstrate that the growth of glioblastoma cancer cells is significantly modified by the presence of graphene edges by comparing growth on films made from two different size graphene nanosheets (larger sheets, L-gra, and smaller sheets, S-gra). This is critical not only for the understanding of cancer growth, but also for developing novel, stable, synthetic substrate systems for cellular studies.

The synthesis and processing of the materials used in this work is described in 2.2.4.1, the characterisation techniques used are described in 2.3.5.1, and details of the cell experiment are included in 2.3.6.1.

### 3.2 Substrate production

Dispersions of various nanomaterials were prepared in-house using LPE. These dispersions were used to perform Langmuir–Schaefer (L–S) deposition. Langmuir films are created by dropping nanomaterial dispersion onto a water subphase; as the solvent evaporates, a thin, monolayer film is left at the air–water interface. Schaefer deposition is the horizontal lowering of a substrate to transfer this film \[261\], as shown in Figure 3.1a.
Figure 3.1: (a) Diagram showing the L–S deposition process. The deposited nanomaterial film is compressed with the trough barriers, then the substrate is brought into contact with the film before being lifted directly upwards (substrate parallel to air–water interface). (b) Representative pressure–area isotherms for each material used. (c) Optical micrographs for each material; the top row is of films deposited at a lower pressure (∼10 mN/m), while the bottom row is of films deposited at a higher pressure (∼25–30 mN/m). Images shown are small areas representative of the whole film. All scale bars = 100 µm.

There are a few main requirements for solvents used for the L–S process. These include that the solvent has a high vapour pressure, so that it evaporates and leaves a film of particulates at the interface [161]; that the solvent spreads on the water surface, to maximize the area over which nanoparticles are spread to minimize reaggregation during solvent evaporation [162]; and that ideally, the dispersion should be water-immiscible.
Cycloketones such as cyclopentanone and cyclohexanone have been shown to be good solvents that satisfy all of these criteria [162]. Additionally, they have been shown to be good solvents for the exfoliation of layered nanomaterials [144, 262]. This removes the need to exfoliate into a different solvent than that used for spreading, avoiding the extra step of redispersion of the material into a suitable spreading solvent. Moreover, redispersion is not always possible because generally good spreading solvents are not good for dispersion of 2D layered nanomaterials, even if transferred [162]. By making use of these solvents, it is possible to process the nanomaterials from powder to completed Langmuir film in a single solvent, greatly simplifying the procedure, and providing the opportunity for bulk processing.

Typical pressure–area isotherms for all material monolayers with aqueous subphases used are given in Figure 3.1b. These isotherms show the ‘phase transitions’ [165] of the monolayer film. At low pressure (the gas phase) the material is sparsely distributed in the trough, creating ‘films’ which appear to be mostly blank substrate with islands of monolayer material. As the pressure increases and flakes of the material come into contact with each other (the liquid phase), the films become denser. This is more apparent in the isotherms for BN and L-gra, where the pressure increases rapidly at smaller trough areas. The variance in behaviour for the different materials is discussed in more detail later. Once the nanomaterial film is at a given surface pressure, measured by use of a Wilhelmy plate, it is deposited onto a glass cover slip. Choosing a range of surface pressures allowed films to range from those which were visibly dense and homogeneous to those which were visibly sparse.

Optical micrographs of typical films for each material after deposition at high and low pressures are shown in Figure 3.1c. It is clear that L-gra and BN differ from the other materials, as they have denser films. Although small sample areas are shown in Figure 3.1c, large film areas (of at least half of each 18 mm × 18 mm sample) were measured and used for the analysis. A simple, two-step post-processing method was used to determine the film surface coverage from the optical micrographs (shown in Figure 2.20).
3.3 Nanosheet characterisation

The aim of this study is to develop a standardised method of creating films with known parameters using a scaleable, commercially viable approach. To this end, the dispersions used were created using standard processes. These dispersions were characterised and shown to demonstrate universal (and expected) size scaling [150].

UV–visible spectroscopy measurements were performed on all dispersions, and representative spectra shown in Figure 3.2a. Metrics that make use of the absorbance feature ratios associated with each different material have been described in detail by Backes et al., and indicate the presence of exfoliated nanosheets [151,202,205,206]. This confirms the successful exfoliation to few-layer nanosheets. Lateral dimensions (length $L$, width $W$) of material nanosheets were measured by atomic force microscope

![Figure 3.2](image)

Figure 3.2: (a) UV–visible spectroscopy of nanomaterial dispersions used; absorbance normalised at 350 nm. (b) Nanosheet length vs width AFM statistics. (c) Sheet-wise $LW$ (length $\times$ width) vs layer number AFM statistics. (d) Plotted histogram for WS$_2$ length data, used to determine an average value $\langle L \rangle = (181 \pm 58)$ nm. Inset shows AFM image of the material.
Sheet thicknesses were measured from AFM profiles, and converted into layer number $N$ using values for monolayer thicknesses from the literature for graphene [248], MoS$_2$ [205], WS$_2$ [151], and BN [206].

The $L$ vs $W$ scaling is given in Figure 3.2b and shows that while all nanosheets follow a power law scaling of the aspect ratio, length scales near linearly with width, i.e. constant aspect ratio. The fit in Figure 3.2b is provided as a guide to the eyes and demonstrates a common aspect ratio. Each material is additionally fitted individually, assuming a linear scaling, to obtain an aspect ratio for each material. Results for all cases are given in Table 3.1. As the plot is on a log$_{10}$ scale, the aspect ratio is calculated by $10^c$, where $c$ is the intercept of the fit. These aspect ratios imply that the shape is independent of the material, and suggests we can approximate the sheets as rectangles.

Table 3.1: Fit results for $L$ vs $W$ plot for each material to obtain values of aspect ratio.

<table>
<thead>
<tr>
<th>Material</th>
<th>Intercept, $c$</th>
<th>Intercept std err, $\sigma_c$</th>
<th>Adj. $R^2$ values</th>
<th>Aspect ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-gra</td>
<td>0.173</td>
<td>0.014</td>
<td>0.79</td>
<td>1.49</td>
</tr>
<tr>
<td>S-gra</td>
<td>0.153</td>
<td>0.014</td>
<td>0.59</td>
<td>1.42</td>
</tr>
<tr>
<td>BN</td>
<td>0.184</td>
<td>0.010</td>
<td>0.88</td>
<td>1.53</td>
</tr>
<tr>
<td>MoS$_2$</td>
<td>0.204</td>
<td>0.010</td>
<td>0.16</td>
<td>1.60</td>
</tr>
<tr>
<td>WS$_2$</td>
<td>0.116</td>
<td>0.009</td>
<td>0.46</td>
<td>1.31</td>
</tr>
</tbody>
</table>

The product of length and width, $LW$, gives an approximation of nanosheet area. Plotting $LW$ against $N$ therefore shows how nanosheet area varies with number of layers. For liquid-exfoliated materials, the lateral size decreases as the thickness decreases; this is due to an increased sonication time leading to the creation of new edges [263,264]. Figure 3.2c shows that the materials used behave as expected. Moreover, all materials generally fall on a universal scaling, although BN shows some scatter. This is broadly consistent with other research into the effect of LPE on the size–thickness relationship [150]. The fit in Figure 3.2c, on all materials, is provided as a guide to the eyes. There are some small differences between materials; results of fitting each material independently are shown in Table 3.2.
Table 3.2: Fit results for $LW$ vs $N$ plot for each material.

<table>
<thead>
<tr>
<th>Material</th>
<th>Intercept, $c$</th>
<th>Intercept std err, $\sigma_c$</th>
<th>Gradient, $m$</th>
<th>Gradient std err, $\sigma_m$</th>
<th>Adj. $R^2$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fitted independently</td>
<td>L-gra</td>
<td>3.577</td>
<td>0.062</td>
<td>0.958</td>
<td>0.072</td>
</tr>
<tr>
<td></td>
<td>S-gra</td>
<td>4.030</td>
<td>0.043</td>
<td>0.301</td>
<td>0.163</td>
</tr>
<tr>
<td></td>
<td>BN</td>
<td>3.882</td>
<td>0.121</td>
<td>0.424</td>
<td>0.172</td>
</tr>
<tr>
<td></td>
<td>MoS$_2$</td>
<td>4.348</td>
<td>0.088</td>
<td>0.406</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>WS$_2$</td>
<td>4.033</td>
<td>0.039</td>
<td>0.758</td>
<td>0.081</td>
</tr>
<tr>
<td>Fitted together</td>
<td>all</td>
<td>3.887</td>
<td>0.034</td>
<td>0.689</td>
<td>0.037</td>
</tr>
</tbody>
</table>

From Figures 3.2b and 3.2c it is ascertained that all materials have a lateral size of less than 1 µm, and are all less than 30 layers thick on average (spread between 1 and 100 layers). Plotting histograms for each sample allows average values to be obtained by analysis of the distributions. Figure 3.2d shows a representative histogram and inset micrograph; additional histograms are available in Appendix B. All histograms are broadly log-normal, as expected [265,266]. The average values are consistent with the positions of clusters in the sheet-wise plots shown in Figures 3.2b and 3.2c and are shown in Table 3.3. The average length and average number of layers, as obtained from fitting the histograms in Appendix B (Figures B.1–B.5), are plotted for each material in Figure 3.3.

![Figure 3.3: Average length plotted against average number of layers for each material.](image-url)
Table 3.3: Average values for nanomaterial length, width, layer number, and approximate area, given with standard errors.

<table>
<thead>
<tr>
<th>Material</th>
<th>$\langle L \rangle$ (nm)</th>
<th>$\sigma_{\langle L \rangle}$ (nm)</th>
<th>$\langle W \rangle$ (nm)</th>
<th>$\sigma_{\langle W \rangle}$ (nm)</th>
<th>$\langle N \rangle$</th>
<th>$\sigma_{\langle N \rangle}$</th>
<th>$\langle LW \rangle$ (nm$^2$)</th>
<th>$\sigma_{\langle LW \rangle}$ (nm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-gra</td>
<td>190</td>
<td>170</td>
<td>119</td>
<td>91</td>
<td>10</td>
<td>15</td>
<td>36000</td>
<td>89000</td>
</tr>
<tr>
<td>S-gra</td>
<td>89</td>
<td>49</td>
<td>63</td>
<td>36</td>
<td>2</td>
<td>1</td>
<td>7000</td>
<td>9000</td>
</tr>
<tr>
<td>BN</td>
<td>200</td>
<td>190</td>
<td>130</td>
<td>130</td>
<td>6</td>
<td>5</td>
<td>50000</td>
<td>160000</td>
</tr>
<tr>
<td>MoS$_2$</td>
<td>370</td>
<td>120</td>
<td>232</td>
<td>75</td>
<td>27</td>
<td>19</td>
<td>91000</td>
<td>56000</td>
</tr>
<tr>
<td>WS$_2$</td>
<td>181</td>
<td>58</td>
<td>139</td>
<td>47</td>
<td>3</td>
<td>2</td>
<td>27000</td>
<td>18000</td>
</tr>
</tbody>
</table>

3.4 Surface pressure–surface coverage relation

The surface pressure of a film can change either through addition of more nanomaterial or by decreasing the trough area. For all materials, and a range of deposition pressures, surface pressure ($\Pi$) was plotted against surface coverage ($\Phi$). Intuitively, the relation can be fitted logarithmically, as $\Pi \propto \log \Phi$. This implies that the denser the film, the slower the rate of change of pressure with respect to the surface coverage. This is represented mathematically by $\frac{d\Pi}{d\Phi} \propto \frac{1}{\Phi}$; i.e., once a film becomes dense, ever-greater increases in pressure are required to produce any further increases in surface coverage.

This fit is shown in Figure 3.4a, and $R^2$ values are included in Table 3.4. Figure 3.4a shows that the materials behave as expected for a Langmuir process and can still be fitted effectively, despite the system being noisy due to factors such as transfer efficiency causing a scatter in data.

Figure 3.4: (a) Surface pressure versus surface coverage plot, fitted with a simple logarithm. (b) Surface pressure–surface coverage plot, fitted with Equation 3.1 [267].
Although this basic relation works well, Fainerman et al. [267] describe a thermodynamic model for the interpretation of pressure-area isotherms of material monolayers. This also accounts for the size difference between material monolayers and solvent molecules. Their equation is

\[
\Pi = \frac{-kT}{\omega_0} \left[ \ln \left( 1 - \frac{\omega}{A} \right) + \left( \frac{\omega}{A} \right) \right] - \Pi_{\text{coh}},
\]

(3.1)

where in the first term \( k \) is the Boltzmann constant, \( T \) is the temperature, \( \omega_0 \) is the molecular area of a solvent molecule, \( \omega \) is the molecular area of a nanoparticle, and \( A \) is the available surface area per nanoparticle.

The first term describes the increase in surface pressure with surface coverage due to entropy terms associated with subphase–nanosheet interactions. The offset term is the cohesion pressure, a term related to enthalpy, which describes long-range interactions between components [267]. In this study it is anticipated that these interactions will be dominated by attraction or repulsion between nanosheets when separated on the water surface, and based on the charge of edge states or van der Waals forces [268]. This information could suggest some phenomenon in the long-range interactions that is associated with the chemistry of materials used, leading to controlled film surface coverage.

The same surface pressure and surface coverage data are plotted in Figure 3.4b, but fitted with the model described in Equation 3.1. Although the model is designed for amphiphilic particles and only in the liquid expanded (low pressure) phase, the fit provides some insight for the layered nanomaterials and the intercept value at minimum pressure should hold true, with deviation expected for the higher pressure phases. The \( R^2 \) values are included in Table 3.4. As for the fitting in Figure 3.4a, although the fit is not ideal, presumably not capturing all of the complex edge interactions, it is an interesting start to capturing the dominant physics in what is a noisy and complex system that has not been previously described.
Table 3.4: $R^2$ values for simple logarithm and Equation 3.1 fitting of surface pressure vs surface coverage plots.

<table>
<thead>
<tr>
<th>Material</th>
<th>Adj. $R^2$ values</th>
<th>Simple log Fainerman (Eq. 3.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-gra</td>
<td>0.90</td>
<td>0.94</td>
</tr>
<tr>
<td>S-gra</td>
<td>0.63</td>
<td>0.75</td>
</tr>
<tr>
<td>BN</td>
<td>0.18</td>
<td>0.55</td>
</tr>
<tr>
<td>MoS$_2$</td>
<td>0.80</td>
<td>0.56</td>
</tr>
<tr>
<td>WS$_2$</td>
<td>0.97</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Values of $\Pi_{coh}$ for each material are given in Table 3.5. Negative values of $\Pi_{coh}$ indicate repulsive forces between the particles. This will be dominated by the edges rather than the surface, due to the unique geometry of the L–S process. Higher interparticle forces lead to greater stability of films on the surface, because this creates a higher surface tension and therefore the films remain assembled without reorganisation or loss to the other phases [268]. Higher interparticle forces also lead to higher 2D film moduli, but will reduce the spreading efficiency of the nanomaterial at low density, and hence the optimisation of the surface coverage efficiency [269].

It is seen from Table 3.5 that BN has the least negative cohesion force and hence forms a film with the least pressure. This might correlate with the micrographs in Figure 3.1c, which show the BN films as being the densest and most uniform. Interestingly, it is still a slightly repulsive interparticle force, counter to bulk dispersion of BN which is usually described as attractive compared to other layered nanomaterials (although $\Pi_{coh} = 0$ mN/m is within error) [270]. However, as the edge effects dominate, the ionic nature of the BN bond leads to charged edges and potential for functionalised edge sites leading to dominant repulsive edges [270,271].

Table 3.5: Cohesion pressure values ($\Pi_{coh}$) and uncertainties ($\sigma_{\Pi_{coh}}$) for each material, as obtained from the fit in Figure 3.4b.

<table>
<thead>
<tr>
<th>Material</th>
<th>$\Pi_{coh}$ (mN/m)</th>
<th>$\sigma_{\Pi_{coh}}$ (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-gra</td>
<td>-11.9</td>
<td>2.1</td>
</tr>
<tr>
<td>S-gra</td>
<td>-10.8</td>
<td>2.3</td>
</tr>
<tr>
<td>BN</td>
<td>-2.0</td>
<td>4.3</td>
</tr>
<tr>
<td>MoS$_2$</td>
<td>-20.3</td>
<td>2.1</td>
</tr>
<tr>
<td>WS$_2$</td>
<td>-14.3</td>
<td>1.6</td>
</tr>
</tbody>
</table>
For the exfoliation and size selection processes used in this experiment a broad range of size values were used, implying that pressure–area behaviour is not solely determined by either nanosheet size or material class. If only nanosheet size had any effect then Figures 3.4a, 3.4b would show L-gra, BN, and WS$_2$ grouped together, with MoS$_2$ to one side of this cluster, and S-gra to the other. If material class alone affected the pressure–area behaviour, then in Figures 3.4a, 3.4b clear distinctions between the graphene, TMD, and BN samples are expected. In practice, S-gra and the two TMD samples are clustered together, with L-gra and BN separated, and the idea that behaviour might rely on a combination of nanosheet size and material class seems logical. Different material classes have different edge functionalities, which will influence film formation in different ways; however, size differences within material classes result in differing numbers of edges per unit area, and hence varying edge–edge interactions. If edge interactions between nanosheets play a significant role, as expected for L–S films, then this emphasises the need to account for nanosheet size before comparing the pressure–surface coverage data of the different samples. This would allow a fair comparison of how the intrinsic chemistry affects the edge–edge interaction between nanosheets.

3.5 Surface coverage normalisation

As discussed previously, the influence of material edge interactions on the film surface pressure can be ascertained more easily by normalising the surface coverage, $\Phi$, to nanosheet size, approximated by $\langle LW \rangle$. In particular, the need for a parameter that scales with the number of nanosheet edges per unit area is apparent. For this purpose, the centre-to-centre interparticle distance, $s$, is used. This is derived in Appendix C, and has the form

$$s = \sqrt{\frac{\langle LW \rangle}{\Phi}}. \quad (3.2)$$
Figure 3.5: (a) Plot of surface pressure vs interparticle distance. (b) Plot of normalised surface pressure vs interparticle distance (y-axis normalised for cohesion pressure).

This new variable represents a normalisation of the surface coverage to account for the influence of the nanosheet size. Plotting surface pressure against this variable therefore represents a normalisation of the surface pressure–surface coverage plot. This is shown in Figure 3.5a, where it can be seen that this reduces the materials to two generic curves. BN shows the closest particle approach, and densest film, as expected due to the stronger cohesion forces between the BN particles (Table 3.5).

If one normalises for the cohesion pressure determined from the fitting in Figure 3.4b, then in principle the long range interaction differences between the different materials is removed. Indeed, it is seen in Figure 3.5b that the BN and graphene converge onto a single curve, but interestingly, the TMDs remain at a distance and with slightly lower slopes. The TMD films have the largest repulsion from the cohesion pressure (Table 3.5); this is expected because the sulfur-terminated edge sites are stable and so the charge distribution at the edge is uneven due the electronegativity difference between the sulfur and the metal [38,272]. This leads to highly polarised edges and therefore strong dipole interactions [38,272]. This effect is noted to be particularly strong for small particles as the edges become proportionally more dominant compared to the particle volume [273]. The WS$_2$ particles in this study are highly exfoliated and of small lateral dimensions, meaning they are likely to have a high degree of edge charge. This explains the strong repulsive cohesion pressure, but also the variation in the interparticle force compared to the other
materials as seen in Figure 3.5. In addition to the long-range edge interactions, the MoS$_2$ in this experiment is particularly large, and multi-layered, so it is likely that there are large enough capillary forces acting on these particles compared to the WS$_2$ particles to start to affect the interactions [268]. The TMD films remain at a further interparticle separation compared to the other layered nanomaterials, due to their more polarised edge states and the comparatively stable nature of the edges. In this way, the class of material can be used to separate out the expected behaviour for monolayer film formation.

In nanomaterial films, rigidity percolation is reached when the particles form a connected bridge from one side of the Langmuir trough barrier to the other. For particles with the least repulsive edge interactions (such as BN) this bridge can form quickly, without total surface coverage, as even small numbers of particles agglomerate together forming branched networks. Conversely, a repulsive film will need to reach almost complete surface coverage to reach rigidity percolation ($\Phi = 0.7$) [274]. This is because the particles will continually rearrange on the subphase surface to minimise their interaction, and therefore will spread out across the entire area until forced by density to form a rigid film. The 2D bulk modulus, $\beta$, is one way of quantifying this behaviour. This is described by

$$\beta = -A \left( \frac{\partial \Pi}{\partial A} \right)_T,$$

where $A$ is the trough area, $\Pi$ is the surface pressure, and $T$ represents that the temperature is kept constant [274]. This is plotted for each material in Figure 3.6a. Although different materials clearly have different maximum bulk moduli, the rigidity onset occurs at varying surface pressures.

However, when normalised to the cohesion pressure, the 2D bulk modulus follows a similar onset and maximisation (Figure 3.6b). This fits well with the concept of rigidity percolation. As expected, the BN has the largest 2D bulk modulus as it has the strongest interparticle interactions and the densest films. The TMDs show very weak modulus behaviours, as the repulsive edge states allow for particle slippage in
the film system, maintaining a ‘liquid-like’ state for far longer, until geometrically ‘jammed’. Therefore, the larger MoS$_2$ particles are locked into place more rapidly than the smaller WS$_2$ particles, or even the exceptionally small S-gra flakes.

This confirms that it is possible to extract interesting information about edge interactions from the cohesion pressure $\Pi_{coh}$ only. This is a useful value beyond just Langmuir, providing insight into films more generally, including hybrid films, printing, heterostructure stitching, etc [275, 276].

The process from Figure 3.2 to Figure 3.6 describes how variable pressure–surface coverage data can be normalised to nanosheet size to account for edge effects. This results in data clustered by material group that Equation 3.1 suggests is related to the area coverage when accounting for the area density of edges. Outstanding differences remaining in Figures 3.5 and 3.6 are due to the chemical nature of the edge functionalities and therefore merit study in further detail. Examining the edge chemistries in more detail should confirm the relative interaction strengths described above. However, even without knowing the detail of the chemistry it is possible to account for nanomaterial class and size across a broad range of materials to determine film density from standard dispersion properties.
3.6 Cell study

To demonstrate the importance of controlled film properties, a cell study comparing different edge densities was conducted. Two sets of films were produced from the same graphene dispersion size-selected to have larger and smaller sized nanosheets. As these films were deposited at similar surface coverages, the L-gra films had a lower area density of edges than the S-gra films.

It is observed that the edge density strongly determines the growth of glioblastoma cells (Figure 3.7). The films with greater edge density have greatly increased (~1.5 times greater) cell proliferation at 12 days compared to those with lower edge density, with increased cell cluster formation and improved cell spreading both at 4 and 12 days. This increased proliferation is likely due to the increased surface roughness.

Figure 3.7: Optical micrographs showing U87 glioblastoma cell growth (a) at 4 days on L-gra substrate, few cells present and not inclined to growth; (b) at 12 days on L-gra substrate, still few proper cell clusters but those present were less confluent compared to other substrates; (c) at 4 days on S-gra substrate, cells had good size extensions; (d) at 12 days on S-gra substrate, many cell clusters observed and overconfluency apparent. All figures have scale bar = 200 μm.
that the cells experience with the greater edge density (as the graphene should itself be relatively inert), allowing them to form more adhesion sites. The cell stability is therefore increased, allowing more effective mobilisation and proliferation. Such a large impact on cell growth is critical when setting up controlled cell studies, and importantly this Langmuir substrate technique allows for the production of large quantities of identical substrates, which are important for the statistical requirements of biological studies [277].

3.7 Conclusions

Liquid-phase exfoliated (LPE) dispersions of BN, MoS$_2$, WS$_2$, and two sizes of graphene were prepared and used to deposit L–S films at a range of different surface pressures. This is the first demonstration of the production of single layer BN films using this method. These dispersions were made by generic processes to facilitate a standardised film creation method. UV–visible spectroscopy measurements showed the dispersions to be exfoliated, while AFM measurements were used to obtain average lateral dimensions and thicknesses for each material. Running a binary threshold on optical micrographs of the films gave quantitative values for the surface coverage of the material films, allowing the surface pressure–surface area relation to be plotted. To gain further insight from these plots, the data were normalised to account for differing sizes of nanosheets between materials. Plotting surface pressure against the interparticle distance begins to account for edge density effects and results in near parallel data. Additionally, the 2D bulk modulus was plotted, and, once normalised to the cohesion pressure, showed a similar rigidity onset and maximum for each material. Increased edge density on a graphene cell substrate increased colony formation and proliferation, demonstrating the importance, and tissue engineering potential, of control over film properties.

These results allow for an improved understanding of the physical and chemical influences on film formation, surface pressure, and surface coverage behaviour in Langmuir films of 2D layered nanomaterials. This straightforward process has been
demonstrated for a range of layered nanomaterials, including BN, which has not yet been used extensively for Langmuir deposition. An approach for analysis has been developed where size effects can be discarded, leaving only the effects due to the inherent chemistry of the material type. This combination of variable surface coverage and edge functionalities makes Langmuir films of layered nanomaterials interesting as substrate modifications for studies of cell growth and proliferation. These results will have uses beyond even cell studies, as this technique can be used to create films for a wide range of applications, such as transparent electrodes, supercapacitors, etc. as well as providing a framework for processing new layered materials into single layer films, with maximised surface area.
Chapter 4

Cell–substrate interactions lead to internalisation and localisation of layered MoS$_2$ nanosheets

Using an ultrathin film substrate, the first internalisation of MoS$_2$ nanosheets through mechanotransduction is demonstrated. The usual method of dispersing nanomaterials in the media limits interactions to random, serendipitous surface contact, and the nanoparticles must be dispersible in media. A substrate approach means that cells directly engage with the nanomaterial, sensing and adhering through sustained interaction and actively internalising the nanomaterial. This activates previously unobserved cell–substrate mechanotransduction mechanisms and receptor-mediated uptake pathways. Moreover, a wide variety of nonsoluble nanomaterials can be used, improving control over the amount of material exposed to a cell through tuneable deposition density. Volumetric Raman mapping demonstrates localisation of material to the endoplasmic reticulum (ER), a historically hard-to-target region. The nanosheets do not cause cytotoxicity, are transferred to daughter cells, and have applicability across multiple cell lines. The innate fluorescence or Raman signal of the nanosheet can be utilised for live cell imaging, and targeted accumulation within specific cellular organelles offers potential for photothermal treatments or
drug delivery vectors. This substrate-mediated approach provides a step change to studying nanomaterial–cellular interactions, taking advantage of the broad palette of available two-dimensional (2D) materials and making use of mechanosensing to stimulate tuneable responses, with potential for therapies and diagnostics.

4.1 Introduction

The properties of nanomaterials are of increasing interest for biological applications. The transition metal dichalcogenides (TMDs), such as molybdenum disulfide (MoS$_2$) and tungsten disulfide (WS$_2$), are of particular interest. These tend to have direct band gaps in the monolayer form, making them key to optoelectronic devices [278–280]. This also means that they are inherently fluorescent [251], which is a useful attribute in cell studies as a probe, if an appropriate target can be identified. TMDs also have accessible chemistries [281] and can be easily functionalised [282–284], which can improve the biological interaction [285,286] or allow the material to be used as a drug vector [107,108,287]. MoS$_2$ has fluorescence in the red [288] in the monolayer form, strong Raman-active phonon modes that are correlated to its geometry [289], and sulfur edge chemistry that is particularly useful for protein binding [290]. In addition, molybdenum and tungsten enzymes are known to be essential to life [291,292], yet how cells access these metals is not well understood, which makes TMDs important materials for investigation.

Previous work on MoS$_2$ in cell studies has always made use of a media dispersion and then measured the uptake and localisation from this system [293–296]. Issues with dispersed nanomaterial studies include the general lack of nanomaterial solubility [295, 296], degradation of the material within the cell [297], and toxicity [298–300]. How cells sense and physically engage with their substrate is critical to a multitude of cellular processes, including mobility, motility, proliferation, nutrient uptake, and stem cell differentiation [301,302]. Development of ultrathin film substrates provides a unique way to present 2D nanosheets to a cell [118], allowing cells to be seeded directly onto the material of interest. This physical interaction between cell and
material leads to different mechanotransduction responses \[303,304\]. These substrate-mediated responses provide an opportunity, through the use of nanomaterials, to access different mechanisms and organelles and stimulate various cellular responses, which has not been possible before \[305\]. Although there is little research into active mechanotransduction of nanomaterials currently, Yeh et al. have shown that when cells are grown on chemical vapour deposition (CVD) WS\(_2\), the cells are able to internalise portions of the single crystal and also present fluorescence in daughter cells \[306\]. However, they did not speculate as to the mechanisms for this uptake or the internal localisation of the materials.

The synthesis and processing of the materials used in this work is described in 2.2.4.2, the characterisation techniques used are described in 2.3.5.2, and the details of the cell experiments are included in 2.3.6.2. The cell lines used are described in more detail in 5.3.

### 4.2 Substrate production

Liquid-phase exfoliation by sonication (LPE) is the most scaleable way to produce large quantities of various layered nanomaterials in dispersion \[120,248\]. By controlling exfoliation and centrifugation parameters and solvent choice, it is possible to tune the morphology and chemistry of the nanosheets produced. Common parameters that have been used in this work yield MoS\(_2\) nanosheets that are \(\sim 200\) nm in length and 8 layers thick, confirmed by atomic force microscopy (AFM) (Figures 4.1a–c)

![Figure 4.1: MoS\(_2\) nanosheet characterisation. (a & b) AFM statistics histograms for MoS\(_2\) nanosheets showing length and thickness data. (c) AFM image of as-produced MoS\(_2\) nanosheets.](image)
Figure 4.2: MoS$_2$ thin film substrate characterisation. (a) Optical micrograph of a typical thin film substrate on glass, a close-up shown in the inset. (b) Map of the layer number, $N$, for a typical MoS$_2$ thin film substrate on glass. (c) Typical resonant Raman spectra (660 nm) taken from the 2D Raman map (b) of the thin film substrate; metrics [203] indicate that (d) the average thickness is 8 layers and that (e) the average length is $\sim$200 nm.

and Raman metrics (Figures 4.2b–e) [203, 212]. MoS$_2$ has Raman-active modes that can be correlated to the thickness and defect densities, and also has strong resonance with a 660 nm laser [203]. Figure 4.2c, in particular, illustrates this as the 2LA(M) mode is not present at other wavelengths [203]. These properties mean that a significant signal can be gathered from a very small sample volume [203]. This provides an excellent way to characterise the MoS$_2$, to track its location throughout a cellular interaction, and also to determine any modifications it undergoes [203, 297].

The Langmuir–Schafer (L–S) deposition technique works by dropping nanomaterial dispersion onto a trough of water, such that the dispersion solvent evaporates, leaving a thin film of material at the air–water interface. It has proven to be highly effective for the production of ultrathin films of a variety of nanomaterials and has been shown to be possible to tune the thin film density, as discussed in Chapter 3 [118, 162]. With careful solvent selection for the LPE process, it is also
possible to deposit films directly without any solvent transfer steps. These thin films can be deposited onto various substrates, but for this work, glass slides have been used to provide compatibility with standard cell culture techniques and equipment, and still enable characterisation by Raman spectroscopy before and after cell growth. The film density is controlled via barriers on the Langmuir trough to provide full or partial surface coverage (Figure 4.2a). Combining LPE and L–S deposition means that these thin film substrates can be produced with various 2D layered nanosheets, in large quantities, repeatably, and therefore can be used for multiple cell growth studies. Figure 4.3 is a scheme that shows the process from bulk MoS$_2$ powder via LPE to the final L–S film.

![Figure 4.3: Bulk MoS$_2$ (structure shown, left) is added to a suitable solvent, in this work cyclopentanone, and exfoliated by ultrasonication (shown centre). After exfoliation is complete, the resulting dispersion is used to create an L–S film (shown right).](image)

### 4.3 Cell studies

When cancer cells (U2OS) are seeded onto the L–S thin film substrates, the cells grow and proliferate with no cytotoxicity evident even after 14 days. In fact, there is a nonsignificant increase in the cell count after growth on the MoS$_2$ substrates compared to that on pristine glass cover slips (Figure 4.4a). Potentially, this is due to the increased surface roughness providing improved adhesion. Of great interest is that after ~3 days of incubation on the substrate, the cells begin to modify
Figure 4.4: Cell–substrate interactions and uptake. (a) Cell count data for U2OS cells grown on MoS2 substrates (blue) and pristine glass controls (orange); error bars calculated as standard error in the mean from three repeat experiments (two samples per type for each experiment). (b) Optical micrograph of U2OS cells (7 days of growth) on a split substrate (left half of the substrate pristine glass and the right half coated in a MoS2 Langmuir thin film of typical density shown in the inset); MoS2 nanosheets have been accreted by the cells (dark regions). The inset scale bar is also 200 µm. (c & d) AFM height images of U2OS cells (3 days of growth) on MoS2 nanosheets, with cytoskeletal extensions and adhesion sites directed toward the MoS2 nanosheets on the substrate. (e & f) Optical micrographs showing the internalisation of the MoS2 nanosheets and the localisation around the nucleus in the ER (taken of the same sample as shown in Figure 4.4b). The inset scale bar is also 20 µm.
the film and internalise the MoS$_2$ nanosheets. After 7 days, the majority of the MoS$_2$ has been accreted under the cells and large proportions of it are internalised (Figures 4.4b, e & f).

Using substrates that are only half-coated in the MoS$_2$ allows this physical interaction to be seen clearly. What was once a complete thin film on the right half of the substrate (typical density shown in Figure 4.4b inset) now forms the dark regions of MoS$_2$ that have been accumulated under and internalised within the cells (Figures 4.4b, e & f). This is due to the action of the cells adhering to, and mobilising on, the substrate. As the nanosheet forms part of the cell substrate, the material is internalised through a mechanotransduction response; the cell senses the substrate and then actively internalises the material. This response is of enormous interest and is little understood; it is an entirely different approach to the internalisation of nanomaterials, which are usually dispersed in the cell media. It can be seen that the majority of the MoS$_2$ nanosheets are internalised in the region around the nucleus (Figures 4.4e & f). Some recent work has shown that the stiffness of the cell substrate influences how much uptake of dispersed nutrients the cell engages in [307, 308]. For the stiffer materials, the cells can be seen to have formed far larger cytoskeletons, with extensions and adhesion sites that spread throughout the substrate. The cells must mechanically interact with their substrate to be able to engage in material uptake. In this work, the cells are actively interacting with the MoS$_2$ nanosheets, adhering to them, and applying forces to remove and move them across the substrate. The cells can be clearly seen to have large spreading areas, lots of cytoskeletal extensions, and adhesion sites associated with migration and motility (Figures 4.4c–f). The atomic force microscope (AFM) images illustrate extensions and adhesion sites specifically grown toward dense regions of MoS$_2$ nanosheets; when grown on sparse film substrates (Figures 4.4c & d), the cells are seeking out the nanosheet material. This in turn means that the cells tend to congregate around denser MoS$_2$ regions, improving their ability to conduct cell-to-cell communication, which is vital for proliferation and viability.
Figure 4.5: Localisation and lifetime of MoS$_2$ nanosheets. (a) 2D Raman map of the intensity of the $\sim$405 cm$^{-1}$ $A_{1g}$ peak of MoS$_2$ (green) overlaid on the optical microscope image of cells; (b & c) metrics [203] indicate the same layer number (8 layers) but reduced length (<200 nm). (d) Top-down optical view of the location of (e) volumetric Raman mapping of the intensity of the $A_{1g}$ peak (green), with some nanosheets inside the cell and some still on the substrate. (f) Cell count data for U2OS cells grown on MoS$_2$ substrates and glass controls for 7 days and then removed from the substrate, split, and reseeded as described in 2.3.6.2; the error bars are calculated from standard error in the mean from two repeat experiments (two samples per type in each experiment). (g) Raman mapping of cells taken from MoS$_2$ substrates and reseeded onto pristine glass controls, indicating that MoS$_2$ nanosheets are still localised within the ER and present in daughter cells, with (h) minimal modification of innate Raman spectra indicating limited degradation.

Raman volumetric mapping is a powerful technique for determining the localisation of MoS$_2$ nanosheets within the cell. Mapping the peak intensity of the $\sim$405 cm$^{-1}$ $A_{1g}$ mode yields detailed spatial information for the nanosheet within the cell. Combining multiple overlapping steps gives a $z$-resolution of $<1$ µm, and therefore, it is possible to confirm the difference between the material internalised within the cell and that above or below the cell. Volumetric (Figures 4.5d, e & D.1)
and 2D (Figures 4.5a, g & D.2) Raman mapping confirms our observations from the optical microscopy (Figures 4.4e & f). The MoS$_2$ nanosheets were presented as a thin film substrate only; no MoS$_2$ was added directly to the cell media, so there is no possibility of the material being on top of the cells, as can be the case for media-dispersed nanomaterials. This volumetric map shows nanosheets that are clearly raised above the substrate, indicating that they are largely internalised in the region around the nucleus (the endoplasmic reticulum, ER), with some material identified in the cytoplasm (most likely in lysosomes) and some still lying at the base of the cell and therefore still on the substrate (Figure 4.5e).

If the cells are harvesting transition metals or sulfur components for internal processes, then it would be interesting to note if the cells are able to break down the MoS$_2$ to access the elemental components. Raman spectroscopy (Figure D.3) indicates that there is little change to the layer number for the MoS$_2$ nanosheets after its internalisation — still 8 layers (Figure 4.5b) — but the average length has decreased (Figure 4.5c). This suggests either that the cells are able to begin to decompose the edge sites (spectra taken from cells after 7 days of growth), as the metrics do not indicate an increase in defect sites, such as holes, in the basal plane; or that cells are selectively internalising any smaller-sized sheets on the substrate, due to a possible limit on the size of the sheets able to be internalised via the caveolin pathway.

Excitingly, the MoS$_2$ is also transferred to daughter cells (Figure 4.5g), as was also seen by Yeh et al. with the CVD WS$_2$ [306]. In an experiment, after 7 days of growth on MoS$_2$ and pristine glass control substrates, the cells reached confluence and were trypsinised, split, and reseeded onto new substrates (Figure 4.5f). The cells were reseeded at the initial (day 0) density to allow room for further proliferation on the new substrates (indicated by the sudden decrease in cell count in Figure 4.5f). The new substrates used were either further fresh MoS$_2$ substrates or pristine glass cover slips (control). In this way, cells that had been initially grown on MoS$_2$ are seeded onto both further MoS$_2$ and clean controls, and cells from the clean controls
are seeded onto further clean controls and onto MoS$_2$. As before, it is seen that this does not affect the proliferation of the cells (Figure 4.5f); if anything (although it is not significant), the MoS$_2$ actually increases the cell proliferation both before and after the substrate change. Importantly, MoS$_2$ is still observed to be internalised within the ER of cells seeded onto the clean substrates (Figure 4.5g), so the MoS$_2$ is retained within the cell and passed on to daughter cells (U2OS doubling time is $\sim$29 hours). Although we have not performed extensive studies to quantitatively determine for how many generations the MoS$_2$ is retained in the cells, we can make some speculation based on the U2OS doubling time. The cell study continued for 7 days after cells initially seeded on MoS$_2$ were reseeded onto pristine glass substrates. As the U2OS doubling time is $\sim$29 hours, and this part of the cell study ran for 7 days, we estimate that the MoS$_2$ remains present in daughter cells for at least five to six generations.

Raman spectroscopy of the MoS$_2$ internalised in the daughter cells again shows similar layer numbers and no further significant decrease in length compared to that of the continuous growth for the same length of time (Figure 4.5h). This has implications for theranostics, as the vector remains within the cell line. As the tumour grows, for example, all cells may contain the vector, which could be used as a therapeutic centre or tag. The nanosheet perseverance is further confirmation of their potential to act as new probes, as the nanosheets can be tracked throughout long live cell imaging experiments, over multiple generations, and also tracked within a co-culture. This paves the way for the next generation of diagnostics and imaging that can investigate the microenvironment and complex tissue development.

4.4 Internalisation mechanisms

The receptor-mediated endocytosis pathways transport material to the ER and Golgi apparatus, whereas more passive processes silo unwanted or unknown material into lysosomes and usually eject it [309]. The ER, in particular, is associated with molybdenum enzymes and sulfur bridges for protein building [291]. Several transition
metals, including molybdenum, are vital for cell function. The molybdenum cofactor is found in all kingdoms of life; five molybdenum cofactor enzymes are known to exist in eukaryotes and four of these are found in humans [310]. A lack of molybdenum in the environment can lead to a deficiency in molybdenum cofactor, a rare disease first identified in 1978; this can lead to neurological damage, seizures, and feeding difficulties [310]. Despite their importance, little is known about how cells access and internalise these materials. The active interaction and accumulation of the MoS$_2$ nanosheets, shown here, may provide a new means to study the cellular uptake of essential transition metals.

Figure 4.6: (a) U2OS cells grown on MoS$_2$ substrates, no nystatin solution (control). (b–d) U2OS cells grown on MoS$_2$ substrates with 20% nystatin solution to limit caveolin uptake.

Other works using MoS$_2$ nanosheets in dispersion have identified the predominance of the caveolin pathway in the internalisation process. This pathway is poorly understood, but recent insights have identified its important role in substrate sensing [311–313], mechanoprotection [314], and cancer growth [311,314]. Nystatin
is used as an inhibitor for the caveolin pathway. In the present study, the use of nystatin did not alter the U2OS cell accretion of the MoS$_2$, but significantly reduced internalisation was observed, implying that this uptake pathway is dominant for MoS$_2$. There was some evidence of MoS$_2$ within the cytoplasm, most likely in lysosomes through passive diffusion or alternate endocytic pathways. Typical optical micrographs of the U2OS cells grown in 20% nystatin solution are shown in Figure 4.6. This fits with the expected uptake mechanism for transition metals in a mechanotransduction substrate response. Additionally, this uptake pathway is a direct route to the ER, explaining the accumulation within that organelle [314,315]. This all adds strong weight to the argument that the cells identify the transition metal and sulfur chemistry within the substrate through sensing mechanisms, and harvest the materials through this directed route for protein and enzyme building. The caveolin pathway is little studied, and so this nanomaterial substrate technique provides a route to probe and understand these fundamental cell processes further.

The unfolded protein response (UPR) is a cellular response to errors in protein folding in the ER; the cell enlarges the ER to accommodate the unfolded material [316]. This response aims to restore normal function to the cell, but, if unsuccessful and ER stress is prolonged, leads to apoptosis. One of the mechanisms of cancer is that it is able to maintain the unfolded protein response (UPR) far longer than a normal cell, thus evading apoptosis. In the present study, a clear increase is seen in the size of the ER in the sarcoma cells grown on the MoS$_2$ (Figure 4.7a), compared to those grown on the control (Figure 4.7b), indicating stress in the ER. Not only does this confirm the localisation of the MoS$_2$ within this organelle, but also this indicates that the exposed sulfur edges and/or molybdenum metal sites of the MoS$_2$ are being identified by the cell as protein or enzyme components, thus activating the UPR. It is interesting to note that this large accumulation of nanosheets within the ER of a cancerous cell could provide novel cancer theranostics, targeting a specific cancer mechanism. The overactive UPR of cancer cells could be utilised to accumulate a critical mass of material in only the cancerous cells, which either drives them to
Figure 4.7: Generalisation of the methodology and applications. (a & b) Optical images of U2OS cells grown on (a) MoS$_2$ and (b) control substrates; a substantial increase in the size of the ER is observed for the cells grown on MoS$_2$ nanosheets. One ER from each image is highlighted with blue dotted circles to give a guide to its size and location within the cell. (c) A cell with internalised MoS$_2$ after irradiation with a 100 mW 660 nm laser showing severe local damage. (d) Cell count data for primary fibroblast cells, showing no toxicity after 7 days; error bars calculated from the experimental error of single experiment. (e & f) Optical micrographs of fibroblast cells grown on MoS$_2$ substrates with nanosheets localised in the ER, but less accretion under the cells compared to U2OS.
apoptosis (although cell death was not seen even after 14 days of incubation) or can be used as a photothermal site. High exposure of the internalised MoS\(_2\) with a resonant laser causes significant localised damage to a cell — the ER is destroyed through local heating of the excited MoS\(_2\) (Figure 4.7c), confirming the possible use of the internalised MoS\(_2\) for photothermal therapy (PTT). Alternatively, drugs that target this organelle could be attached to the material. All the while, the condition and location of the nanomaterial could be tracked by Raman volumetric mapping or indeed by the material’s inherent fluorescence in the case of monolayer TMDs (as demonstrated by Yeh et al. [306]).

A noncancerous cell line (fibroblasts) also shows localisation of MoS\(_2\) within the ER, although the amount is not as significant as for the cancerous cell line (U2OS), and there is more material distributed in the cytoplasm (Figures 4.7e & f). The cells do not seem to actively accrete the nanosheets as the cancer cells do, the multicoloured contrast indicating the presence of MoS\(_2\) still distributed across the substrate in Figure 4.7f. In this work, the fibroblasts proliferated faster than U2OS cells and also have larger spreading areas, so they reached confluence more quickly, limiting the time spent mobilising on the substrate before intercellular interactions started to dominate. Regardless, there is no indication of cytotoxicity up to 7 days of growing on the substrate (Figure 4.7d), however, the slight nonsignificant increase in proliferation as for the U2OS cells was not observed, probably due to the lower mechanical substrate interaction of fibroblasts compared to that of bone-derived cells. This shows that the mechanotransduction-mediated uptake of nanosheets is a potential pathway to internalisation for many different cell types. A new mechanotransduction internalisation route could be used for developing new fluorescent and Raman probes for previously unattained targets. However, there may be more specific implications for cancer cells due to the exaggerated cancer cell–substrate interactions, the ‘hungry’ nature of cancer cells to scavenge materials from the environment, and the overactive UPR. These phenomena could all be exploited through mechanotransduction-mediated uptake of nanosheets to internalise
imageable drug delivery vectors or localise photothermal sites to cancer-specific targets, for example. Therefore, this approach may have exciting potential for cancer theranostics.

### 4.5 Conclusions

The need for the cell to form adhesions, and deform its environment, is critical to its functioning. In this work, the substrate is stimulating the cellular membrane proteins for sensing and recognition, triggering uptake pathways, as well as spreading and proliferation. These mechanotransduction responses provide new insights into the full interaction of a cell with its environment and offer novel approaches to studying cellular mechanisms.

This work presents a completely new internalisation methodology for synthetic nanoparticles. A new technique for targeting receptor-mediated uptake has been demonstrated, and its links to internal structures such as the ER have been shown, which is important in many different diseases [317]. It is well known that accurate internal mapping is difficult; this work aims to improve this by using Raman spectroscopy. While it is possible that the determination of the localisation within the cell could be improved, significant localisation of the nanosheets in specific organelles has been seen, all identified and mapped through volumetric Raman spectroscopy, a non-invasive process that has the potential to be used with live cells. Additionally, novel insights into mechanotransduction responses to substrate-derived nanomaterials have been made and shown to be generic to different types of cells with no toxicity, but of particular interest to cancer cells due to their overactive mechanical substrate interactions and modified UPR. Following this initial study, presented here to introduce the broad scope of this method of looking at cell-substrate interactions, further biological characterisation experiments are needed to further understand this system, including detailed cytotoxicity assays, comparisons of other nanomaterials, and further investigations of the UPR effects.
The sulfur chemistry of the TMDs can be exploited for biological attachment and for drug delivery or more specific targeting [182]. By utilising MoS$_2$ as a cell substrate, there is real potential to use the material’s inherent fluorescence or unique Raman features for tracking, live cell imaging, and potential therapies with photothermal treatment or drug delivery of a mechanotransduction-internalised nanovector. This new approach to nanomaterial–cell interfacing offers an exciting opportunity to develop the next generation of theranostics.
Chapter 5

Internalisation and localisation of transition metal dichalcogenide nanosheets

5.1 Introduction

A clear way to extend the work in Chapter 4 was to consider other transition metal dichalcogenides (TMDs). While MoS$_2$ was shown to be internalised in that work, it had not been ascertained whether the same would be true of WS$_2$, or MoSe$_2$. Yeh et al. [306] had previously shown that cells internalised chemical vapour deposition (CVD) WS$_2$, so it seemed likely that this research would yield positive results. However, this had not yet been studied for liquid-phase exfoliation by sonication (LPE)-produced TMDs other than the work on MoS$_2$ [318].

As discussed in Section 2.1.2, TMDs are compounds described by the chemical formula MX$_2$, where M is a transition metal (e.g. Mo, W) and X is a chalcogen (e.g. S, Se) [8, 38]. The monolayer, formed of a hexagonal plane of transition metal ions sandwiched between planes of chalcogen atoms [39], is not strictly two-dimensional (2D). However, TMDs are often classed as such due to their extremely thin monolayers [38–40]. This standard structure makes different TMDs structural
Figure 5.1: Structure and dispersion of MoS$_2$ (left), WS$_2$ (centre), and MoSe$_2$ (right). The materials are structural analogues of each other.

analouges of each other (Figure 5.1). Dispersions of these materials are made in the same way and have similar solvent compatibilities, but have different band gap structures due to their different components.

The synthesis and processing of the materials used in this work is described in 2.2.4.3, the characterisation techniques used are described in 2.3.5.3, and the details of the cell experiments are included in 2.3.6.3.

5.2 Nanosheet characterisation

Briefly, the WS$_2$ and MoSe$_2$ dispersions used in this study were produced by LPE, and these dispersions were used to create dense Langmuir–Schaefer (L–S) thin films. Raman spectra of the substrates were taken to confirm the presence of the expected material and to show the level of material exfoliation. These spectra can be seen in Figure 5.2, with labelled phonon modes [319–321]. The WS$_2$ Raman spectrum shows a sharp peak assigned to the 2LA(M) mode at 349 cm$^{-1}$, and another peak assigned to the A$_{1g}$ mode at 419 cm$^{-1}$ [319]. The high intensity of the second-order 2LA(M) mode compared to the A$_{1g}$ mode implies the presence of mono- or few-layer (~2–3 layer) WS$_2$ [319]. The MoSe$_2$ Raman spectrum shows a sharp peak assigned to the A$_{1g}$ mode at 242 cm$^{-1}$, and another peak assigned to the E$_{2g}^1$ mode at 292 cm$^{-1}$ [321]. The peak spacing of approximately 50 cm$^{-1}$ indicates the presence of
MoSe$_2$ monolayers [321]. The high degree of exfoliation shows that the methodology is good for optimising the nanoscale properties, and Raman spectroscopy is a simple and effective way to characterise the material. The exfoliation compares well to that of the MoS$_2$ used in Chapters 3 and 4.

### 5.3 Cell lines

The same cell lines were used for this study as for the MoS$_2$ study in Chapter 4, namely 1BR primary fibroblasts and U2OS bone carcinoma. Here, these are described in more detail. Fibroblasts are mesenchymal cells with an elongated, spindle shape [322]. Their branched cytoplasm projections surround a nucleus with two or more nucleoli, and contains an abundance of rough endoplasmic reticulum and a large Golgi apparatus [323]. Fibroblasts are one of the most common cell types in connective tissue [323], and are responsible for maintaining the structural integrity of connective tissues by synthesising extracellular matrix components throughout the body [322]. Additionally, fibroblasts are involved in the wound healing process [322]; when tissue is injured, nearby fibroblasts proliferate, migrate into the wound, and produce large amounts of collagenous matrix [324]. However, it is important to note that there is
variation between fibroblasts from different anatomic sites [322], and that a number of different functions can be displayed even within one system [323]. Figure 5.3a shows control 1BR primary fibroblasts from this study.

U2OS cells are human osteosarcoma cells derived in 1964 from a moderately differentiated sarcoma of the tibia of a 15 year old girl [325]. They have an epithelial morphology. The chromosomes present in this cell line are significantly abnormal [325]. Furthermore, the cells show typical signs of malignancy, including an increased nucleocytoplasmic ratio, pleomorphism and enlarged nucleoli [325]. Figure 5.3b shows control U2OS osteosarcoma cells from this study.

Figure 5.3: Optical micrographs of control (a) 1BR primary fibroblasts and (b) U2OS osteosarcoma cells. Red boxes indicate magnified areas.

5.4 $WS_2$ Substrates

First considering the 1BR primary fibroblasts, the $WS_2$ is visibly internalised within the cells (Figure 5.4b–e). This is confirmed by Raman spectroscopy of one of these dark areas within a cell. Figure 5.4a shows the characteristic Raman modes associated
with WS$_2$ (cf. Figure 5.2a). Because the cells were trypsinised and reseeded onto pristine glass cover slips for 2 days before fixation, any material present must be either within the cells, or loose having been ejected by the cell after reseeding.

Figure 5.4: (a) Raman spectrum taken of a visibly dark area in a 1BR cell which grew on a WS$_2$ substrate for 7 days before trypsinisation and reseeding onto a pristine glass control. WS$_2$ features are still present, indicating the presence of WS$_2$ within the cell. Inset shows a typical 1BR cell with visibly internalised WS$_2$. (b–e) Optical micrographs of 1BR primary fibroblasts grown on WS$_2$ substrates. Red then blue boxes indicate areas undergoing increasing magnification. Red and purple boxes indicate different areas of an image undergoing the same magnification.
There are clear similarities between the amount of internalised WS$_2$ in this work and the amount of internalised MoS$_2$ in Chapter 4. However, while the WS$_2$ is still localised to the cell body, it is not as obviously localised to the endoplasmic reticulum (ER) as MoS$_2$ was previously. Further, the material appears to be occupying spherical vesicles, suggesting that it is likely that the material may be contained within lysosomes.

The work presented by Yeh et al. [306] also shows strong uptake of WS$_2$. However, they use CVD WS$_2$ rather than liquid-phase exfoliated (LPE) WS$_2$, so the size and quality of the material may differ. Further, Yeh et al. use the LMH hepatocellular carcinoma cell line whereas this work considers 1BR primary fibroblasts and U2OS osteosarcoma cells, meaning that direct comparison between cell lines is not possible.

Figure 5.5: Optical micrographs of U2OS osteosarcoma cells grown on WS$_2$ substrates. (a & b) Many cells show no obvious internalisation of WS$_2$, typical examples given here. (c) Some cells did show internalisation, but not to the extent as for the 1BR primary fibroblasts. Red box indicates areas undergoing increasing magnification.
When looking at the U2OS cells, the contrast with 1BR cells is stark; although some cells show WS$_2$ uptake (Figure 5.5c), not as much material is seen within these cells, and several cells do not show any evidence of internalisation (Figure 5.5a & b). The amount of internalisation is also less than that seen for MoS$_2$.

This suggests that the presence of sulfur groups may cause the generic uptake by 1BR primary fibroblasts, as these cells show large amounts of internalisation of both MoS$_2$ and WS$_2$. Because there is not as much internalisation of WS$_2$ in the U2OS cells, it is not so clear that the sulfur groups are the main cause of uptake by these cells.

Therefore, it is possible that the transition metal has more influence on the material internalisation for U2OS cells. While Mo and W are both key elements to cells, only Mo is used in eukaryotic cells; this might explain why less WS$_2$ is internalised by U2OS cells compared to the amount of MoS$_2$ that was internalised in Chapter 4. W may also be internalised by the same process due to the similar chemistries of the two materials [291], but perhaps this would not be as efficient as the uptake of MoS$_2$. However, this is speculative, and more experimentation is required to investigate this aspect.

If these hypotheses are correct, namely that 1BR fibroblasts favour the sulfur groups of the material, and that U2OS favours the Mo transition metal, then it is possible to suggest potential outcomes for the cell study on MoSe$_2$ substrates. The 1BR fibroblasts grown on MoSe$_2$ would not necessarily show much material internalisation because the sulfur groups are not present. U2OS cells may show more internalisation of MoSe$_2$ than WS$_2$ if the Mo transition metal is preferred. However, selenium is known to be toxic to cells, so the selenium groups of MoSe$_2$ may mean that neither cell line shows much internalisation.
5.5 MoSe₂ Substrates

Both cell lines grown on MoSe₂ substrates showed very little internalisation and limited growth, including no growth on one run of U2OS cells (Figure 5.6, 5.7). However, the cell counts can’t be compared directly with those on WS₂ or MoS₂ because the cell density wasn’t controlled at reseeding for this initial experiment, designed to look at internalisation rather than toxicity. Cell extensions are visible, particularly for several of the U2OS cells grown on MoSe₂ (Figure 5.6). Although extensions are seen for U2OS cell growth on WS₂ (e.g. Figure 5.5b), they are far less common than those seen for U2OS cell growth on MoSe₂. This implies a different substrate interaction for the MoSe₂ substrates, perhaps trying to find adhesion sites not associated with MoSe₂ nanosheets (i.e. avoiding the material). This agrees with the earlier suggestion that the sulfur chemistry could be critical to the biocompatibility of the TMDs, with coincidental internalisation of the transition

![Figure 5.6: Optical micrographs of U2OS osteosarcoma cells grown on MoSe₂ Langmuir thin film substrates. Red circles indicate cell extensions; these are enlarged in the insets.](image)
metal. However, this experiment uses reseeded cells; it is possible that the cell lines grown on MoSe$_2$ may have internalised and then expelled the unwanted material within the initial 7 day growth period before trypsinisation and reseeding onto controls.

### 5.6 Conclusions

This work builds on the foundations laid by the initial MoS$_2$ internalisation study presented in Chapter 4 (from [318]). It is shown that the internalisation methodology — utilising cell–substrate interactions instead of passive material uptake from the cell media — is not unique to MoS$_2$, but is also shown for WS$_2$. As in the MoS$_2$ study, the sulfur chemistry is suggested to play an important role in the internalisation process. While MoSe$_2$ is not seen internalised within either cell line, it is still undetermined whether this is due to the material never having been internalised, or due to rapid internalisation and excretion of the material in the 7 day growth window before trypsinisation and reseeding for imaging. This requires further investigation by repeating these experiments at a range of growth windows. U2OS cells on MoSe$_2$ substrates are imaged with cell extensions, showing the active sensing processes at work. This approach has been shown to be generic to other TMDs in addition to MoS$_2$, opening the door to further potential theranostic applications utilising the various TMD materials.

Figure 5.7: Optical micrographs of 1BR primary fibroblasts grown on MoSe$_2$ Langmuir thin film substrates.
Chapter 6
Conclusions and future work

Nanobiotechnology is a flourishing field in which nanotechnological advances are used to explore biological applications. Still, interactions between nanomaterials and cells or tissues are not well understood. To investigate cell–substrate interactions, it is therefore crucial to use well-characterised substrates. Presently, it is hard to ensure that substrates have the same properties from sample to sample because substrate synthesis is not standardised. This observation prompted the first aim of this thesis:

Is it possible to define a methodology to ensure a controlled and tuneable production of two-dimensional (2D) thin film substrates?

The ability to control thin film production would allow properties of the thin film to be known from initial parameters set during synthesis. To become widely adopted, not only would any such method need to be easily applied, but production of the materials required would also need to be simple, scaleable, and standardised.

Work in Chapter 3 tackles this by utilising sonication-assisted liquid-phase exfoliated (LPE) nanomaterial dispersions. As discussed in 2.2.1, liquid-phase exfoliation by sonication (LPE) is known to be an easily scaleable method of nanomaterial production [120] with several standard characterisation metrics, making it a good choice for the development of a standardised film creation methodology. Several LPE materials (hexagonal boron nitride (BN), MoS$_2$, WS$_2$, and two sizes of graphene) were
used for Langmuir–Schaefer (L–S) deposition at a range of surface pressures, with the idea that it might be possible to predict the film surface area from measurements of the surface pressure and the material dimensions alone. Surface pressure–surface area plots were obtained after surface area was quantitatively measured by analysis of binary thresholds of optical micrographs of the films, but because film surface area depends on edge-edge interactions, normalisation for nanosheet size was necessary to assess the effect of intrinsic chemistry on the edge-edge interactions between nanosheets. These edge density effects start to be accounted for by plotting surface pressure against centre-to-centre interparticle distance. The 2D bulk modulus, which can quantify rigidity percolation, showed a similar rigidity onset and maximum for each material after normalisation for cohesion pressure (a term which describes long-range interactions between nanosheets). This straightforward process allows for an improved understanding of the physical and chemical influences on film formation, surface pressure, and surface coverage behaviour in Langmuir films of 2D layered nanomaterials.

The combination of variable surface coverage and edge functionalisation made Langmuir films of layered nanomaterials an interesting candidate for studies of cell growth and proliferation. Cell colonies were shown to form and to proliferate on thin film substrates; furthermore, the cells showed preference for the substrates with increased edge density. This emphasises the importance of control over thin film substrate production. The cell study served as an initial proof of concept for the second aim of this thesis:

Most nano–bio studies introduce the nanomaterial to the cell by dispersion in the cell medium — can nanomaterials be introduced to the cell from the substrate alone? Does this change the effect on the cell?

Langmuir thin films for cell studies needed further exploration to fully address this question. Chapter 4 investigates this further, with focus on MoS$_2$ rather than graphene substrates due to the material’s interesting chemistry. Cells were found
to exhibit mechanotransduction responses to the thin film substrate; stimulation of the cellular membrane proteins for sensing and recognition triggered uptake pathways. This method of introducing the cell to the nanomaterial leads to uptake to cell organelles such as the endoplasmic reticulum (ER); noninvasive 2D and three-dimensional (3D) Raman spectroscopy mapping showed internalisation to this structure. Moreover, different cell lines were tested and these cell–substrate interactions were shown to be generic to different types of cells with no toxicity. This is of particular interest for research into cancer cells due to their overactive mechanical substrate interactions and modified unfolded protein response (UPR).

It is unclear whether it is the molybdenum or sulfur components which are the main target of the uptake mechanism. Certainly, the internalisation of monolayer transition metal dichalcogenides (TMDs) holds particular benefits. The monolayers are inherently fluorescent and have unique wavelength-dependent Raman modes which opens up potential applications such as tracking or live cell imaging. The differing band gaps of the TMDs could provide a way to co-stain samples if the materials localise to different cellular regions. Photothermal or drug delivery therapies could also potentially be made possible due to material localisation, so this new approach to nanomaterial–cell interfacing could provide an exciting opportunity to develop the next generation of theranostics.

One way to test which of the molybdenum or sulfur components had the biggest effect on the internalisation of MoS$_2$ was to conduct further experiments on other TMDs. This is discussed in Chapter 5. Two new materials, WS$_2$ and MoSe$_2$, were used to create similar Langmuir thin film substrates. The same cell lines were tested for these materials as for MoS$_2$, namely, 1BR primary fibroblasts and U2OS osteosarcoma cells. While a reasonable amount of WS$_2$ was seen to be internalised by the 1BR fibroblasts, less appeared to be internalised in the U2OS cells, and very little MoSe$_2$ appeared to be internalised into either cell line.
The results from these initial experiments show that this internalisation methodology, where material uptake is mediated by cell mechanotransduction, is not unique to MoS$_2$, but is also shown in WS$_2$. The fact that little to no MoSe$_2$ internalisation is seen may bolster the hypothesis that the sulfur chemistry is what plays a particularly important role in the internalisation process, rather than the molybdenum. Although there is little MoSe$_2$ uptake, the reasons for this are still unclear and require further exploration. To ensure that any material present is within the cells, the cells were trypsinised at 7 days and reseeded onto pristine glass cover slips. This leaves open the question of whether the MoSe$_2$ was never internalised at all, or whether it was rapidly internalised and excreted within the first 7 days. Moreover, several cells which had grown on MoSe$_2$ substrates were imaged with noticeable cell extensions (although there was some evidence of this in the cells from the WS$_2$ substrates, far fewer cells were observed with this behaviour). This indicates some strong substrate interactions; whether this behaviour is due to cells trying to locate areas without MoSe$_2$, avoiding a toxic material, or due to a rapid attachment process to the nanomaterial is still unclear.

This places emphasis on the need of further study into the mechanotransduction processes. Despite the limitations on this preliminary research, it has suggested that the sulfur chemistry may play the starring role in the uptake pathways associated with this internalisation methodology. Among other tests needed to complete the work presented here, full biological characterisation experiments are required to understand the system; this includes detailed cytotoxicity assays and investigation of the UPR effects for cells grown on MoS$_2$, WS$_2$, and MoSe$_2$ substrates. Additionally, repeat tests at a range of growth windows with WS$_2$ and MoSe$_2$ are required, and comparisons with other nanomaterials may also give insight into the processes at work.

Better understanding of these processes will be key to realising cell imaging and drug delivery possibilities. With uptake and localisation of monolayer TMDs, fluorescent or Raman imaging is possible. Furthermore, this material internalisation
occurs without fixation or permeabilisation of the cells, so there is no reason that live cell imaging would not be possible. For TMDs which show localisation, such as MoS$_2$, the creation of drug delivery vectors is promising. With the known chemistries of these materials, then for example, adding drug groups via simple sulfur chemistry should be straightforward. Some specificity of the drug delivery may even be possible, because uptake seems to differ slightly between cancerous and healthy cells.

Looking ahead, one avenue to explore could be the extension of these initial experiments into the realm of biosensors. TMDs have previously been shown to be capable of sensing bovine serum albumin by surface plasmon resonance [326,327] and of detecting dengue by electrochemical impedance spectroscopy [182]. This dengue biosensor is of particular interest as it also utilises the Langmuir deposition technique (although Solanki et al. use the Langmuir–Blodgett technique as opposed to the L–S technique used throughout this thesis). As mentioned in 2.4.2.3, the need to develop simple, cheap and sensitive biosensors has been brought into sharp relief over the past year since the advent of the ongoing COVID-19 pandemic. It seems possible that biosensors based on MoS$_2$, similar to that for dengue described by Solanki et al., could be developed towards the SARS-CoV-2 virus (or other viruses). This would be an interesting angle to explore in future work.
Bibliography


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[517] J. Huang, B. He, Z. Zhang, Y. Li, M. Kang, Y. Wang et al., 

Appendices

A Tables for Chapter 2

In Chapter 2, a review of low-dimensional materials in diagnostics and therapeutics for life sciences applications is presented. The background, relevant review papers and discussion of common materials is included in 2.4. Here are included tables of the papers reviewed by the author; relevant information is summarised for each application. A list of abbreviations is included for convenience.

List of applications being reviewed:

A.1 Fluorescence imaging. ...................................................... 185
A.2 Live cell imaging. ......................................................... 188
A.3 Biosensing. ................................................................. 192
A.4 Photothermal therapy. ................................................... 195
A.5 Drug delivery. .............................................................. 198
A.6 Stem cell control. .......................................................... 202
A.7 Toxicity studies. ............................................................ 207
A.8 Theranostics. ............................................................... 211
List of Abbreviations used in Appendix A:

| # | A | B | C | D | E | F | G | H | I | J | L | M | N | O | P | Q | R | S | T |
|   | U | V | W |

---

1D one-dimensional. 197
2D two-dimensional. 197, 212, 214
3D three-dimensional. 197
5-FU 5-fluorouracil. 187, 209

A

AA acrylic acid. 212
Ab antibody. 189, 192, 193, 194, 195
AFM atomic force microscope. 210
AgNP silver nanoparticle. 194, 198, 205
AI activity index. 207
AIV avian influenza virus. 192, 193
AMSC adipose-derived mesenchymal stem cell. 203, 204, 205
APTES (3-aminopropyl)triethoxysilane. 198
AuNC gold nanocluster. 186, 190, 196
AuNP gold nanoparticle. 185, 190, 192, 193, 194, 207, 212

B

BMP-2 bone morphogenetic protein 2. 202
BMSC bone marrow-derived mesenchymal stem cell. 202, 203, 204, 206
BP black phosphorus. 212, 214
BSA bovine serum albumin. 186, 192, 194, 213

C
calcein AM calcein acetoxyethyl. 208
CB carbon black. 186, 199, 207, 209
CCK-8 cell counting kit assay 8. 207
CD carbon dot. 185, 186, 188, 189, 198, 211
CD44 cluster of differentiation 44. 190, 196, 200, 213
CD63 cluster of differentiation 63. 196
CEA carcinoembryonic antigen. 194
CNT carbon nanotube. 190, 193, 198, 199, 200, 204, 205, 208, 209
CQD carbon quantum dot. 185, 188, 189, 198, 211
cRGD cyclo Arg-Gly-Asp (RGD) peptide. 212
CT computed tomography. 213
CuNC copper nanocluster. 190, 191
Cy7 cyanine 7. 195, 212
Cys cysteine. 185

D

DA dopamine. 185, 190, 193
DACHPt dichloro(1,2-diaminocyclohexane)platinum(II). 200
DCF dichlorofluorescein. 207
DCF-DA 2’,7’-dichlorofluorescein diacetate. 208
DCFH-DA 2’,7’-dichlorodihydrofluorescein diacetate. 208
DeK death kinetics. 207
DIZ diameter of inhibition zone. 207
DMEM Dulbecco’s modified Eagle’s medium. 209
DMSA meso-2,3-dimercaptosuccinic acid. 197
DNA deoxyribonucleic acid. 188, 189, 191, 193, 207, 208
DOX doxorubicin; doxorubicin hydrochloride. 195, 198, 199, 200, 201, 211, 212, 213, 214
DPPH 2,2-diphenyl-1-picrylhydrazyl. 207

**E**

ELISA enzyme-linked immunosorbent assay. 208

**F**

FeTMPyP Fe(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachlorideporphyrin pentachloride. 187

FITC fluorescein isothiocyanate. 186, 188

**G**

GO graphene oxide. 188, 192, 195, 196, 198, 202, 203, 207, 211, 212

GQD graphene quantum dot. 185, 189, 198, 211, 212

GrK growth kinetics. 207

GSH glutathione. 187, 189, 191

**H**

HA hyaluronic acid. 190, 196, 200, 201, 202, 213

HAP hydroxyapatite. 205, 206

Hb haemoglobin. 185, 207

HER2 human epidermal growth receptor 2. 186

HFW human fibroblast. 189, 208

HMVEC human microvascular endothelial cells. 196

hROS highly reactive oxygen species. 191

HSA human serum albumin. 199

hTERT human telomerase reverse transcriptase. 207
**HUVEC** human umbilical vein endothelial cell. 185, 195, 196, 213

**I**

**IGF-1R** insulin-like growth factor 1 receptor. 195

**IgG** immunoglobulin G. 193

**iRGD** a 9-amino acid cyclic peptide (sequence: CRGDKGPDC). 185

**J**

**JEV** Japanese encephalitis. 192

**L**

**LDH** lactate dehydrogenase. 209

**LED** light-emitting diode. 195

**Lys** lysine. 188

**M**

**MAA** methacrylic acid. 212

**MB** methylene blue. 193, 195, 196, 198

**MBA** methylenebisacrylamide. 212

**MBC** minimum bactericidal concentration. 207

**MDCK** Madin-Darby Canine Kidney cells. 195

**MI-1** 1-(4-Cl-benzyl)-3-Cl-4-(CF3-fenylamino)-1H-pyrrol-2,5-dione. 209

**MIC** minimum inhibition concentration. 207

**miRNA** micro RNA (ribonucleic acid). 188, 190, 191, 196

**MMP** mitochondrial membrane potential. 208

**MPA** mercaptopropionic acid. 186

**MRI** magnetic resonance imaging. 191, 211, 213, 214

**mRNA** messenger RNA (ribonucleic acid). 188
MRSA methicillin-resistant *Staphylococcus aureus*. 203
MSC mesenchymal stem cell. 202, 203, 204, 205, 206
MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium. 210
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. 207, 208, 210
MWCNT multi-walled carbon nanotube. 192, 193, 194, 195, 205, 207, 208
MXene transition metal carbide, nitride, or carbonitride. 194

N

ND nanodiamond. 189, 193, 198, 204
NHDF normal human dermal fibroblasts. 195
NIR near-infrared. 188, 189, 193, 195, 196, 197, 206, 212, 213, 214
NO nitric oxide. 208, 209
NP nanoparticle. 185, 187, 191, 192, 193, 195, 196, 198, 199, 202, 208, 210, 211, 213
NRP-1 neuropilin-1. 185
NRU neutral red uptake. 208
NSC neural stem cell. 204

O

OBNSC olfactory bulb neural stem cell. 205

P

PAI photoacoustic imaging. 212, 213, 214
PBMC peripheral blood mononuclear cell. 202
PCL polycaprolactone. 203
PDA polydopamine. 194, 195, 196, 213
PDMS polydimethylsiloxane. 193, 194
PDT photodynamic therapy. 211, 212, 214
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<td>PEG</td>
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<td>PEI</td>
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<td>PET</td>
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<td>PLGA</td>
<td>poly(lactic-co-glycolic acid). 201, 202, 211</td>
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<td>Q</td>
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<td>rGO</td>
<td>reduced graphene oxide. 192, 195, 203, 204, 207</td>
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<td>RNA</td>
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<td>TEM</td>
<td>transmission electron microscopy. 193</td>
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TMD transition metal dichalcogenide.  200

Tyr tyrosine.  207

U

UV ultraviolet.  187

V

VEGF vascular endothelial growth factor.  205

W

WHO World Health Organization.  208

WST water-soluble tetrazolium salt.  209, 210
Table A.1: Fluorescence imaging.

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<td>Nucleus</td>
<td>HeLa</td>
<td>[329]</td>
</tr>
<tr>
<td>CD–silica NP composites</td>
<td>(Phosphorescence-based rather than fluorescence)</td>
<td></td>
<td>EM-6</td>
<td>[330]</td>
</tr>
<tr>
<td>CQDs</td>
<td>Strong hydrophobic interactions</td>
<td>Hb</td>
<td>Human blood</td>
<td>[331]</td>
</tr>
<tr>
<td>Glucose modified CQDs</td>
<td>Glucose</td>
<td>Cytoplasm</td>
<td>HepG2; HL-7702</td>
<td>[332]</td>
</tr>
<tr>
<td>Gold conjugated CDs nano-assembly</td>
<td>Displacement of CDs from AuNP surface with the addition of Cys</td>
<td>Cys</td>
<td>HeLa</td>
<td>[333]</td>
</tr>
<tr>
<td>CDs (pork as carbon source)</td>
<td>Quenching due to surface absorption</td>
<td>Uric acid</td>
<td>Human serum and urine</td>
<td>[334]</td>
</tr>
<tr>
<td>CQDs (as an antipoisoning drug for heavy metal ions)</td>
<td></td>
<td>Mercury and chemet</td>
<td>Human serum</td>
<td>[335]</td>
</tr>
<tr>
<td>iRGD-decorated carbon nanodots</td>
<td>iRGD peptide</td>
<td>NRP-1 and αvβ3 integrin receptors</td>
<td>4T1; A2780</td>
<td>[336]</td>
</tr>
<tr>
<td>N-doped CDs</td>
<td>Formation of non-fluorescent ground state complex</td>
<td>Cysteamine</td>
<td>MDA-MB-231</td>
<td>[337]</td>
</tr>
<tr>
<td>N-doped CQDs</td>
<td>Tyrosinase</td>
<td>DA; alpha lipoic acid</td>
<td>Human blood and urine</td>
<td>[338]</td>
</tr>
<tr>
<td>CDs</td>
<td>N–H bonds; electronic transfer between CDs and DA</td>
<td>DA (accumulation in intracellular space)</td>
<td>CRL-5822; HUVEC</td>
<td>[339]</td>
</tr>
<tr>
<td>CDs</td>
<td>Reversible transformation between azo and quinone structures induced by protonation–deprotonation</td>
<td>H+ (pH sensor)</td>
<td>HeLa</td>
<td>[340]</td>
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</table>
Table A.1: Fluorescence imaging (continued).

<table>
<thead>
<tr>
<th>Material</th>
<th>Probe</th>
<th>Target</th>
<th>Cell lines/Animal models</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>CDs</td>
<td></td>
<td>Intracellular polarity (accumulate in lysosomes and mitochondria)</td>
<td>HepG2; HL-7702</td>
<td>[341]</td>
</tr>
<tr>
<td>CB and calcium carbonate incorporated with FITC–PEG 2-aminoethyl ether acetic acid solution</td>
<td></td>
<td>Cytoplasm periphery</td>
<td>A549</td>
<td>[342]</td>
</tr>
<tr>
<td>WS₂ QDs</td>
<td>Transformation of Fe²⁺/Fe³⁺ and the enzymatic reaction</td>
<td>Glucose</td>
<td>Human blood and serum</td>
<td>[343]</td>
</tr>
<tr>
<td>Aptamer@AuNCs and aptamer@CdTe QDs cell/tumour-targeting nanostructure</td>
<td></td>
<td>HER2</td>
<td>SK-BR-3; MCF-7 (control)</td>
<td>[344]</td>
</tr>
<tr>
<td>AuNCs/N-doped CDs nanohybrids</td>
<td>Cu²⁺</td>
<td>HepG2</td>
<td></td>
<td>[345]</td>
</tr>
<tr>
<td>AuNC embedded in BSA (BSA–AuNCs)</td>
<td>(Interaction between BSA and flavonoids, e.g. aggregation of AuNCs)</td>
<td>Flavonoids (quercetin, apigenin, nobiletin, baicalein, rutin, and wogonin)</td>
<td>Serum, plasma, proprietary Chinese medicine Rutin Tablets</td>
<td>[346]</td>
</tr>
<tr>
<td>BSA/3-MPA co-modified AuNCs</td>
<td>Quenched by Fe³⁺ due to strong electron transfer ability</td>
<td>Alkaline phosphatase</td>
<td>Saos-2</td>
<td>[347]</td>
</tr>
<tr>
<td>MPA capped CdTe QDs</td>
<td>Quenched (“turned off”) by Fe³⁺, then “turned on” by ascorbic acid</td>
<td>Ascorbic acid</td>
<td>Human plasma</td>
<td>[348]</td>
</tr>
<tr>
<td>Dual-mode probes based on mesoporous silica nanomaterials doped with an aggregation-induced emission dye and Gd³⁺</td>
<td></td>
<td>Accumulation in cytoplasm</td>
<td>L929; DU149</td>
<td>[349]</td>
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Table A.1: Fluorescence imaging (continued).

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<thead>
<tr>
<th>Material</th>
<th>Probe</th>
<th>Target</th>
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<tbody>
<tr>
<td>Coumarin-decorated cationic copolymer functionalised mesoporous silica NPs</td>
<td>5-FU chemotherapeutic agent; p53 tumour suppressor gene (UV-light cross-linked and pH de-cross-linked)</td>
<td>H⁺ (triggered by acidic environment)</td>
<td>MCF-7; COS-7</td>
<td>[350]</td>
</tr>
<tr>
<td>Oxidised, porous graphitic carbon nitride nanosheets (CNNSs)</td>
<td>Fluorescence quenching of CNNSs due to overlap of FeTMBPyP absorbance band and CNNSs emission band; quenched fluorescence “turned on” in response to H₂O₂ (caused by decomposition of FeTMBPyP)</td>
<td>H₂O₂</td>
<td>RAW 264.7</td>
<td>[351]</td>
</tr>
<tr>
<td>Manganese-fullerenes core–shell nanocomposites</td>
<td>Collapse of the outer MnO₂ shell leading to reconstruction of the nanoprobes after exposure to GSH</td>
<td>GSH</td>
<td>HeLa</td>
<td>[352]</td>
</tr>
<tr>
<td>Dual QDs-based fluorescence-linked immunosorbent assay</td>
<td>Amphiphilic oligomers (PMAH)</td>
<td>Inflammation biomarkers (serum amyloid A and C-reactive protein)</td>
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<td>[353]</td>
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Table A.2: Live cell imaging.

<table>
<thead>
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<th>Material</th>
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<th>Target</th>
<th>Cell lines/Animal models</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>GO/Fe$_3$O$_4$ hybrids with functionalised polymer</td>
<td>FITC</td>
<td>Fluorescence</td>
<td>BxPC-3</td>
<td>[354]</td>
<td></td>
</tr>
<tr>
<td>Ternary complex of GO, Al ions, and alizarin red (GO–Al–AR)</td>
<td></td>
<td>Fluorescence</td>
<td>Lys</td>
<td>Human retinal pigment epithelium</td>
<td>[355]</td>
</tr>
<tr>
<td>Multiple-targeted GO nanocarrier</td>
<td>Manganese superoxide dismutase (Mn-SOD); β-actin; control DNA with random sequence</td>
<td>Fluorescence</td>
<td>Intracellular mRNA</td>
<td>MDA-MB-231</td>
<td>[356]</td>
</tr>
<tr>
<td>N-doped CDs</td>
<td></td>
<td>Fluorescence</td>
<td>Fe$^{3+}$</td>
<td>HeLa</td>
<td>[357]</td>
</tr>
<tr>
<td>Fluorine and nitrogen co-doped CDs</td>
<td></td>
<td>Fluorescence/STED nanoscopy</td>
<td>Nucleolus; tunnelling nanotubes</td>
<td>4T1</td>
<td>[358]</td>
</tr>
<tr>
<td>Carbon nanodots</td>
<td>Hairpin-structured/amo group modified DNA</td>
<td>Fluorescence</td>
<td>miRNA</td>
<td></td>
<td>[359]</td>
</tr>
<tr>
<td>CDs</td>
<td></td>
<td>Fluorescence</td>
<td></td>
<td>HeLa</td>
<td>[360]</td>
</tr>
<tr>
<td>NIR emissive CDs</td>
<td></td>
<td>Fluorescence</td>
<td>Fe$^{3+}$; accumulation in cytoplasm</td>
<td>AGS; K562; MGC-803</td>
<td>[361]</td>
</tr>
<tr>
<td>N-doped carbon nanodots</td>
<td>Carboxyl/hydroxyl or nitrogen functional groups</td>
<td>Fluorescence</td>
<td>Fe$^{3+}$</td>
<td>HCT-116</td>
<td>[362]</td>
</tr>
<tr>
<td>N-doped CDs</td>
<td>Carboxyl/hydroxyl/amine functional groups</td>
<td>Fluorescence</td>
<td>Au$^{3+}$</td>
<td>A549</td>
<td>[363]</td>
</tr>
<tr>
<td>N-rich metal-free and metal doped CQDs</td>
<td>Phenolic hydroxyl groups</td>
<td>Fluorescence</td>
<td>Fe$^{3+}$</td>
<td>HeLa</td>
<td>[364]</td>
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</table>
Table A.2: Live cell imaging (continued).

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<tr>
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<tr>
<td>N-doped CDs</td>
<td>Hydroxyl/amine groups</td>
<td>Fluorescence</td>
<td>Fe&lt;sup&gt;3+&lt;/sup&gt;</td>
<td><em>C. albicans</em>; Clone 9 hepatocytes</td>
<td>[365]</td>
</tr>
<tr>
<td>N-doped CQDs</td>
<td></td>
<td>Fluorescence</td>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt;; GSH</td>
<td>MCF-7</td>
<td>[366]</td>
</tr>
<tr>
<td>N,P-dual doped CDs (A-NPCDs)</td>
<td>Nitrogen, phosphorus and oxygen containing functional groups [for Hg(II) target]; broad absorption band overlapping the emission and excitation spectrum of A-NPCDs [for Cr(VI) target]</td>
<td>Fluorescence</td>
<td>Hg(II); Cr(VI)</td>
<td>HEK-293</td>
<td>[367]</td>
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<tr>
<td>N,S co-doped aminated ligninsulfonate/GQDs</td>
<td>Coordination between sulfonic acid group and Ag&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Fluorescence</td>
<td>Ag&lt;sup&gt;+&lt;/sup&gt;</td>
<td>A549</td>
<td>[368]</td>
</tr>
<tr>
<td>Alkyne-functionalised hyperbranched-polyglycerol-coated fluorescent NDs</td>
<td></td>
<td>Fluorescence</td>
<td>Azide-modified membrane proteins; azide-modified Abs of membrane proteins</td>
<td>HeLa; HFW</td>
<td>[369]</td>
</tr>
<tr>
<td>Oligonucleotide-functionalised DNA–SWCNTs</td>
<td>(GT)&lt;sub&gt;n&lt;/sub&gt; oligonucleotides (observed intracellular fluorescence intensities shown to increase with increasing oligonucleotide length); confocal Raman spectroscopy</td>
<td>Visible and NIR fluorescence</td>
<td>RAW 264.7</td>
<td>[370]</td>
<td></td>
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</table>
Table A.2: Live cell imaging (continued).

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>Prussian blue (PB) microcubes-decorated graphenated CNTs (g-CNTs)-modified electrode</td>
<td>Polycrystalline structure of PB allows small H$_2$O$_2$ molecules to pass through but screens other species such as DA and ascorbic acid; negatively charged surface of g-CNTs able to repel negatively charged ascorbate anions</td>
<td>Amperometric experiment</td>
<td>H$_2$O$_2$</td>
<td>RAW 264.7</td>
<td>[371]</td>
</tr>
<tr>
<td>C$_{60}$-poly(aminio acid) composites</td>
<td>5'-fluorophore (FAM)-labelled ssDNA probes</td>
<td>Fluorescence</td>
<td>miRNA-21</td>
<td>CD44-positive colorectal cancer</td>
<td>[372]</td>
</tr>
<tr>
<td>HA-coated gold nanodot-decorated hollow carbon nanospheres</td>
<td>Functionalised hairpin nucleic acid, Cy3 fluorophore</td>
<td>Fluorescence</td>
<td>RNA-induced silencing complex (RISC)</td>
<td>A549; HeLa; MCF-7</td>
<td>[373]</td>
</tr>
<tr>
<td>AuNP-based probe</td>
<td>Highly metallophilic Hg$^{2+}$–Au$^+$ interactions leading to a significant change of conjugate electron structure in the Hg–Au complex</td>
<td>Fluorescence</td>
<td>Hg$^{2+}$</td>
<td>NIH-3T3</td>
<td>[374]</td>
</tr>
<tr>
<td>Peptide–gold nanoclusters</td>
<td>COO$^-$ groups on CuNCs@GC surface; Zn$^{2+}$ acting as a crosslinker to re-aggregate dispersed nanoclusters</td>
<td>Photoluminescence</td>
<td>Zn$^{2+}$</td>
<td>A549</td>
<td>[375]</td>
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</table>
Table A.2: Live cell imaging (continued).

<table>
<thead>
<tr>
<th>Material</th>
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<th>Cell lines/Animal models</th>
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<tr>
<td>GSH-coated CuNCs</td>
<td></td>
<td>Fluorescence</td>
<td>H(^+) (pH sensor)</td>
<td>HeLa; L-132</td>
<td>[378]</td>
</tr>
<tr>
<td>CuInS(_2)/ZnS QDs</td>
<td>Biotin-binding protein traptavidin</td>
<td>Fluorescence</td>
<td>Biotinylated cell surface</td>
<td>MCF-7</td>
<td>[379]</td>
</tr>
<tr>
<td>Benzoperylene probe–ZnS QDs nanocomposite</td>
<td></td>
<td>Photoluminescence</td>
<td>hROS (ONOO(^-), ·OH, H(_2)O(_2), O(_2)^{−}, ClO(^-))</td>
<td>HeLa</td>
<td>[380]</td>
</tr>
<tr>
<td>Core–shell QDs containing environmentally-benign transition metal ion Mn(II)-doped ZnS as a core material encapsulated within different thickness ZnS shell layers</td>
<td></td>
<td>Fluorescence</td>
<td></td>
<td>HeLa, HEK-293; Hg(^{2+}), Pb(^{2+})</td>
<td>[381]</td>
</tr>
<tr>
<td>ZnO NPs</td>
<td></td>
<td>Fluorescence</td>
<td></td>
<td>T47D</td>
<td>[382]</td>
</tr>
<tr>
<td>ZnO NPs</td>
<td>Hairpin-structured DNA</td>
<td>Fluorescence</td>
<td>miRNA-21; miRNA-373</td>
<td>HeLa; HepG-2; L-O2</td>
<td>[383]</td>
</tr>
<tr>
<td>Gadolinium cluster encapsulated metallofullerene Gd(<em>3)N@C(</em>{80})</td>
<td>MRI probe [IL-13-TAMRA-Gd(<em>3)N@C(</em>{80})(OH)(_{30})- (CH(_2)CH(<em>2)COOH)(</em>{20})]</td>
<td>Fluorescence</td>
<td>Overexpressed IL-13Rz2 receptors</td>
<td>RAW 264.7</td>
<td>[384]</td>
</tr>
<tr>
<td>Gadolinium phosphate (GdPO(_4)) nanoprisms doped with lanthanide (Eu(^{3+}), Tb(^{3+})) ions</td>
<td></td>
<td>Fluorescence</td>
<td></td>
<td>HeLa; L-O2</td>
<td>[385]</td>
</tr>
</tbody>
</table>
Table A.3: Biosensing.

<table>
<thead>
<tr>
<th>Material</th>
<th>Probe</th>
<th>Detection method</th>
<th>Target</th>
<th>Cell lines/Animal models</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Synthetic and biomolecular functionalised graphene-based sensing array</td>
<td>Functionalised graphene nanocomposites</td>
<td>Ultrasensitive electrochemical impedance signal (electrostatic and hydrophobic interactions between probe and target)</td>
<td>Phospholipids, membrane proteins and carbohydrates of the cell surface</td>
<td>A549; HeLa; HepG2; K562; MCF-7; MDA-MB-231; MCF-7/ADR; NIH-3T3; PC-12; HEK-293T; PBMCs</td>
<td>[386]</td>
</tr>
<tr>
<td>Carboxy functionalised graphene on Si/SiO$_2$ substrate</td>
<td>Amine functionalised Abs (anti-JEV for JEV and anti-AIV for AIV) covalently coupled with carboxyl activated graphene</td>
<td>Field-effect transistor-based (monitoring the change in resistance of the graphene channel as a function of time)</td>
<td>Monoclonal Abs of JEV and AIV</td>
<td>JEV; AIV</td>
<td>[387]</td>
</tr>
<tr>
<td>Monolayer nanosheets of graphene- and WS$_2$-coated commercial Au chips</td>
<td>BSA</td>
<td>SPR</td>
<td>Anti-BSA</td>
<td></td>
<td>[327]</td>
</tr>
<tr>
<td>ZnO nanowires and graphene nanoplates synthesis on an Au interdigitated electrode</td>
<td></td>
<td>Resistivity-based</td>
<td>Glucose</td>
<td></td>
<td>[388]</td>
</tr>
<tr>
<td>Cuprous oxide (Cu$_2$O) NP and acid-functionalised GO composite</td>
<td></td>
<td>Cyclic voltammetry; amperometry</td>
<td>8-hydroxydeoxyguanosine (8-OHdG)</td>
<td>Biological fluids (blood serum and urine)</td>
<td>[389]</td>
</tr>
<tr>
<td>Amine-terminated MWCNTs–rGO–polyaniline–AuNPs modified screen-printed carbon electrode</td>
<td></td>
<td>Cyclic voltammetry; amperometry</td>
<td>Glucose</td>
<td>Human blood serum samples</td>
<td>[390]</td>
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</table>
Table A.3: Biosensing (continued).

<table>
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<tr>
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<th>Target</th>
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<tbody>
<tr>
<td>Single-chirality DNA–CNT complex (CNTs noncovalently functionalised with specific ssDNA oligonucleotides)</td>
<td>Lipid binding to the ss(GT)6-(8,6) complex reduces the water density near the nanotube surface, thereby lowering the effective local solvent dielectric; the lower dielectric environment corresponds to a blue-shift of the nanotube emission wavelength</td>
<td>NIR hyperspectral microscopy</td>
<td>Lumen of endolysosomal organelles</td>
<td>Fibroblasts from a Niemann–Pick type C patient</td>
<td>[391]</td>
</tr>
<tr>
<td>Molybdenum NPs self-supported functionalised MWCNTs (MoNPs@f-MWCNTs)-based core–shell hybrid nanomaterial</td>
<td></td>
<td>Electrochemical</td>
<td>DA</td>
<td>Rat brain, human blood serum, and dopamine hydrochloride injection samples</td>
<td>[392]</td>
</tr>
<tr>
<td>Magnetised CNT-based lateral flow strip biosensor</td>
<td>Ab-modified magnetised CNTs</td>
<td>Visual detection</td>
<td>Rabbit IgG</td>
<td>Whole blood</td>
<td>[393]</td>
</tr>
<tr>
<td>Patterned nano-arrays of carbon nanofibers forming a nanosensor–cell construct</td>
<td>Maltotriose-conjugated polypropylenimine dendrimers</td>
<td>Electrochemical</td>
<td>MB (testing access to cytoplasm)</td>
<td>RAW 264.7</td>
<td>[394]</td>
</tr>
<tr>
<td>Ab-conjugated NDs</td>
<td>DNA detection probes with single-stranded sticky end for ssDNA detection</td>
<td>TEM; fluorescence</td>
<td>Nuclear pore complex</td>
<td>HeLa</td>
<td>[395]</td>
</tr>
<tr>
<td>Uniform Pd nanosheets</td>
<td></td>
<td>Fluorescence</td>
<td>Single-stranded circulating tumour DNA (ctDNA)</td>
<td></td>
<td>[396]</td>
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<td>PDMS herringbone structures made with a ZnO nanorod template</td>
<td>Monoclonal Ab against the hemagglutinin of H5 AIV</td>
<td>AuNP-based colorimetric detection with a silver enhancer</td>
<td>Hemagglutinin of H5 AIV</td>
<td>AIV</td>
<td>[397]</td>
</tr>
<tr>
<td>Material</td>
<td>Probe</td>
<td>Detection method</td>
<td>Target</td>
<td>Cell lines/Animal models</td>
<td>Ref.</td>
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<tr>
<td>Gold-coated fiber modified with MoS$_2$ nanosheets followed by</td>
<td>Anti-BSA Abs</td>
<td>SPR</td>
<td>BSA protein</td>
<td></td>
<td>[326]</td>
</tr>
<tr>
<td>bio-functionalisation with anti-BSA Abs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ti$_3$C$_2$ MXene-based sensing platform and MWCNTs–PDA–AgNPs signal</td>
<td>Monoclonal anti-CEA antibody (Ab$_1$) through its Fc region</td>
<td>SPR</td>
<td>CEA</td>
<td></td>
<td>[398]</td>
</tr>
<tr>
<td>enhancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AuNP–PDMS composite film</td>
<td>Direct assay and sandwich assay</td>
<td></td>
<td>BSA</td>
<td></td>
<td>[399]</td>
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</table>

Table A.3: Biosensing (continued).
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<tr>
<th>Material</th>
<th>Probe</th>
<th>Method</th>
<th>Target</th>
<th>Cell lines/Animal models</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positively charged graphene/Fe₃O₄/PEI nanocomposite</td>
<td>(Positive surface charge increased cellular uptake)</td>
<td>808 nm laser irradiation</td>
<td>MCF-7; U14; mice</td>
<td>[400]</td>
<td></td>
</tr>
<tr>
<td>GO–MB nanocomposite</td>
<td>Red LED irradiation</td>
<td>MDA-MB-231</td>
<td>[401]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO functionalised with an amphiphilic polymer containing [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (SBMA) brushes and loaded with IR780</td>
<td>808 nm NIR laser irradiation</td>
<td>MCF-7; NHDF</td>
<td>[402]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rGO–PEG–folic acid functional nanomaterials</td>
<td>NIR laser irradiation</td>
<td>MDA-MB-231; HUVEC</td>
<td>[403]</td>
<td></td>
<td></td>
</tr>
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<td>PDA-functionalised rGO nanosheets and bimetallic AuPd NP composites</td>
<td>915 nm NIR laser irradiation</td>
<td>MDA-MB-231; L929</td>
<td>[404]</td>
<td></td>
<td></td>
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<tr>
<td>rGO functionalised with oxidised polyphenols</td>
<td>808 nm NIR laser irradiation</td>
<td>A549; MDCK</td>
<td>[405]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy7 dye-conjugated SWCNTs bound with targeting IGF-1R Abs</td>
<td>Targeting IGF-1R Ab</td>
<td>785 nm NIR laser irradiation</td>
<td>BXPC-3; PANC-1; ASPC-1; SW1990; BALB/c male mice</td>
<td>[406]</td>
<td></td>
</tr>
<tr>
<td>TAT-chitosan functionalised MWCNTs-based drug delivery system for DOX</td>
<td>NIR irradiation</td>
<td>DOX entered cell nucleus; MWCNTs remained in cytoplasm</td>
<td>Bel7402</td>
<td>[407]</td>
<td></td>
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<tr>
<td>Fluorescent-labelled fullerene C₆₀</td>
<td>Visible light irradiation</td>
<td>Jurkat; L1210</td>
<td>[408]</td>
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Table A.4: Photothermal therapy (continued).

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<th>Target</th>
<th>Cell lines/Animal models</th>
<th>Ref.</th>
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<tr>
<td>Water soluble malonodiserinolamide [60]fullerene (C$_{60}$-ser)</td>
<td></td>
<td>Radiofrequency exposure</td>
<td>(Smaller cells more susceptible)</td>
<td>HUVEC; HMVEC; primary dermal fibroblast; L939; HeLa; 4T1</td>
<td>[409]</td>
</tr>
<tr>
<td>conjugated to the surface of mesoporous silica NPs (SiO$<em>2$–C$</em>{60}$)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HA-modified SWCNTs, GO, and fullerene</td>
<td></td>
<td>Visible light irradiation</td>
<td>CD44 receptors</td>
<td>MCF-7; female KM mice</td>
<td>[410]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gold nanocages</td>
<td></td>
<td>Acoustic radiation; 780 nm</td>
<td></td>
<td>MDA-MB-231</td>
<td>[411]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>laser irradiation</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Core–shell multifunctional nanomaterial-based all-in-one nanoparticle</td>
<td>mucin1 (MUC1) aptamer; ssDNA</td>
<td>808 nm NIR laser irradiation</td>
<td>mucin1 (MUC1); miRNA-21</td>
<td>MCF-7; HepG2; L02</td>
<td>[412]</td>
</tr>
<tr>
<td>composed of gold nanobipyramids@PDA and AuNCs</td>
<td></td>
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<td></td>
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<tr>
<td>MB photosensitisser-trapped AuNCs</td>
<td></td>
<td>Dual light irradiation</td>
<td></td>
<td>CT26; female BALB/c mice</td>
<td>[413]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(660 nm for MB, 808 nm for gold)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Exosomes mildly functionalised by integration with aptamers and Au</td>
<td>aptamer 1 (comprising the HepG2-recognition aptamer (TLS11a) and CD63-</td>
<td>808 nm diode NIR laser</td>
<td></td>
<td>HepG2; MCF-7; A549; HCT116; L02</td>
<td>[414]</td>
</tr>
<tr>
<td>nanorods to assemble Apt–Exos–AuNRs combination</td>
<td>recognition aptamer) and aptamer 2 (comprising the nucleolin-recognition</td>
<td>irradiation</td>
<td></td>
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<tr>
<td></td>
<td>aptamer (AS1411) and CD63-recognition aptamer)</td>
<td></td>
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<tr>
<td>Gold–curcumin nanostructure</td>
<td></td>
<td>808 nm NIR laser irradiation; 650 nm laser irradiation</td>
<td></td>
<td>4T1</td>
<td>[415]</td>
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<tr>
<td>Material</td>
<td>Probe</td>
<td>Method</td>
<td>Target</td>
<td>Cell lines/Animal models</td>
<td>Ref.</td>
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<tr>
<td>Au/MnO₂ nanocomposite</td>
<td></td>
<td>808 nm NIR laser irradiation</td>
<td>C540 (B16/F10); male BALB/c mice</td>
<td>[416]</td>
<td></td>
</tr>
<tr>
<td>DMSA-modified iron oxide-decorated MoS₂ nanosheets with double PEGylation</td>
<td></td>
<td>808 nm laser irradiation</td>
<td>4T1; RAW 264.7; BALB/c mice</td>
<td>[417]</td>
<td></td>
</tr>
<tr>
<td>Ultrasmall single-layer MoS₂ nanodots with different phases</td>
<td></td>
<td>1064 nm NIR-II laser irradiation</td>
<td>A549; HeLa</td>
<td>[418]</td>
<td></td>
</tr>
<tr>
<td>(1T-MoS₂ nanodots more effective than 2H-MoS₂ nanodots)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>TaO₂ deposited onto chitosan-coated MoS₂ nanosheet</td>
<td></td>
<td>808 nm NIR laser irradiation</td>
<td>MCF-7; HBL-1000</td>
<td>[419]</td>
<td></td>
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<tr>
<td>Monocomponent hollow Pt nanoframe (“Pt spirals”), whose superstructure is assembled with three levels (3D frame, 2D layered shells, and 1D nanowires)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Silica nanostructures on Cu₉S₅ core–shell nanostructures</td>
<td></td>
<td>808 nm NIR laser irradiation</td>
<td>EC109; TE8</td>
<td>[421]</td>
<td></td>
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<tr>
<td>Bismuth sulfide (Bi₂S₃) nanorods with retractable zinc protoporphyrin IX molecules</td>
<td>Zinc protoporphyrin IX</td>
<td>808 nm NIR laser irradiation</td>
<td>oxygenase-1 (overexpressed in cancer cells)</td>
<td>4T1</td>
<td>[422]</td>
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</table>
Table A.5: Drug delivery.

<table>
<thead>
<tr>
<th>Material</th>
<th>Probe</th>
<th>Drug</th>
<th>Target</th>
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<tr>
<td>Nano GO</td>
<td>MB</td>
<td>Cell cytoplasm</td>
<td>NIH-3T3</td>
<td></td>
<td>[423]</td>
</tr>
<tr>
<td>Polyacrylate-grafted GO</td>
<td>“Albumin” surface terminations</td>
<td>DOX</td>
<td>SH-SY5Y</td>
<td></td>
<td>[424]</td>
</tr>
<tr>
<td>PEG-modified GO and cobalt NP composites</td>
<td></td>
<td>DOX</td>
<td>L929; 3T3; MG63</td>
<td></td>
<td>[425]</td>
</tr>
<tr>
<td>GO–AgNP nanocomposites</td>
<td></td>
<td>Cisplatin</td>
<td>HeLa</td>
<td></td>
<td>[426]</td>
</tr>
<tr>
<td>Drug-conjugated CQDs</td>
<td></td>
<td>Metronidazole</td>
<td>P. gingivalis</td>
<td></td>
<td>[427]</td>
</tr>
<tr>
<td>GQDs-grafted dextran/glycol-polymeric hydrogel</td>
<td>Charge-reversal APTES</td>
<td>Buprenorphine</td>
<td>Stromal cells (noteworthy infiltration)</td>
<td>L929 (mouse fibroblast); female SD rats with chronic contraction injury</td>
<td>[428]</td>
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<tr>
<td>GQDs</td>
<td></td>
<td>DOX</td>
<td>Nucleus</td>
<td>MDA-MB-231</td>
<td>[429]</td>
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<tr>
<td>Nitrogen and sulfur co-doped CDs</td>
<td></td>
<td>Mitoxantrone</td>
<td>Cytoplasm</td>
<td>HeLa</td>
<td>[430]</td>
</tr>
<tr>
<td>ND-based composites</td>
<td></td>
<td>DOX</td>
<td>Cytoplasm mainly, some cell nuclei</td>
<td>HepG2</td>
<td>[431]</td>
</tr>
<tr>
<td>DOX conjugated with NDs</td>
<td></td>
<td>DOX</td>
<td>Human glioblastoma</td>
<td></td>
<td>[432]</td>
</tr>
<tr>
<td>Carbon nanofibers; CNTs</td>
<td></td>
<td>Carboplatin; cisplatin</td>
<td>DU-145; PC-3; EJ28</td>
<td></td>
<td>[433]</td>
</tr>
<tr>
<td>Smart freestanding CNTs with hydrophilic core and chitosan surface functionalisation</td>
<td></td>
<td>DOX</td>
<td>MDA-MB-231 TXSA; human foreskin fibroblast</td>
<td></td>
<td>[434]</td>
</tr>
<tr>
<td>Material</td>
<td>Probe</td>
<td>Drug</td>
<td>Target</td>
<td>Cell lines/Animal models</td>
<td>Ref.</td>
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<tr>
<td>PEG-coated CNTs</td>
<td></td>
<td>ABT737 nanodrug</td>
<td>Mitochondria (via early endosomes)</td>
<td>A549; NHFB</td>
<td>[435]</td>
</tr>
<tr>
<td>C₆₀ fullerene</td>
<td></td>
<td>Cisplatin</td>
<td>Three principal plasma membrane transporters involved in the efflux of anticancer drugs (P-gp, MRP-1, and MRP-2)</td>
<td>Human isogenic p54-null, Bax-null, and wild-type HCT-116; HeLa; human Jurkat T-leukaemia; KB-3-1 and KBC-1; HL-60/adr; HL-60/vinc; Lewis lung carcinoma (LLC) C57BL/6J male mice</td>
<td>[436]</td>
</tr>
<tr>
<td>C₆₀ fullerene and HL1, HL2</td>
<td></td>
<td>HL1 (dimorfolido-N-trichloroacetylphosphorylamide); HL2 (dimorfolido-N-benzoylphosphorylamide)</td>
<td></td>
<td>Human Jurkat T-leukaemia; Molt-16; CCRF-CEM</td>
<td>[437]</td>
</tr>
<tr>
<td>Glycine-tethered C₆₀ fullerenes conjugated with N-desmethyl tamoxifen</td>
<td></td>
<td>Tamoxifen</td>
<td></td>
<td>MCF-7</td>
<td>[438]</td>
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<tr>
<td>C₆₀ fullerene</td>
<td></td>
<td>DOX, cisplatin and landomycin A</td>
<td></td>
<td></td>
<td>[439]</td>
</tr>
<tr>
<td>HSA attached to pore openings of mesoporous carbon NPs surface-modified with PEG</td>
<td></td>
<td>DOX</td>
<td>Cytoplasm</td>
<td>4T1; Female BALB/c mice</td>
<td>[440]</td>
</tr>
<tr>
<td>CB</td>
<td>Acoustic waves</td>
<td>Calcein</td>
<td>Cell membrane</td>
<td>DU145</td>
<td>[441]</td>
</tr>
<tr>
<td>Oxidised carbon nanohorns</td>
<td></td>
<td>Meso-tetra(4-N-methylpyridyl) free base (H₂TMPyP₄) porphyrin; platinum (Pt₇TMPyP₄) porphyrin</td>
<td></td>
<td>HeLa</td>
<td>[442]</td>
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Table A.5: Drug delivery (continued).

<table>
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<tr>
<th>Material</th>
<th>Probe</th>
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<th>Target</th>
<th>Cell lines/Animal models</th>
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<tr>
<td>Oxidised and unoxidised carbon nanohorns; CNTs</td>
<td></td>
<td>Minocycline</td>
<td></td>
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<td>[443]</td>
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<td>Non-covalently functionalised carbon nano-onions functionalised with a</td>
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<td></td>
<td>Cells overexpressing CD44; digestive tract of the zebrafish larvae</td>
<td>MDA-MB-231 (overexpressing CD44); A2780 (control); adult zebrafish</td>
<td>[444]</td>
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<td>HA–phospholipid conjugate (CNO:f-HA–DMPE)</td>
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<tr>
<td>MoS$_2$ nanosheets functionalised with lipoic acid-modified PEG</td>
<td></td>
<td>DOX; SN-38; chlorine e6</td>
<td>Folic acid</td>
<td>HeLa; KB</td>
<td>[107]</td>
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<tr>
<td>Single-layer MoS$_2$ nanosheets decorated with chitosan</td>
<td></td>
<td>DOX</td>
<td></td>
<td></td>
<td>[108]</td>
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<td>TMDs and phosphocholine liposomes hybrids</td>
<td></td>
<td>DOX; calcein</td>
<td></td>
<td>HeLa</td>
<td>[287]</td>
</tr>
<tr>
<td>Nano-radiogold-decorated composite bioparticles</td>
<td></td>
<td>DOX</td>
<td></td>
<td></td>
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<tr>
<td>Platinum(II) drug-loaded gold nanoshells</td>
<td></td>
<td>DACHPt</td>
<td>Cytoplasm; endo/lysosomes</td>
<td>HCT116; HT29; LS174T</td>
<td>[445]</td>
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<tr>
<td>Rodlike mesoporous silica coated with gold nanoshells, modified with</td>
<td></td>
<td>Ultrasmall gadolinium-chelated supramolecular photosensitizers</td>
<td></td>
<td></td>
<td>[446]</td>
</tr>
<tr>
<td>ultrasmall gadolinium-chelated supramolecular photosensitisers</td>
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<td>Layered zinc hydroxide</td>
<td></td>
<td>Naproxen</td>
<td></td>
<td>HFFF2</td>
<td>[448]</td>
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Table A.5: Drug delivery (continued).

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<th>Material</th>
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<th>Target</th>
<th>Cell lines/Animal models</th>
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<tr>
<td>Hybrid inorganic–organic nanocolloids electrostatically conjugated with DOX</td>
<td></td>
<td>DOX</td>
<td>Cytosol; nucleus</td>
<td>Glioblastoma</td>
<td>[449]</td>
</tr>
<tr>
<td>Nanofibrous silica microparticles/polymer hybrid aerogels</td>
<td></td>
<td>Camptothecin</td>
<td></td>
<td>HeLa; SiHa; C33A; HaCaT; L929 cells, ATCC® CCL1; Vero, ATCC® CCL81</td>
<td>[450]</td>
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<tr>
<td>Hydroxy boron nitride nanosheets and Pd nanohybrids</td>
<td></td>
<td>DOX</td>
<td>Cytoplasm; nucleus</td>
<td>MCF-7; female BALB/c mice</td>
<td>[451]</td>
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<tr>
<td>Silver sulfide QDs</td>
<td></td>
<td>^14C metformin</td>
<td></td>
<td>SK-Hep-1; HEK293T; male C57/Bl6 mice</td>
<td>[452]</td>
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<tr>
<td>Copper oxide QDs-coated HA/PLGA</td>
<td></td>
<td>Piperine</td>
<td></td>
<td>hCMEC/D3; male Wistar rats</td>
<td>[453]</td>
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Table A.6: Stem cell control.

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<tr>
<th>Material</th>
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<th>Key conclusions</th>
<th>Ref.</th>
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<tr>
<td>Graphene substrates with controlled domain size</td>
<td>Human MSCs</td>
<td>Upregulated neuronal differentiation of human MSCs on small domain graphene</td>
<td>[454]</td>
<td></td>
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<tr>
<td>Pristine GO nanosheets</td>
<td>Rat BMSCs</td>
<td>Pristine GO nanosheets at a concentration of 0.1 µg/mL provide benefits to promote BMSCs proliferation and osteogenesis under a sequential-seeding method</td>
<td>[455]</td>
<td></td>
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<tr>
<td>GO; BMP-2–GO</td>
<td>Rat bone marrow stem cells and chondrocytes; osteoarthritic rats</td>
<td>GO may be potentially used to control the release of carrier materials; BMP-2–GO slowed the progression of NF-κB-activated degenerative changes in osteoarthritis</td>
<td>[456]</td>
<td></td>
</tr>
<tr>
<td>A nonviral vector based on Fe3O4 NP-decorated GO complexed with PEI (GO–Fe3O4–PEI complexes)</td>
<td>Three individual episomal plasmids (pCXLEhOCT3/4-shp53, pCXLE-hSK, and pCXLE-hUL) encoding pluripotent-related factors of Oct3/4, shRNA against p53, Sox2, Klf4, L-Myc, and Lin28</td>
<td>Human PBMCs</td>
<td>Combined effect of magnetic targeting and photothermal stimulation promoted the transfection efficiency of suspension cells; transfected cells show positive expression of the pluripotency markers and have potential to differentiate into mesoderm and ectoderm cells; GO–Fe3O4–PEI complex provides a safe, convenient, and efficient tool for reprogramming PBMCs into partially induced PSCs able to rapidly transdifferentiate into mesodermal lineages without full reprogramming</td>
<td>[457]</td>
</tr>
<tr>
<td>Phase-engineered GO</td>
<td>Human BMSCs</td>
<td>Transformation results in clustering of oxygen atoms on the GO surface, greatly improving its ability toward substance adherence; results in enhanced differentiation of human BMSCs toward the osteogenic lineage</td>
<td>[458]</td>
<td></td>
</tr>
<tr>
<td>BMP-2 immobilised on GO-incorporated PLGA/HA biodegradable microcarriers</td>
<td>MC3T3-E1</td>
<td>The immobilisation of BMP-2 onto the GO–PLGA/HA microcarriers resulted in significantly greater osteogenic differentiation of cells in vitro (bone tissue engineering application)</td>
<td>[459]</td>
<td></td>
</tr>
<tr>
<td>Material</td>
<td>Probe</td>
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<td>Key conclusions</td>
<td>Ref.</td>
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<tr>
<td>Laser-printed GO surfaces with both a local photothermal GO reduction and the formation of nano-wrinkles along precise geometric patterns</td>
<td></td>
<td>Calvarial-derived MSCs (cMSC); MRSA</td>
<td>The morphology of the designed surfaces guided stem cell orientation and the reduction accelerated differentiation; reduced sharp nano-wrinkles were able to enhance the GO antibacterial activity against MRSA</td>
<td>[460]</td>
</tr>
<tr>
<td>Micro-sized (1–10 µm) and nano-sized (100–300 nm) GO sheets (MGO; NGO)</td>
<td></td>
<td>Human AMSCs</td>
<td>Human AMSCs grown on the MGO-coated substrates show enhanced cell spreading and proliferation rate when compared with those of NGO counterpart, regardless of their densities</td>
<td>[461]</td>
</tr>
<tr>
<td>Bioactive GO-modified titanium implant surface</td>
<td></td>
<td>Rat BMSCs</td>
<td>Compared with the surface type in common use, the GO-modified surface favoured cell adhesion and spreading, and significantly improved cell proliferation and osteogenic differentiation of rat BMSCs in vitro; GO modification on titanium implant surface has potential applications for achieving rapid bone-implant integration through the mediation of FAK/P38 signalling pathways</td>
<td>[462]</td>
</tr>
<tr>
<td>PCL, rGO/PCL, and GO/PCL nanofibrous meshes via electrospinning</td>
<td></td>
<td>NIH-3T3; human osteosarcoma; MG-63</td>
<td>rGO/PCL fibrous meshes supported improved cell adhesion, spreading and proliferation of fibroblasts and osteoblast-like cell lines; rGO/PCL fibrous meshes enhanced in vitro calcium deposition in the extracellular matrix produced by osteoblast-like cells, and human MSCs grown onto the same substrates had an increased expression of the osteogenic markers necessary for mineralisation (bone tissue engineering application)</td>
<td>[463]</td>
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</table>
Table A.6: Stem cell control (continued).

<table>
<thead>
<tr>
<th>Material</th>
<th>Probe</th>
<th>Cell lines/Animal models</th>
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<th>Ref.</th>
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<tr>
<td>Nanostructured rGO microfibers</td>
<td>RAW 264.7</td>
<td></td>
<td>rGO microfibers inhibit the proliferation of RAW 264.7 macrophages without affecting their viability and cell cycle profiles; rGO microfibers able to support the formation of a highly interconnected neural culture composed of both neurons (map2+ cells) and glial cells (vimentin+ cells)</td>
<td>[464]</td>
</tr>
<tr>
<td>Gelatin from cold water fish skin (FG) electrospun microfibers loaded with NDs</td>
<td>Human AMSCs</td>
<td></td>
<td>Increased viability and proliferation profile of human AMSCs in contact with FG/NDs, correlated with very low cytotoxic effects of the materials; human AMSCs developed an elongated cytoskeleton, suggesting that addition of NDs to FG materials encouraged cell adhesion</td>
<td>[465]</td>
</tr>
<tr>
<td>Pervasion of beta-tricalcium phosphate (βTCP) with ND particles</td>
<td>Sheep BMSCs; (female merino sheep bone tissue engineering application)</td>
<td></td>
<td>βTCP carrying 4% ND resulted in enhanced attachment of MSCs in vitro; ND in βTCP resulted in a consistently steady bone formation when compared to pure βTCP in guided-bone regeneration after lateral augmentation of the mandible in sheep</td>
<td>[466]</td>
</tr>
<tr>
<td>ND monolayers with varied surface functionalisations</td>
<td>Human NSCs</td>
<td></td>
<td>Confluent cellular attachment occurs on oxygen terminated NDs (O–NDs), but not on hydrogen terminated NDs (H–NDs)</td>
<td>[467]</td>
</tr>
<tr>
<td>Multivalent polyanion-dispersed CNTs for nanostructured fibrous scaffolds</td>
<td>Induced PSCs</td>
<td></td>
<td>Biocompatible platform for promoting the adhesion and proliferation of induced PSCs; immunostaining shows that the nanostructured fibrous scaffolds induce higher neural differentiation efficiency compared to the control substrates; the aligned fibrous scaffolds can guide the orientation of generated neurites</td>
<td>[468]</td>
</tr>
</tbody>
</table>
Table A.6: Stem cell control (continued).

<table>
<thead>
<tr>
<th>Material</th>
<th>Probe</th>
<th>Cell lines/Animal models</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Co-engrafted CNTs and human OBNSCs</td>
<td></td>
<td>Human OBNSCs</td>
<td>Engrafted human OBNSCs–CNTs restored cognitive deficits and neurodegenerative changes associated with trimethyltin-induced rat neurodegeneration model; the CNTs seemed to provide a support for engrafted OBNSCs, increasing their tendency to differentiate into neurons rather than into glia cells</td>
<td>[469]</td>
</tr>
<tr>
<td>MWCNTs; nano-HAP</td>
<td></td>
<td>Human AMSCs</td>
<td>Cell attachment strength and proliferation on the MWCNTs were better than on the nano-HAP; MWCNTs induced osteogenic differentiation of the human AMSCs better than the nano-HAP; MWCNTs could induce ectopic bone formation in vivo while the nano-HAP could not</td>
<td>[470]</td>
</tr>
<tr>
<td>Chemically crosslinked CNTs (of various diameters), graphene nanoplatelets, and graphene nanoribbons coatings</td>
<td></td>
<td>Primary human AMSCs</td>
<td>The greatest autodeposition of calcium was observed on graphene nanoribbon surfaces, while MWCNTs of high diameter had the greatest influence on stem cell fate</td>
<td>[471]</td>
</tr>
<tr>
<td>Hexagonally arranged gold nanostructures of three different dimensions on silicon</td>
<td></td>
<td>Human AMSCs</td>
<td>Nanostructure size and distance affects the spreading of human AMSCs; an increase of nanostructure feature dimensions corresponded to a decrease of cell size and to the expression of focal adhesions and presence of the small GTPase RhoA</td>
<td>[472]</td>
</tr>
<tr>
<td>Metal ion (Zn, Ag and Cu)-doped HAP nano-coated surfaces</td>
<td></td>
<td>Human AMSCs</td>
<td>Results demonstrate that the viability and osteogenic differentiation of the human AMSCs and cell adhesion capability are higher on nanocoated surfaces that include Zn, Ag and/or Cu metal ions than commercial HAP</td>
<td>[473]</td>
</tr>
<tr>
<td>AgNPs incorporated VEGF</td>
<td></td>
<td>Human MSCs</td>
<td>AgNPs with VEGF molecules promoted the cell adhesion and proliferation of human MSCs; AgNPs incorporated VEGF material is highly favourable to fracture healing and blood vessel repair</td>
<td>[474]</td>
</tr>
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</table>
Table A.6: Stem cell control (continued).

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<tbody>
<tr>
<td>Hydrogenated black TiO$_2$ (H-TiO$_2$) coating with hierarchical micro/nano-topographies</td>
<td></td>
<td>Rat BMSCs</td>
<td>The fabricated H-TiO$_2$ coating possessed excellent and controllable photothermal effect in inhibiting tumour growth under 808 nm NIR laser irradiation <em>in vitro</em> and <em>in vivo</em>; the hierarchical hybrid micro/nanostructured surface and Ti-OH groups improved the adhesion, proliferation, differentiation and osteogenic gene expressions of rat BMSCs</td>
<td>[475]</td>
</tr>
<tr>
<td>Nanopatterned Ti surfaces (by electrochemical nanopattern formation, ENF)</td>
<td></td>
<td>Human MSCs</td>
<td>Human MSCs on ENF surfaces exhibit increased proliferation and enhanced osteogenic differentiation compared to the ordered TiO$_2$ nanotubular and compact TiO$_2$ surfaces</td>
<td>[476]</td>
</tr>
<tr>
<td>Phosphorylated Ti coating (TiP-Ti) with a micro/nano hierarchically structured topography on commercially pure Ti implants</td>
<td></td>
<td>Rat BMSCs; female Sprague Dawley rats</td>
<td>The improved osteointegration mainly benefited from the better spread and adhesion of rat BMSCs on the micro/nano hierarchically structured TiP-Ti surfaces compared to HAP coated Ti (the positive control) and untreated Ti (the negative control)</td>
<td>[477]</td>
</tr>
<tr>
<td>Polyether ether ketone (PEEK); Ti$_6$Al$_4$V (smooth Ti); macro-micro rough Ti$_6$Al$_4$V (Endoskeleton®); macro-micro-nano rough Ti$_6$Al$_4$V (nanoLOCK®)</td>
<td></td>
<td>Human MSCs</td>
<td>Incorporation of a hierarchical macro-micro-nano roughness on titanium produces a stellate morphology typical of mature osteoblasts/osteocytes, rapid and random migration, and improved osteogenic differentiation in seeded human MSCs</td>
<td>[478]</td>
</tr>
</tbody>
</table>
Table A.7: Toxicity studies.

<table>
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<tr>
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<tr>
<td>Fabricated nano GO (NGO) sheets</td>
<td>Extrinsic and synchronous fluorescence spectroscopy; far circular dichroism spectroscopy; molecular docking investigation; cellular assays (trypan blue exclusion, cellular uptake, ROS, cell cycle, and apoptosis); molecular assay</td>
<td>Secondary and quaternary structural alterations of human Hb; human lymphocyte cells</td>
<td>NGOs can unfold the quaternary structure of Hb in the vicinity of Tyr residues; α-helicity of Hb experienced substantial alteration upon interaction with increasing concentrations of NGOs; NGOs interacted with polar residues of Hb; NGOs lead to ROS formation, cell cycle arrest, and apoptosis through the BAX and BCL2 pathway</td>
<td>[479]</td>
</tr>
<tr>
<td>AuNPs-decorated rGO nanocomposite</td>
<td>MTT assay; DCF assay; bacterial toxicological evaluation; DIZ and AI evaluation; MIC and MBC determination; GrK and DeK evaluation</td>
<td>A549; HCT116</td>
<td>The synthesised nanocomposite showed significant anti-cancer activity towards A549 cell line and Gram-negative bacterial strain E. coli compared to the rest</td>
<td>[480]</td>
</tr>
<tr>
<td>rGO; O. sanctum rGO (ORGO)</td>
<td>Test for phenols; test for flavonoids; DPPH assay; IC_{50} value determination; haemolysis assay; CCK-8</td>
<td>Hb; Balb 3T3</td>
<td>GO showed higher haemolytic activity of 6.9% and higher inhibition of growth of 3T3 cells at all tested concentrations than ORGO</td>
<td>[481]</td>
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<tr>
<td>SWCNT; MWCNTs; ultrafine CB; crocidolite asbestos</td>
<td>Primary human small airway epithelial cells immortalised with hTERT (SAECs)</td>
<td></td>
<td>Chronic exposure to carbon nanomaterials and asbestos caused substantial DNA damage and p53 dysregulation in human SAECs; exposed lung cells exhibited neoplastic and cancer stem cell (CSC)-like properties, as indicated by anchorage-independent colony formation, spheroid formation, anoikis resistance, and CSC marker expression; high aspect ratio materials including SWCNT, MWCNT and asbestos exhibited strong neoplastic and CSC-like properties as compared to low aspect ratio ultrafine CB particles</td>
<td>[482]</td>
</tr>
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</table>
Table A.7: Toxicity studies (continued).

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<tr>
<td>Unfunctionalised MWCNTs–ultrahigh molecular weight polyethylene (MWCNT–UHMWPE) nanocomposites</td>
<td>Cellular assays (Trypan blue, calcein AM, cell proliferation, cell adhesion)</td>
<td>HFW</td>
<td>Good cell viability on the surface of MWCNT–UHMWPE composites even after 72 h; nanocomposites showed better cell attachment for fibroblasts than pristine UHMWPE; overall, the results showed that MWCNT–UHMWPE composites displayed good cellular growth and biocompatibility indicating another way CNTs can be nontoxic</td>
<td>[483]</td>
</tr>
<tr>
<td>Raw and functionalised MWCNTs (RMWCNTs; FMWCNTs)</td>
<td>Cytotoxicity assays (MTT, NRU); cellular morphology (phase contrast microscope); intracellular ROS generation (DCFH-DA); MMP (Rhodamine-123 fluorescent dye)</td>
<td>MCF-7</td>
<td>Concentration-dependent decrease in cell viability of MCF-7 cells; RMWCNTs- and FMWCNTs-exposed cells found to alter the normal morphology of MCF-7 cells; cells showed significant induction in ROS generation and reduction in MMP level</td>
<td>[484]</td>
</tr>
<tr>
<td>Pristine and functionalised MWCNTs</td>
<td>Cell viability (PI); intracellular ROS (DCF-DA, confocal microscopy); MMP (Rhodamine 123); DNA damage (comet assay); phlogistic effect (gene expression analysis and ELISA tests)</td>
<td>Differentiated SH-SY5Y</td>
<td>The neurotoxic and neuroinflammation properties of the examined MWCNTs assessed in vitro highlighted the potential pathogenic mechanisms triggered by these engineered NPs; impairment of several molecular pathways could contribute to the onset and progression of neurodegenerative diseases</td>
<td>[485]</td>
</tr>
<tr>
<td>Carboxylated SWCNTs and MWCNTs (SWCNT-COOH and MWCNT-COOH)</td>
<td>Cell viability (MTT test); cell motility (evaluated by WHO guidelines); ROS production; NO production</td>
<td>Human spermatozoa</td>
<td>Neither MWCNT-COOH nor SWCNT-COOH exerted negative effect on viability, but motility was significantly dropped in a dose-dependent manner; no significant effect of the type, dose and exposure time of the CNT-COOH on NO production; exposure of sperm cells to both examined types of CNTs at concentrations as low as 0.1 µg/ml caused a significant increase in ROS levels</td>
<td>[486]</td>
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Table A.7: Toxicity studies (continued).

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<tbody>
<tr>
<td>Eight commercially available CNTs</td>
<td>WST-1 measurement; LDH detection; ROS detection; inflammatory cytokine detection</td>
<td>RAW 264.7</td>
<td>Cellular uptake of CNTs by RAW 264.7 macrophages depends on their sizes, specifically on the widths of their bundles in dispersion, regardless of type or manufacturer; uptake of CNTs increased linearly with dynamic size, and cytotoxicity increased with uptake</td>
<td>[487]</td>
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<tr>
<td>SWCNTs with physicochemical alterations in structure resulting from ball-milling</td>
<td>Evaluation of protein components (proteomics)</td>
<td>Normal and cholesterol-rich mouse serum</td>
<td>Increased ball-milling time of SWCNTs resulted in enhanced structural defects; following incubation in normal mouse serum, differences in the biomolecular content of the biocorona due to the ball-milling process were identified; incubation in cholesterol-rich mouse serum resulted in the formation of unique biocoronas compared to SWCNTs incubated in normal serum</td>
<td>[488]</td>
</tr>
<tr>
<td>Pristine C$<em>{60}$ fullerene aqueous solution (C$</em>{60}$FAS); 5-FU and pyrrole derivative MI-1 cytostatic drugs</td>
<td></td>
<td>Colorectal cancer</td>
<td>The number of tumours and total lesion area decreased significantly under the action of C$_{60}$FAS and MI-1; because these drugs have different mechanisms of action, their simultaneous administration can potentially increase the effectiveness and significantly reduce the side effects of antitumour therapy</td>
<td>[489]</td>
</tr>
<tr>
<td>CB particles with primary diameter 56 nm (CB56) and 95 nm (CB95)</td>
<td>Cytotoxicity (trypan blue); 8-nitroG formation (immunocytochemistry); ROS generation (flow cytometry); NO release (Griess method); cellular uptake (DMEM treatment)</td>
<td>RAW 264.7; A549</td>
<td>Both types of CB induced 8-nitroG formation, mainly in the nucleus of RAW 264.7 and A549 cells; CB95 tended to induce more 8-nitroG formation than CB56; CB95 generated larger amounts of ROS than CB56 in RAW 264.7 cells; CB95 produced significantly larger amount of NO than CB56; CB95 was more efficiently internalised into the cells than CB56</td>
<td>[490]</td>
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Table A.7: Toxicity studies (continued).

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<tr>
<td>MoS₂ functionalised with thiobarbituric acid (TBA)</td>
<td>Cell viability assays (MTT; WST-8)</td>
<td>A549</td>
<td>MoS₂–TBA is less toxic to cells compared to MoS₂</td>
<td>[491]</td>
</tr>
<tr>
<td>Boron nitride nanotubes (BNNTs)</td>
<td>Cytotoxicity (WST-8, MTS, MTT assays); cardiomycyte beating behaviour (novel cardiomycyte AFM model)</td>
<td>NB4; HepG2; U87</td>
<td>Correlation between tube length and cytotoxicity with longer tubes having higher cytotoxicity; BNNTs exhibit concentration and cell-line dependent cytotoxic effects</td>
<td>[492]</td>
</tr>
<tr>
<td>Titanium dioxide NPs modified with noble metals (Au, Ag, Pd, Ag₂O, and PdO)</td>
<td>Cell viability (MTT; flow cytometry, Annexin V-FITC)</td>
<td>Caco-2; BJ; L929</td>
<td>Irrespective of cell line and assay used, NPs of unmodified titanium dioxide as well as those with addition of gold and palladium have a slight impact on cell viability at the investigated concentration range (10–200 µg/mL); NPs with addition of silver (Ag and Ag₂O) were found to have significantly higher toxic effect, the level of which varied depending on the cell line and assay used</td>
<td>[493]</td>
</tr>
</tbody>
</table>
Table A.8: Theranostics.

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<tr>
<td>Highly fluorinated GO (FGO) as a carrier for linoleic acid–curcumin conjugate (FGO–Lino–CUR)</td>
<td>T$_2$-weighted MRI</td>
<td>Chemotherapy</td>
<td>Therapy (linoleic acid–curcumin conjugate as an anticancer drug)</td>
<td>MCF-7; MCF10A; 4T1-induced BALB/c mice</td>
<td></td>
<td>[494]</td>
</tr>
<tr>
<td>Cyclodextrin-functionalised GO bound to carbon-coated iron NPs (GO-CD/Fe@C)</td>
<td>MRI</td>
<td>Chemotherapy (DOX)</td>
<td>Nuclei (mainly); lysosomes, mitochondria (some amount)</td>
<td>MDA-MB-231</td>
<td></td>
<td>[495]</td>
</tr>
<tr>
<td>Fluorescent CQDs</td>
<td>Fluorescence imaging</td>
<td>PDT; chemotherapy (rose bengal)</td>
<td>Mitochondria</td>
<td>MCF-7; HeLa; HepG2; AT II; L02; RAW 264.7</td>
<td></td>
<td>[496]</td>
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<tr>
<td>Protein (urokinase-type plasminogen activator)–CD nanohybrids</td>
<td>Fluorescence imaging</td>
<td>Thrombolysis ability</td>
<td></td>
<td>Human blood clots; male BALB/c mice subjected to 3 h transient middle cerebral artery occlusion (tMCAO) or sham-operated</td>
<td></td>
<td>[497]</td>
</tr>
<tr>
<td>PLGA–CDs hybrid</td>
<td>Fluorescence imaging</td>
<td>Chemotherapy (irinotecan); PTT (810 nm laser irradiation)</td>
<td></td>
<td>MDA-MB-231</td>
<td></td>
<td>[498]</td>
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<tr>
<td>Pristine GQDs; GQDs irradiated with gamma rays; GQDs doped with N and N,S atoms</td>
<td>GQDs (GQDs functionalised with urea better for imaging than pristine GQDs)</td>
<td>Fluorescence imaging</td>
<td>PDT (GQDs-U)</td>
<td>HeLa</td>
<td></td>
<td>[499]</td>
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<tr>
<td>Phycocyanin-functionalised single-walled carbon nanohorns (PC@SWNHs) hybrid</td>
<td>Phycocyanin</td>
<td>PAI; thermal imaging</td>
<td>PDT (SWNHs); PTT (SWNHs)</td>
<td></td>
<td>4T1; MDA-MB-231; mice</td>
<td>[500]</td>
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<tr>
<td>2D BP nanosheets functionalised with positively charged PEG–NH₂</td>
<td>NIR (Cy7)</td>
<td>Chemotherapy (DOX)</td>
<td>Lysosomes</td>
<td>HeLa</td>
<td></td>
<td>[501]</td>
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<td>Hollow carbon nanospheres</td>
<td>PAI; NIR</td>
<td>PTT (NIR-triggered)</td>
<td></td>
<td>HeLa; HepG2; MCF-7; male nude BALB/c mice</td>
<td></td>
<td>[502]</td>
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<tr>
<td>C₆₀ fullerene-based tumour-targeted PET imaging probe</td>
<td>⁶⁴Cu; cRGD</td>
<td>Fluorescence imaging; PET</td>
<td>Radiotherapy; radiopharmaceuticals</td>
<td>Integrin α₅β₃</td>
<td>U87 MG (integrin α₅β₃ positive); MCF-7 (integrin α₅β₃ negative)</td>
<td>[503]</td>
</tr>
<tr>
<td>GO–GQDs hybrid</td>
<td>Fluorescence imaging</td>
<td>PTT (NIR-triggered)</td>
<td></td>
<td>MDA-MB-231; L929</td>
<td></td>
<td>[504]</td>
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<tr>
<td>PEI- and PEG-modified carbon nano-onion clusters (CNOCs)</td>
<td>Optical (uptake so high that fluorescence labelling is not required); dual-modal PTI/PAI</td>
<td></td>
<td>PTT</td>
<td>AT II; A549; mice</td>
<td></td>
<td>[505]</td>
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<tr>
<td>AuNPs-functionalised hollow P(MAA-co-MBA-co-AA) nanocontainers</td>
<td>Fluorescence imaging</td>
<td>Chemotherapy (DOX)</td>
<td></td>
<td>MCF-7; HEK-293</td>
<td></td>
<td>[506]</td>
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<td>Material</td>
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<td>Therapy</td>
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<td>Structured NP (GNR@HPMO@PVMSN) composed of a gold nanorod (GNR) core,</td>
<td>PAI; infrared thermal imaging</td>
<td>PTT (808 nm NIR laser irradiation);</td>
<td>GNR@HPMO@PVMSN is mainly distributed in the liver, spleen and lung</td>
<td>7721; H22 tumour-bearing female BALB/c nude mice</td>
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<td>[507]</td>
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<tr>
<td>hollow periodic mesoporous organosilica (HPMO) shell and PDA-doped</td>
<td></td>
<td>chemotherapy (DOX)</td>
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<td>virus-like mesoporous silica NP (PVMSN) outer shell</td>
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<tr>
<td>Fe₃O₄–Au nano-heterostructures</td>
<td>MRI</td>
<td>Magnetic fluid hyperthermia</td>
<td></td>
<td>Huh7; PLC/PRF/5-Alexander</td>
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<td>[508]</td>
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<td>Folate-modified vincristine-loaded PDA-coated Fe₃O₄ superparticles</td>
<td>MRI</td>
<td>Chemotherapy (vincristine); PTT</td>
<td></td>
<td>HaCat; T24; T24 tumour-bearing BALB/c nude mice</td>
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<td>[509]</td>
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<tr>
<td></td>
<td></td>
<td>(808 nm laser irradiation)</td>
<td></td>
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<tr>
<td>PDA-modified Fe₃O₄ nanocomposites</td>
<td>T₂-weighted MRI</td>
<td>PTT (808 nm NIR laser irradiation)</td>
<td></td>
<td>4T1; 4T1 tumour-bearing BALB/c mice</td>
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<td>[510]</td>
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<tr>
<td>Fe₃O₄@PDA@BSA–Bi₂S₃ composite</td>
<td>MRI; CT</td>
<td>ROS-induced chemodynamic therapy</td>
<td>Bi ions largely accumulated in the liver, spleen, and kidneys on the</td>
<td>L929; HT29 tumour-bearing BALB/c mice</td>
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<td>[511]</td>
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<tr>
<td></td>
<td></td>
<td>(CTD); PTT (808 nm NIR laser</td>
<td>evidence of excretion over 14 days</td>
<td></td>
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<td></td>
<td></td>
<td>irradiation)</td>
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<tr>
<td>HA-modified porous carbon-coated Fe₃O₄ NPs</td>
<td>HA</td>
<td>PTT; chemotherapy (DOX)</td>
<td></td>
<td>CD44 receptors</td>
<td>HeLa; HUVEC; nude mice</td>
<td>[512]</td>
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</table>
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<tr>
<td>Boron nitride nanosheets functionalised with tannic acid–Fe$^{3+}$ coordination complex (TA–Fe/BNNS)</td>
<td>T$_1$-weighted MRI</td>
<td>PTT (808 nm NIR laser irradiation)</td>
<td></td>
<td>KB tumour cells; 5-wk-old female BALB/c nude mice</td>
<td>[513]</td>
<td></td>
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<tr>
<td>2D PEGylated antimonene nanosheets</td>
<td>Multi-modal fluorescence imaging/PAI/PTI</td>
<td>PTT; chemotherapy (DOX)</td>
<td></td>
<td>MCF-7</td>
<td>[514]</td>
<td></td>
</tr>
<tr>
<td>Gd$^{3+}$-doped monolayered-double-hydroxide (MLDH) nanosheets</td>
<td>Dual-modal NIR fluorescence imaging/MRI</td>
<td>Chemotherapy (coloading of DOX and indocyanine green)</td>
<td></td>
<td>hepG2; HepG2 tumour-bearing mice</td>
<td>[515]</td>
<td></td>
</tr>
<tr>
<td>Ultrathin nanosheets of CoFe-selenide with a finely controlled structure followed by surface modification with PEG</td>
<td>T$_2$ MRI</td>
<td>PTT (808 nm NIR laser irradiation)</td>
<td></td>
<td>HeLa; HeLa tumour-bearing mice</td>
<td>[516]</td>
<td></td>
</tr>
<tr>
<td>Aggregation-induced emission (AIE) photosensitisers and 2D BP nanosheet compound</td>
<td>Fluorescence imaging (NIR region); PTI (808 nm NIR laser irradiation)</td>
<td>PTT (808 nm NIR laser irradiation); PDT (ROS generation under 808 nm laser irradiation)</td>
<td></td>
<td>4T1; 4T1 skin-tumour-bearing male BALB/c nude mice</td>
<td>[517]</td>
<td></td>
</tr>
<tr>
<td>Glucose oxidase-armed manganese dioxide nanosheets (MNS–GOx)</td>
<td>MRI; PAI</td>
<td>PTT (808 nm NIR laser irradiation); self-oxygenation</td>
<td></td>
<td>A375 tumour-bearing mice</td>
<td>[518]</td>
<td></td>
</tr>
</tbody>
</table>
In Chapter 3, average values of length ($L$), width ($W$), $LW$ (length $\times$ width), and number of layers ($N$) for each material were needed to normalise the data for nanosheet size. To obtain these average values, the sheetwise data was binned to allow analysis of the distributions. Figures B.1–B.5 show the histograms of $L$, $W$, $LW$, and $N$ obtained from AFM statistics for each material. The insets show example AFM images of a nanosheet for each material; Figure B.6 shows enlarged versions of these AFM images.

Figure B.1: Histograms of L-gra (i) length; (ii) width; (iii) $LW$; (iv) $N$. 
Figure B.2: Histograms of S-gra (i) length; (ii) width; (iii) $LW$; (iv) $N$.

Figure B.3: Histograms of BN (i) length; (ii) width; (iii) $LW$; (iv) $N$. 
Figure B.4: Histograms of MoS\(_2\) (i) length; (ii) width; (iii) \(LW\); (iv) \(N\).

Figure B.5: Histograms of WS\(_2\) (i) length; (ii) width; (iii) \(LW\); (iv) \(N\).
Figure B.6: AFM images of (i) L-gra; (ii) S-gra; (iii) BN; (iv) MoS$_2$; (v) WS$_2$. 
C Derivation of interparticle distance variable
from Chapter 3

The system on the air–water interface can be approximated as shown in Figure C.1. It is assumed that the film comprises uniformly distributed square nanosheets, and that the trough area, \( A \), is also square.

![Figure C.1: Top-down diagram of a Langmuir trough with a uniformly distributed film on the air–water interface.](image)

The centre-to-centre interparticle distance, \( s \), and the mean nanosheet diameter, \( d \), are related such that

\[
\frac{d^2}{s^2} = \frac{A_f}{A},
\]

where \( A_f \) is the total film area. This is apparent when considering the square formed by \( s^2 \) (shown by the dashed blue line in Figure C.1). As the total film area per trough area gives the surface coverage of the film, \( \Phi \), this ratio can be rewritten as

\[
\frac{d^2}{s^2} = \Phi.
\]

Since the area of a nanosheet has been approximated as \( \langle LW \rangle \), the diameter can be taken as \( \sqrt{\langle LW \rangle} \). Substituting in this value and rearranging, the interparticle distance can be written as

\[
s = \sqrt{\frac{\langle LW \rangle}{\Phi}}.
\]
D Additional Raman spectroscopy and mapping for Chapter 4

By mapping the peak intensity of the 405 cm$^{-1}$ $A_{1g}$ mode, and using Raman volumetric mapping, it is possible to determine detailed spatial information for the nanosheet within the cell. Combining multiple overlapping steps gives a $z$-resolution of $< 1 \mu m$, and therefore it is possible to confirm the difference between material internalised within the cell and that above or below the cell. This is shown in Figures 4.5d, 4.5e & D.1.

Figure D.1: Raman volume map of the $A_{1g}$ Raman mode for cells after 7 days growth on a MoS$_2$ substrate.
2D Raman mapping confirms our observations from optical microscopy, showing that the nanosheets are largely internalised in the region around the nucleus (the ER), with some material identified in the cytoplasm. This is seen in Figures 4.5a, 4.5g & D.2.

![2D Raman map of the $A_{1g}$ mode showing MoS$_2$ localised around the nucleus.](image)

As discussed in the paper, Raman spectroscopy was used to ascertain whether the internalisation process modified the MoS$_2$. The results shown in Figure D.3 show that there is little to no change to the material.

![Raman spectrum from MoS$_2$ internalised in cells after 7 days growth on a MoS$_2$ substrate.](image)