A Combined Electron Microscopy and Computational Study on Cellular Uptake and Stability of Carbon Nanotubes

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Author’s Declaration

I declare that this thesis is a product of the work I have carried out at the Department of Materials at Imperial College London. All the work described herein is my own, unless specified otherwise.

Some of the work contained in this thesis has been published in the following journal articles.


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Abstract

The aims of the PhD project were to understand the mechanisms of cellular uptake as well as the intracellular biostability of oxidised functionalised multi-walled carbon nanotubes (f-MWNTs).

Firstly, high resolution transmission electron microscopy (HR TEM) imaging and 3D electron tomography were applied to study the pathways of f-MWNTs into non-phagocytic cells, and more specifically, to study the interaction between f-MWNTs and the cell membrane. After exposing epithelial cells to f-MWNTs for 24 hours, two distinct uptake processes by which NH$_3^+$ f-MWNTs can enter epithelial cells were observed. Then, the study was complemented using a combination of TEM imaging and coarse-grained molecular dynamics simulations, to provide insight into the interaction of f-MWNTs with cell membranes and the effect of surface charge on this interaction.

Secondly, the question of whether f-MWNTs can be degraded by the body’s own defence mechanisms was addressed. HR TEM techniques were used to assess the graphitic structure and morphology of f-MWNTs injected into the _murine_ brain and after exposure to human monocyte-derived macrophages (HMMs), with the aim of understanding the mechanisms underlying the degradation process. F-MWNTs were found to have a reduced biostability in the brain tissue and in the HMMs. Inside the brain tissue, the degradation occurred rapidly with signs of advanced f-MWNT degradation present after 2 days exposure. In the HMMs, f-MWNT walls were found to delaminate from individual f-MWNTs inside lysosomes after 24 hours exposure. Similar events were observed after 14 days exposure in the cell cytoplasm inside the HMMs. Furthermore, a loss of the graphitic structure was observed. By using scanning TEM electron energy loss spectroscopy (STEM EELS) to compare the near-edge structure of the carbon K-edge prior to, and post injection, graphitic f-MWNTs could be distinguished from the graphitic debris and the amorphous cell background. The combination of HR TEM and STEM EELS techniques provided information about the individual steps, leading to the disintegration of the f-MWNTs, and the morphology of the degradation debris. In order to study the effect of the functionalisation on the biostability, the study was repeated using pristine MWNTs and HMMs. No signs of degradation of the pristine MWNTs were observed after 14 days exposure to the HMMs.
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Chapter 1

Introduction

The first description of filaments of carbon reported in the literature date back more than a century [1–3] and the first experimental evidence of the filaments was achieved using TEM imaging as early as 1952 by Radushkevich and Lukyanovich [4]. However, it was only after Iijima imaged the carbon nanotubes (CNTs) in 1991 [5], that CNTs were hailed as the new material for future nanotechnology. Today, CNTs-based industries form an important part of nanotechnology due to their outstanding electronic and mechanical properties, which make them relevant to a wide range of applications. CNTs are investigated for many applications, ranging from carbon nanotube–polymer composites [6] to nanoelectronics [7, 8]. As a consequence, they are amongst the most commercially important nanostructures, with annual production capacities in the order of thousands of tonnes.

CNTs, especially multi-walled nanotubes (MWNTs), are receiving increasing attention in medical applications since MWNTs are attractive candidates for diagnostic as well as therapeutic applications. As such, MWNTs form part of a new generation of ‘smart drugs’, which are being investigated for delivery of diagnostic as well as therapeutic agents in a targeted manner. In cancer therapy, MWNTs can be used in various ways to overcome the challenge of attacking tumour cells without harming normal cells and tissues [9–15]. Other applications include the use of MWNTs for vaccine [16–18], Nucleic Acid [19–22] and drug delivery [23, 24], as well as for scaffolding in bone tissue engineering [25–27].

The key advantages of using MWNTs in medical applications include:

- MWNTs can enter cells, and therefore can act as delivery vehicles for diagnostic and therapeutic agents.
- MWNTs have a high surface area, which provide numerous attachment sites for functional groups.
- Certain classes of MWNTs have been found to be non-cytotoxic [28–31].
- The unique electrical, thermal and spectroscopic properties of MWNTs offer numerous possibilities for advancing the detection and treatment of disease.
CHAPTER 1. INTRODUCTION

The potential applications in medicine are investigated at numerous academic as well as commercial laboratories. However, there are discrepancies in research results about the exact mechanism by which CNTs enter cells. Furthermore, little is known about the long term fate of CNTs.

Despite the rapid advances in nanoscience, the pathways by which MWNTs enter cells and their intracellular behaviour remain mostly unknown as the findings of research groups are often ambiguous and sometimes even contradictory. There are several routes by which MWNTs enter cells, including active energy-dependent uptake e.g. endocytosis, and passive diffusion through the cell membrane. Several research groups have suggested that the uptake of CNTs occurs via active energy-dependent processes [32, 33], but other experimental evidence suggests that CNTs can pass across cell membranes via diffusion [19, 34–36]. Electron microscopy images from several groups [19,35,36] show what the authors claimed to be functionalised multi-walled carbon nanotubes (f-MWNTs) directly piercing through the membrane of the cells. However, the experimental evidence is not convincing due to the low resolution of the images and the absence of any 3D evidence. Understanding the pathways by which MWNTs enter the cells is crucial to their application as delivery vehicles into the cells, since their applications often rely on the MWNTs entering the cell cytoplasm. In addition, understanding the interactions between MWNTs and cell membranes and the components of the cytoplasm could provide information about their potential toxicity which could in turn lead to improvements in design to gain better control over the processes involved.

Another critical question that remains to be answered concerns the long term fate of the MWNTs inside the cells after uptake and ultimately inside the human body. Finding out whether the MWNTs are trafficked after uptake is crucial for their potential as delivery vehicles. Furthermore, little is known to date about whether the MWNTs can be oxidised or degraded by the body’s natural mechanisms. In order to get a full understanding of the cytotoxic effect of CNTs in the long term and thereby assess their potential as drug delivery vehicles as well as in order for the medical applications of MWNTs to become feasible in the future, one has to gain a full understanding of what happens to the MWNTs once inside the cells. Experimental evidence has shown that f-CNTs can be degraded by enzymatic action outside of the cellular environment [37–40] and by mimicking the environment of the phagolysosomes [41]. Recent evidence also suggested that f-MWNTs can be degraded by neutrophil cells. However, all of the studies relied on Raman spectroscopy, which is a bulk technique and provides no information about the mechanism of degradation. Therefore, the precise mechanism by which the CNTs degrade inside cells has not been analyzed experimentally and remains elusive. Only a fundamental understanding of the uptake processes and interactions of MWNTs with cell components can lead to a fair assessment of the risks involved with this technology.

The aim of the work presented here was to address the controversies around cellular uptake and the long term fate of f-MWNTs inside cells. More specifically, this study aimed to address the following questions:

1. How do MWNTs enter non-phagocytic cells?
2. Does the surface charge modify the cellular uptake?
3. What is the long term fate of MWNTs?

In order to address these questions, a combination of electron microscopy and molecular dynamics simulations were used in this thesis.

First, the interactions of f-MWNTs with non-phagocytic epithelial cells were studied. Since epithelial cells form the epithelium which lines the surfaces of structures, such as lungs and blood vessels, they are important for toxicological and pharmacological studies. Transmission electron microscopy (TEM) imaging was applied to establish whether f-MWNTs can enter via alternative pathways into the cells, including active uptake routes, as well as by direct insertion through the plasma membrane. The study was complemented by use of 3D electron tomography reconstructions of the cellular uptake of f-MWNTs by epithelial cells to gain a better understanding of the position of the cell membrane with respect to the f-MWNTs.

Then, the role of surface charge in the interaction of f-MWNTs with the plasma membrane of cells was studied using a combination of TEM imaging and computational methods. By using molecular dynamics simulations, individual factors, such as the presence of surface charges and the surface charge distribution, which could potentially affect the translocation of f-CNTs through the plasma membrane, were studied in isolation.

Lastly, the long term fate of f-MWNTs in tissues as well as in cell cultures was studied. In a first study, the biostability of oxidised, NH$_3^+$ f-MWNTs, injected directly into the murine brain, was assessed after f-MWNTs exposures of up to 2 days in the tissue. The aim here was to establish whether the f-MWNTs disintegrate inside tissues. The structural integrity of the f-MWNTs after exposure in vivo was studied using high resolution (HR) TEM and electron energy loss spectroscopy (EELS) techniques. In a second study, the oxidised, NH$_3^+$ f-MWNTs were exposed to human monocyte-derived macrophages (HMMs) in vitro for up to 14 days in order to study the mechanism of degradation. Again, the structural integrity of the f-MWNTs with exposure time was studied using high resolution (HR) TEM and EELS analysis. In addition, the changes in intracellular distribution were monitored using TEM imaging. In a third study, the stability of f-MWNTs was compared to the stability of pristine non-oxidised MWNTs (p-MWNTs) inside HMMs in culture. The aim here was to study the effect that the oxidising functionalisation procedure has on the biostability in vitro of f-MWNTs. HMMs were chosen for the study of the long term fate of MWNTs since they constitute the first line of defense of the body by forming part of the immune and inflammatory response.

This thesis presents the work that was carried out for this PhD thesis. Chapters 2 to 4 present an overview of the background and a review of the literature relevant to this project. Chapters 5, 6, 7, 8 and 9 describe the experimental techniques and instrumentation used in the work. Chapter 10 presents the results from the characterisation of the MWNTs material used. Chapter 11 presents the results from the studies on cellular uptake of f-MWNTs. Chapters 13 and 14 present the results from the studies on the biostability and intracellular trafficking of MWNTs. Finally, conclusions and future work are presented in chapter 15.
Part I

Background and Literature Review
Chapter 2

The Cell and Cellular Uptake

In this section some basic cell biology concepts will be introduced. The aim is not to give an exhaustive list of the properties and processes taking place in cells, but to introduce the basic cell biology concepts relevant to the subsequent chapters. More detailed information can be found in [42].

2.1 The Cell

Here the basic cell components of eukaryotic cells (cells containing a cell nucleus), the organelles, will be described.

Cytoplasm  The cytosol is the gel-like fluid found inside of the cell, but not contained within cell organelles. The cell cytoplasm is the collective term for the cytosol plus all the cell organelles contained within the cytosol, with the exception of the cell nucleus. The cytoskeleton, composed of a network of fibres which extend throughout the cytoplasm, is responsible for the movement and stability of the cell.

The Nucleus  The cell nucleus is the defining feature of eukaryotic cells. It controls the cell functions and contains the cell’s genetic material in the form of deoxyribonucleic acid (DNA). The nucleus is enclosed by a double membrane, the nuclear envelope, which forms a barrier impermeable to most particles and substances. Transport in and out of the cell nucleus, which is crucial to the normal functioning of the cell, occurs through nuclear pores which have an effective diameter of ~10nm. A detailed overview of the role and functions of the cell nucleus is given in the review by Lamond et al. [43].

Mitochondria  The mitochondria are responsible for the generation of energy for the cells in the form of adenosine triphosphate (ATP) [44]. The mitochondria play a role in the regulation of the cell metabolism, cell cycle control, antiviral responses and cell death [45]. A mitochondrion
is enclosed by a double membrane, where the inner membrane is highly folded, with the infoldings of the membrane being called cristae. The diameter of the mitochondria varies largely and ranges from 0.5 – 10μm.

**Endoplasmic Reticulum** The endoplasmic reticulum (ER) is an extensive network of membranes, forming a sac-like structure called cisternae which is held together by the cytoskeleton. The role of the ER depends strongly on the cell type. There are two types of ER: the rough ER (RER) and the smooth ER (SER).

The presence of ribosomes on the surface of the membrane of the RER gives it a "rough" appearance. The RER is responsible for the synthesis of the proteins inside the ribosomes bound to the surface of the membrane. However, these ribosomes are only bound to the membrane if they are in the process of synthesizing proteins, and hence, are not a permanent part of this organelle.

The SER plays a role in several metabolic processes in the cell: synthesis of lipids and steroids, regulation of the calcium concentration, metabolism of steroids and carbohydrates and attachment of cell membrane proteins.

**Golgi Apparatus** The Golgi apparatus is formed by membrane bound structures, a cisterna. Its role is to do the sorting and packaging of macromolecules in transit. The vesicles leaving the RER empty their contents into the lumen of the Golgi apparatus where it is sorted and packaged for further transit.

## 2.2 The Plasma Membrane

The plasma membrane forms the interface between the cell and its environment, and as such, it separates the chemically distinct areas of the cell cytoplasm and the extracellular fluid. Our understanding of the plasma membrane is based on the ‘Fluid-Mosaic’ model (figure 2.2.1a), which was developed by Singer et al. [46]. According to the model, the plasma membrane is composed of two layers of lipid molecules, the lipid bilayer (LB), and embedded proteins.

**Phospholipids** The amphipathic phospholipids in the plasma membrane are composed of a hydrophilic head group and two hydrophobic fatty acid tails (figures 2.2.1a and 2.2.1b). The hydrophobic polar head group is composed of glycerol (C₃H₆O₃) and a phosphate group (PO₄³⁻). Furthermore, the hydrophilic head groups are orientated towards the water whereas the hydrophobic tails are lined up on the inside of the LB forming a hydrophobic core. Thus, the lipids inside the LB form a barrier separating different environments. However, since the LB is subject to rapid movement of the lipids, it is not a rigid structure and hence allows some passage of substances through the membrane itself.
Uptake Mechanisms

Figure 2.2.1: According to the Fluid-Mosaic model, developed by Singer et al. [46], the lipid bilayer is composed of a double layer of lipids and embedded proteins as shown in (a). A phospholipid is composed of a hydrophilic headgroup and two hydrophobic fatty acid tails, as seen in (b) and (c). The figure was adapted from Singer et al. [46].

Proteins The membrane proteins are responsible for most functional properties of the cell. There are four major groups of membrane proteins regulating the exchange of information or substances between the inside and outside of cells. The pumps, transporters and ion channels are responsible for the exchange of molecules across the LB. Whereas the receptors transmit information from the outside of the cells to the inside. Lastly, the identifiers are a group of membrane proteins acting as labels for the cells. The immune system uses these markers to distinguish between their own cells and foreign organisms. Identifier proteins are important for targeted drug-delivery.

Hence, the plasma membrane forms a selectively permeable barrier between the intracellular environment and the extracellular environment. In addition, it contains labels to identify the cells as well as other markers signaling the processes taking place inside the cell.

2.3 Uptake Mechanisms

All trafficking in and out of the cells occurs through the plasma membrane of the cell. Endocytosis has been labelled as cell ‘eating’ and ‘drinking’, but it extends far beyond these processes. A particle can often enter the cell via several different entry mechanisms, depending on what signal triggered the uptake, as well as the exact surface properties of the particle, the cell type and various other factors. The different uptake processes are shown in figure 2.3.1. In general, the uptake of substances into cells can be divided into ‘active’ uptake processes, which require energy under the form of ATP from the mitochondria of the cell, and ‘passive’ uptake processes, which do not require ATP [47].
2.3.1 Active Uptake through Phagocytosis

Phagocytosis in humans and other mammals occurs only in specialized cells. One such cell type is the macrophage, which are white blood cells. These form part of the immune defense system and function as a clearance mechanism for cell debris, dead cells and micro-organisms. By phagocytosing debris and pathogens, macrophages stimulate lymphocytes and other immune cells to respond to attack.

During phagocytosis, the cytoplasm membrane folds inwards to form a pocket engulfing the matter inside a phagosome [48]. This phagosome then delivers the engulfed matter to the lysosome, which in turn is responsible for breaking the matter down. The digested components are then either released via exocytosis, transporting them out of the cell, or they will undergo further digestion inside the cell.

Phagocytosis is an active process which is particle dependent and highly regulated through the presence of receptors on the surface of the plasma membrane. When the receptors on the surface of the plasma membrane recognize anti-bodies on the surface of micro-organisms, for example bacteria, a cascade of signals is triggered causing the cell to activate the inflammatory response. Furthermore, after break-up of the engulfed matter, any antibodies present are regurgitated and presented on the outside of the plasma membrane of the cell which successively triggers the larger immune response. Since the cells are specific in their phagocytic uptake, the inflammatory response is only triggered when the matter engulfed is recognized as foreign and not when, for example, dead cells are engulfed. If the cells fails to engulf the targeted matter in its entirety, the process is referred to as ‘frustrated phagocytosis’. This could result in release of toxic substances into the cell and potentially damage, or kill the cell and damage surrounding cells.

As such, macrophages act in a non-specific manner by phagocytosing foreign bodies, but also in a specific manner against recognized pathogens, forming the first line of defense of the body by triggering and generating an immune and an inflammatory response.

2.3.2 Active Uptake through Pinocytosis

The vesicles formed through pinocytosis are called endosomes. These transport matter into the cell and fuse with lysosomes [49]. Pinocytosis is not specific to a cell type and can take place in most cells.

Macropinocytosis  Macropinocytosis is preceded by ruffling of the plasma membrane [48, 50, 51]. Membrane protrusions form and collapse onto the cell membrane to fuse with the cell membrane, surrounding the cargo. This leads to the formation of a macropinosome, which engulfs cargo with a diameter $> 1\mu m$ [47]. Macropinosomes have no coating or specific receptors, and hence this process describes a non-specific uptake route of large cargo into the cell.
Uptake Mechanisms

Figure 2.3.1: Schematic representation of cellular uptake mechanisms, adapted from Conner et al. [47]. Phagocytosis is specific to cells such as macrophage and neutrophil cells, that function to clear large debris and pathogens. Different types of pinocytosis include: macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and clathrin- and caveolin-independent endocytosis.

Clathrin-mediated Endocytosis Small clathrin coated pits, found along the plasma membrane [52], regulate clathrin-mediated endocytosis. The presence of receptors in the coated pits result in specific uptake of cargo. During uptake into the cell, the pits in the membrane pinch off the membrane to form vesicles containing the cargo of up to 120 nm in size. The resulting vesicles are called endosomes.

Caveolin-mediated Endocytosis Small flask-shaped caveolae pits in the plasma membrane, composed of the protein caveolin, cholesterol and glycolipids, regulate the uptake via caveolin-mediated endocytosis. This uptake route is believed to be mediated via specific receptors. Furthermore, the process is cholesterol dependent; cholesterol depletion suppresses this uptake route [53].

Clathrin- and Caveolin-independent Endocytosis Uptake can occur through cholesterol-rich rafts of 40-50nm diameter in the plasma membrane which are free to move along the membrane [47]. Clathrin- and caveolin-independent endocytosis is poorly understood, but similarly to caveolin-mediated endocytosis, it is also sensitive to cholesterol depletion [53].

2.3.3 Passive Uptake Processes

According to the equipartition of energy theory, if in thermal equilibrium, all free particles possess the same amount of energy associated with each translational degree of freedom. The contribution from each degree of freedom is $\frac{1}{2}k_B T$, where $k_B$ is the Boltzmann constant and
T is the temperature, and there are three translational degrees of freedom in 3D. Hence, the average translational kinetic energy of a particle of mass \( m \) moving at velocity \( v \) is described by,

\[
K.E_{\text{average}} = \frac{1}{2} m \bar{v}^2 = \frac{3}{2} k_B T
\]  

(2.3.1)

Note, in this relation the particles are treated as point-masses and therefore rotational degrees of freedom are not accounted for.

As a result of this kinetic energy, the molecules inside the cells and surrounding the cells move in a random motion, described by ‘Brownian Motion’. In addition, the molecules have a tendency to move from areas with higher concentration to lower concentration. Molecules, for example, might transit, through the lipid bilayer, down the concentration gradient, from regions of higher concentration to regions of lower concentration; this process is called diffusion. In contrast, transport against the concentration gradient requires energy and is therefore an ‘active’ process.

The most well-known diffusion process through the pores in the membrane of the cell is ‘osmosis’ [42], where water molecules pass through the membrane down the concentration gradient, to balance the concentration of water molecules on both sides of the membrane.

### 2.4 Cell Types

In the following section, the cell types used in this work are presented.

#### 2.4.1 Macrophage Cells

Macrophage cells are white blood cells and constitute the first line of defense, forming the immune and inflammatory response of the body. As such, macrophage cells form part of the wider immune defense system. Macrophages act in a non-specific manner by phagocytosing foreign bodies, but also in a specific manner, against recognized pathogens as described in section 2.3.1. Due to the important role that macrophage cells play in the immune and inflammatory defense of the body, it is crucial to understand their response to carbon nanotube (CNT) exposure. Important questions about how the CNTs are taken up and if they can be digested by the macrophage cells, need to be addressed to establish the effects that exposure to CNTs has on the physiology of the body.

#### 2.4.2 Epithelial Cells

Epithelial cells line the cavities and surface structures throughout the body in animal tissue, such as the lungs and blood vessels. These cells are tightly bound together to form the epithelium. Junctions, which act as communication portals, form between adjacent cells. Since
epithelial cells line the lung and other organs, this cell type forms the first point of contact when CNTs are inhaled or injected. Hence, epithelial cells are often employed in toxicology studies.

2.4.3 Murine Brain Tissue

The brain tissue of the peripheral nervous system (PNS) and the central nervous system (CNS) contain several different types of cells including: neurons and glial cells. The neurons are composed of a cell body which contains the nucleus, cell cytoplasm and organelles - including a single axon and several dendrites. The axon is responsible for the transmission of signals from one nerve cell to another and the dendrites are responsible for receiving and integrating signals from other nerve cells. Myelinated axons, as opposed to unmyelinated axons, are enveloped by myelin which increases the speed of the signal propagation. The myelin is produced by glial cells - Schwann cells in the PNS and oligodendrocytes in the CNS. These cells are mainly responsible for the support and insulation of the axons of the nerve cells [54].
Chapter 3

Carbon Nanotubes: Structure, Production & Functionalisation

In 1985, the Buckminster fullerene, comprised of sixty carbon atoms, was discovered. The carbon atoms are sp² hybridized, arranged in hexagons and pentagons to form the “Buckyball” shape [55]. This discovery led to a new field of carbon chemistry that led, in 1991, to the discovery of multi-walled carbon nanotubes (MWNTs) by Iijima et al. [5] using transmission electron microscopy (TEM) imaging. In 1993, Iijima imaged a single-walled carbon nanotube (SWNT) for the first time [56].

3.1 Electronic Structure of Carbon

In order to gain insight into the electronic structure of carbon nanotubes, one may begin by studying the electronic structure of carbon.

The carbon atom has six electrons [57] and its electronic configuration is 1s² 2s² 2p². Hence, only two electrons are free to bond with other atoms. However, when in the presence of other carbon atoms, enough energy is available to cause unpairing of the 2s electrons. This leads to an electronic configuration of 1s² 2s¹ 2p³ - now four electrons are available to undergo bonds.

Following the unpairing, the electrons in the 2s orbitals hybridise with one, two or all three of the p orbitals- undergoing sp, sp² and sp³ hybridisation respectively as shown in figure 3.1.1. The actual hybridisation of the 2s orbitals is thermodynamically unfavourable, but the subsequent bonding the carbon atom can undergo makes it thermodynamically favourable overall.

In the diamond structure [58], the 4 valence electrons singly occupy 4 sp³ hybrid orbitals. In this configuration, four equivalent covalent σ bonds, with a length of 0.15 nm, are formed between atoms. These four bonds, oriented at an angle of 109° to one another in the tetrahedral directions, form a 3D structure. The σ bond is the strongest covalent bond which gives the diamond structure its mechanical strength.
Figure 3.1.1: Electronic configurations of carbon: The ground state of carbon is 1s^2 2s^2 2p^2, where only two carbon atoms are free to undergo bonds. If the 2s electrons unpair, the configuration becomes 1s^2 2s^1 2p^3, resulting in sp, sp^2 and sp^3 hybridisation.

In graphene [59], 3 of the valence electrons from each atom singly occupy 3 sp^2 hybrid orbitals, forming three in-plane covalent σ bonds and an out-of-plane π orbital. The resulting structure is a planar (flat) hexagonal structure, where the C-C bonds have a length of 0.142 nm. In both graphite and diamond, the relatively short interatomic distances lead to the remarkable properties exhibited. In graphite, several layers of graphene are held together by Van der Waals forces, with each layer separated by 3.354 Å.

The CNT structure can be viewed as a rolled up sheet (or several sheets) of graphene. Hence, the bonding is mostly sp^2 hybridised. However, the curvature causes quantum confinement and the re-hybridisation of σ − π bonds, resulting in the three σ bonds being slightly out of plane and the π bond being more delocalised outside of the cylindrical structure. This delocalisation increases the mechanical strength as well as the thermal and electrical conductivity, and makes the CNTs more chemically and biologically reactive than the graphite structure. Even so, this effect becomes less and less important with increasing CNT diameter, as the degree of curvature of the CNT structure decreases.

SWNTs are composed of one layer of graphene and as such they can be visualised as hollow cylinders [55]. Depending on their chirality, the SWNTs can be metallic or semiconducting. In contrast, MWNTs are typically formed from 2-50 layers of graphene, concentrically stacked or rolled-up.

### 3.2 Production & Defects of Carbon Nanotubes

There are several processes by which SWNTs can be produced, including arc discharge, laser ablation of carbon or chemical vapor deposition (CVD) using catalyst particles. The CNTs purchased for this work were produced via the last production route. Detailed information about the production routes mentioned above, can be found in [60].

For the CVD production method, a substrate is prepared with a layer of catalyst particles (commonly nickel, cobalt or iron). The diameter of these catalyst particles determines the diameter of the CNTs that are grown on them. The substrate is heated to temperatures of 700°C while two gases are blended in the reactor: a process gas and a carbon-containing gas. The carbon from the gas deposits on the catalyst particles, thereby forming the CNTs. A disadvantage of
this production method is that the catalyst particles may remain at the tip of the CNTs. Hence, there can be a significant amount of impurities left in the CNT sample. In addition, the production of CVD CNTs results in a mixture of different types of CNTs, e.g. various lengths and diameters, present within a single production batch. Although this method does not produce the purest and most defect-free CNTs, it is the production route favoured for commercially available CNTs.

From experimental observation it is known that CNTs can be bent, capped [62], or branched [63], which provide an indication that the CNT structure comprises inherent topological defects. Defect A in figure 3.2.1 shows the existence of five- or seven-membered rings, instead of the usual six-membered rings, which leads to CNT bending. Defect B in figure 3.2.1 shows the appearance of sp³-hybridised defects which can occur in strongly curved SWNTs [61,64]. Hence, defects can be introduced during the production process and may not always be the result of a functionalisation processes.

Other types of damage can occur on the CNTs structure during certain functionalisation or purification procedures. The appearance of the COOH groups on the sidewalls of the tubes (figure 3.2.1, defect C) has been explained by the CNT structure breaking open [65, 66]. This formation of holes, with COOH groups attached along the edges, can be caused by oxidative conditions. Furthermore, the existence of capped CNTs, resulting in a -COOH groups terminus

Figure 3.2.1: Schematic of defects on the CNT surface, adapted from [61]. (A) The existence of five- or seven-membered rings instead of the usual six-membered rings will lead to bending of the CNT structure. (B) The appearance of sp³-hybridised defects (R=H and OH) can occur in strongly curved CNTs, such as SWNTs. In addition, certain functionalisation and purification procedures can introduce defects to the structure: COOH groups can be attached to sidewalls (C) and terminus (D).
(figure 3.2.1, defect D), can be a result of the removal of catalysts, often found in the tip of the CNTs, through oxidative methods [65–69].

In addition to the topological defects, there are often structures present within CNT samples that exhibit more extensive defects. These can have a bamboo-like structure or discontinuous and cone-shaped walls [60].

In general, the SWNTs contain fewer structural defects than the MWNTs. However, all CNTs can tolerate a certain number of defects in their structure without it affecting their electronic and mechanical properties [69]. Therefore, it is acceptable to add defects into the CNT structure in order to provide further attachment sites for functional groups.

### 3.3 Functionalisation

It is beyond the scope of this thesis to explain in detail all the routes by which CNTs can be functionalised. Instead, the aim of this section is to give a brief introduction to the processes involved. A more detailed description can be found in reference [64].

Functionalisation of CNTs does not only offer a possibility to modify the chemical properties of the CNTs, but it can also increase their solubility [70]. This is an important property since the use of CNTs for biomedical applications is strongly dependent on the CNTs being soluble.

There are several ways of functionalising the CNTs (figure 3.3.1), including: defect group functionalisation (figure 3.3.1A), sidewall functionalisation (figure 3.3.1B), sidewall functionalisation with molecules (such as surfactant) through \( \pi \)-stacking (figure 3.3.1C), non-covalent exohedral functionalisation with polymers (figure 3.3.1D) and endohedral functionalisation (figure 3.3.1E).

In general, functionalisation routes are classed as covalent and non-covalent.

**Covalent Functionalisation**  The strongly curved region of the endcaps of SWNTs (with a low diameter of 1-2nm) have a local chemistry comparable to fullerene molecules. These regions are susceptible to addition reactions resulting in covalent sidewall functionalisation of the SWNTs (figure 3.3.1B) [71]. These direct addition reactions are accompanied by a change in \( \text{sp}^2 \) to \( \text{sp}^3 \) hybridisation of the carbon atom. MWNTs have a larger diameter and therefore do not undergo covalent sidewall functionalisation via direct additions.

For defect functionalisation (figure 3.3.1A), defects present in the CNT structure can be utilised as attachment sites. Alternately, defects can be introduced to the structure by oxidative purification of the CNTs which introduces carboxylic groups (considered to be defects) to their surface. This oxidative purification of the CNTs can be achieved using sulfuric acid [72], nitric acid [73–76], a mixture of sulfuric acid and nitric acid [76], sulfuric acid-hydrogen peroxide [67], ozone [66, 77, 78], or by heating the CNTs to high temperatures in air [79, 80], to name just a few of the methods.
The introduction of carboxylic groups creates anchor groups for further modification. Methods occurring through transformation and modification of the carboxylic functions include amidation [67–69] and fluorination [81–83] of the CNTs. Other modifications include hydrogenation [84], addition of radicals [85] and cycloadditions [68, 86, 87]. One of these cycloaddition methods using the 1,3-dipolar addition of azomethine ylides, developed by Prato et al. [86], was used to functionalise the NH$_3^+$ f-MWNTs used in this thesis.

**Non-covalent Functionalisation**  Non-covalent functionalisation of MWNTs is largely based on the formation of complexes using Van der Waals forces and π-stacking interactions. The prospect of functionalising the CNTs without changing the CNTs structure itself through, for example, destructive oxidative treatments, is appealing. By dispersing the CNTs in surfactant for example, one can shield the hydrophobic surface of the CNTs with a hydrophilic terminated dispersant, aiding their solubility in water [88]. Remarkably, the surface of the surfactant coated CNTs can be imaged using TEM [89]. Furthermore, CNTs, in the presence of conjugated polymers in organic solvent, were found to form supramolecular complexes with the polymers [90, 91]. This can be seen in figure 3.3.1D, where the polymers are wrapped around the CNTs.
where the CNT is filled with small molecules or atoms [75, 92]. Hence, functionalisation of the CNTs enables not only improvement of the solubility of this material, but also the combination of its intrinsic unique properties with the properties of other materials. This creates numerous potential applications for CNTs. Medicine is just one of the industries that holds great hope for CNT applications. Some examples of these potential medical applications will be given in the following chapter in section 4.1.
Chapter 4

Applications of Carbon Nanotubes

4.1 Medical Applications of Carbon Nanotubes

Functionalised CNTs (f-CNTs), single-walled nanotubes (SWNTs), double-walled nanotubes (DWNTs) and multi-walled nanotubes (MWNTs) are all receiving increasing attention for medical applications. One advantage of using f-CNTs, rather than other nanoparticles (NPs), is the high surface area of the f-CNTs which provide numerous attachment sites for functional groups. In addition, the f-CNTs can carry cargo inside the tube. Again, the volume inside the f-CNTs is large compared to other NPs. Furthermore, certain classes of f-CNTs have been found to be non-cytotoxic [93]. Also, f-CNTs may be able to translocate across the plasma membrane [34] and enter the cell cytoplasm directly. The controversy in the literature surrounding the latter phenomena will be discussed in detail in section 4.2.

Overall, f-CNTs are attractive candidates for diagnostic, as well as therapeutic applications. In cancer therapy, f-CNTs can be used in various ways to overcome the challenge of selectively killing tumour cells without harming the normal cells and tissue [9–15]. Other applications include the use of f-CNTs for delivery of vaccines [16–18], Nucleic Acids [19–22] and drugs [23, 24], and also as scaffolds in bone tissue engineering [25–27].

Cancer Treatment Several groups have been working on developing cancer therapies using f-CNTs. The f-CNTs are most commonly used to deliver drugs or other anti-cancer agents into the tumour cells in order to selectively target and kill cancerous cells. The following section summarises the main findings of each of these studies.

- Zhang et al. [9] studied the use of f-SWNTs carrying complexed small interfering RNA (siRNA) for cancer therapy. More specifically, the aim was to determine whether the siRNA could be delivered into the cancer cells by the f-CNTs. They found that the complexed siRNA was not only carried into the tumour cells by the f-SWNTs, but was also released from the f-SWNTs, silencing the targeted genes. This lead to the suppression of tumour growth.
• An in vivo study by Liu et al. [13] was based on using SWNTs, functionalised with the cancer chemotherapy drug paclitaxel (SWNT-PTX), to deliver the drug directly to the tumour site. It was shown that even at relatively low doses, the SWNT-PTX resulted in a reduction of the tumour growth rate. The SWNT-PTX showed better results in suppression of tumour growth than the common cancer treatment drug Taxol. According to the authors, this was due to the prolonged circulation times of SWNT-PTX as well as a greater retention rate of the SWNT-PTX by the tumour sites. Furthermore, no toxic side effects were observed.

• Podesta et al. [14] used f-MWNTs and cationic liposomes (as a control) for delivery of cytotoxic siRNA into human lung carcinoma as well as murine cells. They found that only the f-MWNTs-mediated delivery of siRNA lead to suppression of the tumour growth and a reduction in tumour volume after direct injection into the tumour site.

• Bhirde et al. [15] used multi-functionalised SWNTs to selectively destroy cancer cells by targeted drug delivery. The SWNTs were bioconjugated with anticancer agents and targeting ligands to specifically attack the cells that exhibited the receptors to these targeting ligands: the cancerous cells. As a control, the study was repeated using SWNTs bioconjugated with the anticancer agents, but without the targeting ligands. The cell culture study showed that the complexes containing targeting ligands were taken up more readily by the cancer cells than the complexes not containing targeting ligands. Furthermore, they injected the SWNTs into live mice and showed that the complexes containing targeting ligands were found to selectively enter cancer cells and kill them. Importantly, the complexes containing targeting ligands was found to reduce the tumour in size. Both effects were not observed in the control experiments.

Cancer Imaging Another approach for using f-CNTs in cancer treatment is to selectively target cancer cells with the aim of selectively labelling them for diagnostic purposes or further treatment. Several examples are given below.

• In order to obtain an improved understanding of the targeted accumulation of f-CNTs in the body, Liu et al. [11] used PEGylated SWNTs 1 to study the biodistribution of f-SWNTs in vivo. Using positron emission tomography, ex vivo biodistribution and Raman spectroscopy it was found that the f-SWNTs were largely retained in the body and remained in circulation for 24 hours. In addition, the PEGylated SWNTs were conjugated with peptides that have a high affinity for binding to specific receptors of the tumour neovascular, and to a lesser extent, of tumours, in order to target tumours in mice. An accumulation of the targeted f-SWNTs in the tumour tissue was observed. Hence, by functionalising the SWNTs one can specifically target tumour sites in vivo.

• The tumour targeting properties of f-SWNTs were used for diagnostic purposes in a study by De La Zerda et al. [12], since photoacoustic imaging relies on the use of contrast

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1PEGylation of CNTs describes the process of covalent attachment of polyethylene glycol polymer chains to the CNTs.
agents as most diseases do not show photoacoustic contrast otherwise. De La Zerda et al. [12] studied the use of targeted SWNTs as contrast agent for imaging tumours in living subjects. The SWNTs were conjugated with peptides similar to the f-SWNTs used by Liu et al. [11]. The photoacoustic signal of the targeted f-SWNTs was found to be linearly proportional to the f-SWNT concentration. In addition, mice injected with the targeted f-SWNTs were found to have eight times higher photoacoustic signal in the tumorous region than the mice injected with untargeted SWNTs. This result showed that targeted f-SWNTs could be used to image and monitor tumours, especially in the early stages of tumour development when the natural photoacoustic contrast is low.

- Gannon et al. [10] discovered that SWNTs heat up when in a radiofrequency field (RF). A technique based on this property was developed to selectively kill cancer cells in vitro, that contain f-SWNTs, through application of a RF. It was found that the tumours injected with f-SWNTs and treated with RF became necrotic, whereas tumours that were not injected with f-SWNTs, but treated with RF remained unaffected. Future developments combining this technique with the use of targeted f-SWNTs, would provide a powerful, but non-invasive, method to selectively attack cancerous cells in living subjects.

**Vaccine Delivery** In a study by Pantarotto et al. [16,17], peptides of the Foot-and-Mouth virus were attached to the CNTs, and presented to the immune system of mice. The mice treated with the complex showed a higher antibody count compared with the mice exposed to the free peptide. The enhanced antipeptide immune response showed the potential of f-CNTs as new vaccine delivery vehicles.

Meng et al. [18] investigated the use of f-MWNTs for antitumour immunotherapy by attempting to mount an anti-cancer immune reaction. They reported that the tumour ablation rate was improved by the use of f-MWNTs conjugated to tumour lysate proteins. The immune response against the tumour was also found to be enhanced. However, the exact mechanism of how the tumour lysate protein acts to enhance the antitumour immune reactions remains to be explored.

**Nucleic Acid Delivery** The introduction and expression of foreign DNA by cells is of great medical interest and the use of f-CNTs as delivery vehicles of the foreign DNA into the cells is being investigated by several groups.

- Pantarotto et al. [19] used covalently functionalised CNTs as intracellular gene delivery systems. The f-SWNTs were further functionalised using plasmid DNA to form large complexes [21]. It was found that using the f-SWNTs as delivery vehicles to chinese hamster ovary (CHO) cells enhanced the DNA uptake by the cells, leading to a higher gene expression, compared to using the DNA alone. However, little is known about how these complexes enter the cells and whether they are trafficked inside the cells.

- Lu et al. [20] investigated the use of f-SWNTs as vectors to transport RNA polymer poly(rU) into breast cancer cells. Translocation of the fluorescently labeled SWNT-poly-
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(rU) hybrids into the breast cancer cells in culture was observed using confocal microscopy.

- Synthetic oligodeoxynucleotides containing CpG motifs (ODN CpGs), which are short single-stranded synthetic molecules of DNA, are known to increase protection against various pathogens intracellularly by enhancing the immune response. As such, they show great promise in vaccines, cancer therapy and allergy treatment. Bianco et al. [22] investigated whether the f-CNTs could bind with ODN CpGs, acting as delivery vehicles to enhance the immunostimulatory properties. The f-CNTs increased uptake of the ODN CpGs and also enhanced their immunostimulatory potential substantially. The authors suggested that the surface charge of the complex was more favourable for cellular uptake than the surface charge of the molecules alone. Again, the exact pathway(s) by which the complexes entered the cells remain(s) unknown.

Drug Delivery Using f-CNTs for drug delivery applications has the advantage that their surface can be functionalised using different compounds, resulting in multi-functionalised CNTs.

In the in vitro study by Wu et al. [23], MWNTs were functionalised with fluorescent probes for tracking after uptake, as well as with active molecules, amphotericin B (AmB), an antifungal drug. The drug AmB on its own caused overt toxicity to mammalian cells, as the molecules tend to aggregate into large clusters in water. However, by conjugating the f-MWNTs with AmB, this problem can be overcome. Using epifluorescence and confocal microscopy, it was found that AmB bound to f-MWNTs had a high fungal activity without causing any toxic effect to human Jurkat lymphoma T cells.

Yinghuai et al. [24] investigated the use of f-SWNTs as drug delivery vectors for boron in boron neutron capture therapy (BNCT), a form of radiotherapy. The SWNTs were functionalised by attaching carborane cages via nitrene cycloaddition to their sidewalls. It was found that there was a higher concentration of boron in the tumour regions, suggesting that f-SWNTs have great potential as boron delivery vehicles for BNCT.

Despite the great potential of using f-CNTs for medical applications, all of the applications described above rely on the body being exposed to the f-CNTs and the safety risks of exposure to this class of nanomaterial are currently unknown. Furthermore, several of the applications depend on the f-CNTs entering the cell cytoplasm. Although there have been several reports which have shown that the f-CNTs enter cells, it is also necessary to understand the pathways by which the f-CNTs enter cells to direct their development as targeted drug delivery vectors in medical applications.

In order to ensure the safe commercial development of CNTs in therapeutic applications it is essential to have a full understanding of their interactions with the cells in the human body. This can only be achieved by investigating the interactions of CNTs with the cells in a mechanistic manner.
4.2 Controversy around Cellular Uptake

One of the challenges that scientists face when using CNTs are the many factors that potentially influence the interactions between CNTs and the cells in biomedical applications. Varying experimental conditions, different CNT preparations and the use of different toxicity assays make comparison between experimental studies difficult.

In recent years, several groups have been working on studying the cellular uptake of carbon nanotubes. Pantarotto et al. [34] were the first to report uptake of f-SWNTs by cells through passive non-endocytic uptake routes. Using epifluorescence and confocal microscopy the group detected no difference in uptake between exposures at 37°C and 4°C with the addition of endocytotic inhibitors. Therefore they deduced that the uptake must be an energy-independent process. In a second paper [19], Pantarotto et al. used HeLa cells and exposed them to f-SWNTs and f-MWNTs to study the use of f-SWNTs as carriers for DNA gene delivery. They noted an increase in DNA gene delivery when using f-SWNTs or f-MWNTs as drug delivery agents. In addition, the study was complemented by use of bright field (BF) TEM imaging showing single f-MWNTs at the plasma membrane.

In the same year, Cherukuri et al. [32], using spectrofluorometry and fluorescence microscopy, reported a steady uptake of SWNTs coated in pluronic surfactant in macrophage-like cells at 37°C, which was reduced at low temperatures of 27°C. The authors deduced that the uptake must be an active, energy-dependent process and after assessing the size of the clusters of SWNTs inside the cells, suggested that the uptake is most likely to occur via phagocytosis.

In the following year, Kam et al. [33] undertook an extensive study on the uptake of f-SWNTs. In a first step, a temperature of 4°C and the addition of the endocytosis suppressing Sodium Azide (NaN₃) were used. At these conditions the authors noted a significant reduction in uptake and deduced that the uptake must be an energy-dependent process. In a second step, the authors introduced a K⁺ depletion aiming to suppress the clathrin-mediated uptake. This resulted in the suppression of f-SWNTs uptake, leading the authors to suggest that f-SWNTs enter cells via clathrin-mediated endocytosis. In order to exclude the possibility of caveolin-mediated uptake, the effect of cholesterol depletion on uptake of f-SWNTs was studied using filipin and Nyastin. No change in uptake was observed and hence the uptake via the caveolae sites was thought to play a negligible role in the uptake of f-SWNTs.

All three groups proposed different uptake pathway of CNTs into cells. These conflicting results highlight the complexities underlying the intracellular uptake of CNTs or the lack of consistency in design of these studies.

4.2.1 The Possibility of Non-endocytotic Uptake

Since the process of passive diffusion or spontaneous insertion of f-CNTs into the plasma membrane constitutes an energy-independent pathway into the cells, the occurrence of this process could have important consequences for the medical applications of f-CNTs as well as their toxicity to cells. If f-CNTs can cross the plasma membrane directly, they may also be
Controversy around Cellular Uptake

Figure 4.2.1: BF TEM images of f-MWNTs at the plasma membrane showing inconclusive evidence for the non-endocytic uptake mechanism. Image (a), adapted from Pantarotto et al. (2004) [19], shows a gradual increase in electron-density of the material at the membrane (red arrows), suggesting increasing cell material thickness from position A to B. The relative z-height position of f-MWNT is unknown. Image (b), adapted from Kateb et al. (2007) [35], shows no evidence for piercing of the membrane (red arrows). Image (c), adapted from Mu et al. (2009) [36], shows an electron-dense area surrounding the f-MWNT (circled in red), suggesting that the plasma membrane is wrapped around the f-MWNT, or that the f-MWNT is interacting at an incurvature in the plasma membrane.

capable of crossing other membranes, including the membranes surrounding the mitochondria and the cell nucleus. Furthermore, by entering the cell cytoplasm directly, the f-CNTs would escape intracellular trafficking by the cells, with important consequences for their biostability.

Passive diffusion or spontaneous insertion of f-CNTs into the plasma membrane of cells have been studied both via experimental and computational approaches.

Experimental Studies  As described above, Pantarotto et al. [19,34] were the first to suggest that f-CNTs enter cells via passive diffusion processes. Shortly afterwards, Lu et al. [20] used confocal fluorescence microscopy to analyse the uptake of RNA polymer f-SWNTs by cancer cells. The group presumed uptake by direct passive translocation of the f-SWNTs through the plasma membrane. However, due to the lack of resolution and the fact that the f-SWNTs were conjugated to large fluorescent molecules, which modify their surface chemistry, one cannot exclude endocytic uptake routes such as clathrin- and caveolin-mediated uptake as possible mechanisms of entry.

After the studies by Pantarotto et al. and Lu et al., other groups [35, 36] followed suit and assumed that passive diffusion of f-CNTs through the cell membrane was not only possible, but that it constitutes the main pathway of f-CNTs into cells.

The results described above [19,34–36] support the hypothesis that f-CNTs can enter cells via non-endocytic pathways. Nevertheless, the cells used in these studies also internalise substances by active energy-dependent processes, therefore active uptake of the f-CNTs into the cells cannot be excluded as a possibility, especially since the evidence presented in favour of the passive uptake is often insufficient and inconclusive.

Bright field (BF) TEM images from several groups [19,35,36] showed that the f-MWNTs appear
Controversy around Cellular Uptake

Figure 4.2.2: Using single-particle tracking (SPT) of the band gap fluorescence of DNA-SWNTs in 3T3 cells, Jin et al. (2008) [94] observed SWNTs associating with the plasma membrane as a function of time.

to pierce through the membrane of the cells. However, the resolution of the TEM images used was too low to identify the relative position of individual tubes and the plasma membrane (figure 4.2.1).

The TEM image in figure 4.2.1a from Pantarotto et al. [19] was acquired at low resolution and is only a 2D projection image, which makes it impossible to determine the relative position of the plasma membrane and the f-MWNT, whether the membrane is wrapped around the f-MWNT or whether an actual ‘piercing’ has taken place. In addition, it is not clear whether the f-MWNT has pierced the plasma membrane, since the gradual increase in electron-density of the material at the membrane (red arrows) suggests that the membrane in position A is at a different height along the z-axis compared to the position of the f-MWNT in B. This study would benefit from 3D evidence which could be acquired through for example, 3D electron tomography studies. The image in figure 4.2.1b from Kateb et al. [35] shows an individual f-MWNT at the plasma membrane, it is not clear however if this f-MWNT has pierced through the membrane or not. It appears the membrane has an incurvature at the site, as there is an electron dense line visible and curving inwards on both sides of the f-MWNT (figure 4.2.1b, red arrows). BF TEM imaging was not sufficient here to determine whether the f-MWNT had pierced the membrane or not. The image in figure 4.2.1c from Mu et al. [36] also shows a f-MWNT at the plasma membrane. However, the electron-dense area (circled in red) surrounding the site of interaction suggests that the membrane was wrapped around the f-MWNT and/or that the f-MWNT was interacting at an incurvature of the membrane. Hence, there is no conclusive evidence that the f-MWNT was in the process of translocating across the membrane into the cell cytoplasm.

A weakness of the studies mentioned above [19, 35, 36] is the absence of any 3D evidence. This is important since the f-MWNTs may be lying on top of the sections, or might be an artifact from the microtome knife pushing the f-MWNTs across the section. It would be premature to draw conclusions about the exact uptake mechanism based on this evidence alone, especially since studies by Jin et al. [94] clearly showed that f-SWNTs have an affinity for the membrane and a tendency to associate strongly with it (figure 4.2.2). This could lead to misinterpretation of the TEM images.

Complementary studies are needed to establish the relative positions of the f-MWNTs in relation to the plasma membrane in 3D and to establish any disruption of the membrane at the site.
of interaction. Only then could one conclusively say that the mechanism is a passive uptake process as described in 2.3.3 rather than an active process mediated by the many caveolae and pits in the plasma membrane described in 2.3.2.

**Computational Studies**  Computer simulation plays an important role in understanding the interactions of CNTs and membranes and their components. Experimental techniques can give indications of the mechanical and electrical properties of CNTs. Nevertheless, the interactions with biological systems remain difficult to investigate experimentally due to technical limitations and the large number of permutations of f-CNTs properties present in one sample. Imaging at the nanoscale can be challenging as electron microscopy only gives us snapshots of the interactions rather than a dynamical picture. Quantum mechanical, classical molecular dynamics and Monte Carlo techniques have been employed to study CNTs in cell environments as complementary techniques. This section will focus on the molecular dynamics (MD) simulations, as they are relevant to the work presented in the chapter 12.

The parameterisation of the CNT models can be divided into two general categories: the atomistic and the coarse-grained approach. The atomistic approach for classical MD simulations ignores the electronic properties of the CNTs as well as the CNT polarisation interactions. Due to the large computational costs only short time and length scales have been achieved to date. The coarse-grained approach reduces the system size substantially by grouping several atoms together and replacing them by one ‘grain’. This allows for longer simulation time and length scales. However, this coarse-graining is a balance between the efficiency of the model and the reduced accuracy introduced through the ‘graining’.

Lopez et al. [95] performed a coarse-grained molecular dynamics study of a ‘nanosyringe’ and a lipid bilayer (LB) model membrane. Spontaneous insertion of the nanosyringe into the LB was observed. The nanosyringe was represented by a generic cylindrical transmembrane molecule and was either hydrophobic with hydrophilic functionality at the end of the tube or purely hydrophilic. A later study by the same group [96] found that the length of the tubes played an important role in the stability of the system. If the length of the hydrophobic region of the tubes exceeded the width of the LB, the latter swelled, and if it was shorter, the membrane compressed. Furthermore, the hydrophilic caps appeared to stabilize the structure. Additionally, the hydrophobic tubes were found to insert at an angle such as to maximise the contact between the hydrophobic tube and the inside of the membrane.

Wallace et al. [97] used a steered coarse-grained method to model the usage of CNTs of different diameters as nano-injectors into cells. The model was based on the model developed by Marrink et al. [98], whereby one particle represents four heavy atoms. In the Marrink model particles interact via screened Lennard-Jones and Coulombic potentials while maintaining the bond lengths and angles. In this study by Wallace et al. [97], a spring was connected to the hydrophobic SWNTs to pull it at constant velocity across the LB model membrane in order to simulate the entry and exit of a SWNT from the LB. It was found that lipids from the LB stuck to the inside as well as the outside of the tubes and some were even carried across the LB. The lipids aligned such as to chaperone the CNT through the LB, by maximising the contact between
the hydrophobic tails of the lipids with the CNT surface and orientating the polar headgroups outwards to the aqueous environment. These lipids could possibly hinder the transport of cargo inside the tubes as they could "block" the CNTs. Nevertheless, the LB remained intact during the simulation and even exhibited "self-healing" properties.

Shi et al. [99] found that CNTs with diameter of 1-5nm were taken up into the cells via wrapping mechanisms and CNTs with a smaller diameter, of approximately 0.5 nm, entered via "piercing" through the plasma membrane. The authors proposed that there is a cut-off diameter for the piercing mechanism. The wrapping of the CNTs by the LB occurs where the interaction of the CNTs with the LB is strong enough to overcome the energy required to increase the curvature of the LB. The energy needed is larger for smaller CNTs hence the piercing mechanism becomes energetically favourable. Shi et al. [99] also found that the CNTs exiting the LB were covered in lipids. The results by both Wallace et al. [97] and Shi et al. [99] suggested that the shielding of the hydrophobic surfaces of the CNTs by the lipids is essential to the uptake mechanism. However, both groups used CNTs with an overall neutral surface charge in their studies. Results by experimental studies using charged NPs found a strong dependence of NP surface charge on uptake [100], as well as on cytotoxicity [101]. Hence, f-CNTs cannot be approximated as electrostatically neutral, as their surface charge may play an important role in their interactions with the LB.

Recent work by Podogin et al. [102] attempted to answer the question of whether the translocation of CNTs is possible from an energetic point of view. The single-chain field method used is based on the description of the interaction of molecules by a mean molecular field. In the work, the translocation of CNTs across the LB was assumed to be driven mainly by thermal motion. Hence, the energetic barrier that the CNT had to overcome was assumed to be approximately equal to the energy of thermal motion. The second assumption made was that the energy barrier depended strongly on the orientation of the CNT with respect to the LB and the perpendicular position was assumed to be energetically favourable. Next, the effect of hydrophobicity/hydrophilicity was studied by comparing the translocation of hydrophobic with hydrophilic CNTs. The authors suggested that translocation of hydrophilic CNTs would be impossible as it would require too much energy. They also suggested that the hydrophobic CNTs would enter the LB but would not translocate across it, but instead, would remain inside the LB. However, in the simulations, the authors represented the CNTs by cylindrical shapes with the centre inaccessible for lipids and water molecules. This presents a problem as the lipids are thought to interact with the inside of the CNTs as well as the outside as described by Wallace et al. [97]. Thereby playing an essential role in the translocation process. Furthermore, the LB was restricted in position by non-interacting walls at the top and at the bottom of the LB. This prevented the LB from wrapping around or moving away from the CNTs, depending on their interaction. This restricted movement of the LB could have lead to an overestimation of the energetic barrier as the LB was artificially kept in position and was prevented from for example, maximising the contact area between CNT and LB to facilitate the translocation.

In 2010, Yang et al. [103] investigated the effect of the nanoparticle shape on the translocation across the membrane. By measuring the external force required to insert different shapes into the LB, the ability of NPs of different shapes to translocate the LB were compared. The group
found that the size of the contact area between NPs and LB appeared to play a crucial role, whereas the NPs size affected the ability to translocate the LB only indirectly. Therefore, the shape anisotropy and the orientation of the NPs at the beginning of the translocation process were found to be vital. A shortcoming of this work was that solid filled shapes were used, since previous work [97] showed that the lipids enter the CNTs and chaperone them into the LB. This important factor was not accounted for by Yang et al.

4.2.2 Role of Functional Groups in Cellular Uptake

A paper by Kostarelos et al. [104] on cellular internalisation of different types of cells suggested that neither the nature of the surface functionalisation of the f-CNTs nor the cell type affected the uptake rate of f-CNTs. Even functional groups that were of neutral charge did not inhibit the uptake. However, the study relied on confocal microscopy where fluorescein was attached to the f-CNTs for tracking, which is likely to have changed the surface chemistry of the f-CNTs. Furthermore, the study used Kaiser tests [105] to ensure that functionalisation of the f-CNTs had occurred and to determine the loading of amino functional groups of the f-CNTs. The authors failed to characterise the zeta-potential of the different types of f-CNTs. In reality, the actual loading of the f-CNT may have varied immensely within a sample. In addition, the authors did not describe the medium that the f-CNTs were dispersed in prior to cell exposure, which may have further modified the functional groups on the surface of the f-CNTs. The cell medium used was bovine serum albumin (BSA), which contains countless proteins that may have coated all of the f-CNTs in a similar manner prior to cellular uptake, shielding the functional groups present and thereby altering the surface charge of the f-CNTs. Hence, the addition of fluorescein combined with the lack of characterisation of the different f-CNTs make this study unreliable.

Whereas Kostarelos claimed that the surface functionalisation plays no role in the uptake of the CNTs, there is also evidence supporting the opposite claim. Pantarotto et al. [34] suggested that the cationic f-CNTs used were able to bind to the plasma membrane and subsequently undergo a "spontaneous insertion". According to their hypothesis there is a strong dependence on charge for the translocation mechanism to occur. In a study using gold nanorods Hauck et al. [100] assessed the effect of surface charge on cellular uptake of gold nanorods. Gold NPs that were coated in a negatively charged layer exhibited the lowest uptake, whereas gold NPs coated in a positively charged layer exhibited the highest uptake. This suggested that uptake of gold NPs is highly charge dependent and hence the uptake of f-CNTs might also be highly surface charge dependent.

A factor that is often overlooked is the interactions with the extracellular milieu that the f-CNTs and other NPs undergo prior to uptake. Firstly, the NPs are dispersed in solution, often dextrose or surfactants, in order to improve the dispersion prior to exposure. Secondly, the NPs are added to the cell medium which consists of ions and proteins. At both stages there is a strong possibility that other molecules and particles interact with the NPs to form complexes of a different chemical composition. To date, these reactions are difficult to assess, let alone
predict. Work by Walczyk et al. [106] attempted to raise awareness of the fact that the actual corona surrounding the NPs might be more important when determining their interactions with the cells than the bare material prior to exposure. Further, the review by Nel et al. [107] gives an extensive overview of the protein corona and the NPs interactions and the underlying complexities.

Another difficulty is the presence of a vast range of surface charge distributions within a sample of f-CNTs. This adds another layer of complexity to assessing the effect of charge on cellular uptake of f-CNTs experimentally, especially as the location of the defects, introduced before as well as after the functionalisation of the CNTs, are unknown. There is currently no direct technique to assess the number as well as the location of the functional groups added to the surface of the individual CNT prior to exposure.

It may be possible to compare directly the effect of surface charge on the cellular uptake by using computational methods. Unfortunately, there has been very little work published on this matter. Only Shi et al. [99] did work on the entry and exit mechanism of SWNTs and the LB using "functionalised" CNTs. They found that the main forces governing the uptake were the Van der Waals (VdW) forces and hydrophobic forces. The group took the functionalisation into account by increasing the interactions between the CNTs and the head groups. These interaction parameters were set artificially and might have, as the authors pointed out, overestimated the interactions between the CNTs and the LB. A further concern of this particular model is the use of a reduction of the interaction in the neck region of the LB when wrapped around the CNT. This was added to mimic the actions of the transmembrane proteins which facilitate the separation of cargo surrounded by the LB to form vesicles [108]. Even so, the question of whether the CNT would actually translocate the LB if this reduction of interaction were not included was not addressed by the authors.

It is crucial to explore the effects that functional groups attached to the CNTs could have on the transport properties of the CNTs in order to explore the possibility of using CNTs for drug delivery applications. If the surface charge plays a significant role in uptake, one could tailor the surface properties of f-CNTs in order to control their uptake into cells.

### 4.3 Controversy around Long Term Fate of Carbon Nanotubes

In order for the applications of CNTs in medicine to become feasible in the future one has to not only understand how the CNTs enter the cells, but also what happens to the CNTs once inside the cells. The question of the intracellular fate of CNTs needs to be addressed in order to acquire a full understanding of their cytotoxic effect in the long term and thereby assess their potential as drug delivery vehicles. The issues of the final destination of CNTs as well as their intracellular biopersistence are discussed in this section.
4.3.1 Trafficking of Carbon Nanotubes

Elucidating the final destination of CNTs inside the cell is crucial to determine their long term effects on human health. However, relatively little work has been done to establish how CNTs are trafficked inside the cells.

**Intracellular Trafficking**  One of the few studies on intracellular trafficking was by Jin et al. [94], who observed endocytosis and exocytosis of CNTs using single-particle tracking (SPT) of the band gap fluorescence of DNA-SWNTs inside 3T3 cells (fibroblast cells from culture). The medium around the cells was kept under a steady flow to ensure that the f-SWNTs that were not absorbed to the cells were washed away. The authors claimed that the exocytosis and the endocytosis rates were found to be similar due to the self-regulation of the cells. In addition, no signs of cytotoxicity were observed. Unfortunately the technique used in this study does not provide information about the exact intracellular location of the f-SWNTs.

Whereas Jin et al. [94] found that SWNTs can be exocytosed, a recent study by Wang et al. [110] found the opposite. Recombinant protein toxins, ricin A-chain (RTA), were tagged fluorescently which in turn were attached to the MWNTs to form f-MWNT complexes. The fluorescent tagging allowed the tracking of the complexes in the cell using laser scanning confocal microscopy and flow cytometry. They found that the f-MWNTs were taken up via clathrin-mediated endocytosis, leading to the formation of intracellular vesicles. These endosomes then transported the cargo to the endoplasmic reticulum, where the f-MWNTs were passed to the cytosol. This resulted in a cytotoxic response due to the presence of the RTB protein toxins. The study was complemented by use of TEM imaging. The images do not conclusively show the intracellular locations of the f-MWNTs and the use of the fluorescent tagging may have interfered with the processes the authors were trying to probe in the first place, due to the change in surface chemistry of the complexes.

**Trafficking inside the Body**  In the in vivo study by Sato et al. [109], the authors claimed to have found aggregates of MWNTs inside the cytoplasm which would be a sign that the MWNTs can exit the lysosomes. They implanted f-MWNTs into the subcutaneous tissue of rats to study the influence of f-MWNTs length on cytotoxicity. However, the conventional TEM images in figure 4.3.1 from [109] were acquired at low magnification which makes it difficult to determine whether the f-MWNTs are surrounded by a membrane or not. These f-MWNTs could also be inside intracellular vesicles. Furthermore, MWNTs reaching the cells through inhalation or through trafficking to the site may interact very differently from the MWNTs implanted into the tissue. Large aggregates may never even reach these locations through trafficking.
Controversy around Long Term Fate of Carbon Nanotubes

In a study on trafficking using NPs, Faraj et al. [111] used a combination of $^3$He and proton magnetic resonance imaging (MRI) of magnetically labelled magnetite biodistribution in a two week in vivo study. The study attempted to address the issue of trafficking of NPs inside organisms. One of the flaws of the technique was that MRI is not suitable for lung tissue imaging due to the low proton density. Since most of the particles in the study seemed to remain in the lung tissue, the technique seems to have been unsuited. Additionally, the magnetically labelled NPs may have behaved differently from non labelled NPs, due to the altered properties. High resolution TEM imaging of the sample at different time points would give a better insight into the fate of the NPs once inhaled or injected.

4.3.2 Trafficking and Toxicity

A paper published by Poland et al. [112] in May 2008 caused significant controversy in the nanotechnology community. The group claimed that high aspect ratio CNTs induce toxic effects in the abdominal cavity of mice comparable to asbestos. In their in vivo study, 50 μg of non-functionalised MWNTs of different lengths was directly injected into the abdominal cavity of mice. The MWNTs with a higher length-diameter aspect ratio were found to be more toxic, causing lesions. The reason for their toxicity was presumed to be that macrophage cells cannot engulf the large particle aggregates, resulting in “frustrated phagocytosis" where the cells fail to degrade the long and rigid nanotubes. However, several factors were not taken into account in the study. For example, it was not tested whether the different preparation methods of the NTs contributed to the difference in toxic response. This is an important factor as remainders of catalyst or other chemicals inside, or on the surface of the f-MWNTs, can cause cytotoxicity [113,114]. The inflammatory response of abdominal cavity of mice might not be comparable to the effect CNTs might have when inhaled. Further, there was no direct observation of the CNTs actually causing the formation of cancer as was the case for the asbestos sample. The highest inflammatory response was measured from the longest CNTs, but it needs to be tested whether a sufficient number of CNTs of similar aspect ratio would reach the lung tissue when inhaled. In a second study, Donaldson et al. [115] stated that only CNTs with a high aspect ratio, with a length of 15 μm and longer, can cause inflammatory response, due to the cells undergoing “frustrated phagocytosis". However, these large CNTs are not strictly speaking "nanosized“ anymore and their toxicity should be assessed as such. In addition, as already mentioned above, it is questionable whether these high aspect ratio CNTs can even reach the lung tissue through inhalation. It is therefore premature to draw parallels between the cytotoxicity caused through exposure to asbestos and CNTs.

Recent work by Ryman-Rasmussen et al. [116] aimed to study whether the CNTs can reach the lung tissue after inhalation, and found that some f-MWNTs can reach the subpleural tissue in mice. The authors claimed that the f-MWNTs used for this study were of a length of 0.5 μm to 50 μm long, which has to be noted, is a large size distribution within a single sample. Again, at the higher end of the length spectrum, these CNTs cannot be considered to be "nanosized". Furthermore the evidence presented showed only f-MWNTs of shorter length (<500nm) inside the tissue. The f-MWNTs might have been shortened during the preparation process prior to
exposure or only the shorter f-MWNTs were able to reach the subpleural tissue - one cannot conclusively say which was the case. Nevertheless, this study showed that short f-MWNTs can enter the body through inhalation and might accumulate in the tissue. This highlights the need for assessing the biopersistence of the f-MWNTs inside the human body.

4.3.3 Biopersistence of Carbon Nanotubes

Before the potential of CNTs as drug delivery vehicles can be realised, it is crucial to address the issue of biopersistence of the CNTs. It is difficult to predict the possible side-effects the CNTs could have on the human body if the CNTs remain in circulation inside the body after administration as drug delivery vehicles. Hence, understanding the biopersistence of CNTs is crucial when determining the long term effects to the human health of CNTs exposure.

Most research on the biopersistence of CNTs has been based on enzymatic degradation of CNTs outside of the cellular environment. Recent evidence by Allen et al. [37,38] indicated that oxidised f-SWNTs disintegrate by natural enzymatic catalysis when incubated with horseradish peroxidase. Using visible-near-infrared (vis-NIR) absorption spectroscopy and Raman spectroscopy, the degradation of the SWNTs was monitored over 12 weeks. There have also been further reports that f-SWNTs lose graphitic structure in the presence of the human neutrophil enzyme myeloperoxidase (hMPO) and low concentrations of hydrogen peroxide [39]. This work suggested that the presence of carboxylic acid groups and defects on the graphitic surface of the f-SWNTs provide a site for interaction with oxidising agents (e.g. superoxide radicals) leading to unzipping of the f-SWNT walls. A recent report has shown that the diameter of purified, oxidised and nitrogen–doped MWNTs was reduced in the presence of horseradish peroxidase and hydrogen peroxide [40]. The authors suggested that the MWNTs can be degraded in a layer-by-layer degradation process. In addition, the stability of f-SWNTs has also been studied in vitro by mimicking the oxidative environment of phagolysosomes [41].

The only work on biostability of CNTs in cells from culture was done by Kagan et al. [117], who demonstrated that the f-SWNTs degrade in the presence of the human neutrophil enzyme myeloperoxidase (hMPO) and low concentrations of hydrogen peroxide. They also found that neither hMPO nor H$_2$O$_2$ alone degraded the CNTs, which suggests that reactive superoxide radical intermediates, generated by the action of hMPO on H$_2$O$_2$, catalyze the degradation of the CNTs. Molecular modeling indicated that interactions between basic amino acids of the enzyme and the carboxyl groups on the CNTs, position the CNTs near the catalytic site. Kagan et al. also suggested that pristine CNTs were less susceptible to biodegradation.

The studies described above all relied on bulk techniques such as Raman spectroscopy, providing no insight into the detailed mechanisms leading to the loss of graphitic structure. And therefore, the precise mechanism by which the CNTs degrade inside cells has not been analysed experimentally and hence remains elusive.

Several groups have studied the mechanisms of degradation using computational methods. The mechanism of layer-by-layer degradation of f-MWNTs, as suggested by Zhao et al. [40], has been supported by first-principles quantum-mechanical simulations [118], showing that
Controversy around Long Term Fate of Carbon Nanotubes

Figure 4.3.2: Using first-principles quantum-mechanical simulations, Li et al. (2006) [118] investigated the formation of epoxy bridges. (a) The oxygen atoms acts like a wedge, pushing the carbon atoms apart. (b) If the epoxy bridges align on opposite sides of the hexagonal structure, they have a lower energetic barrier to overcome. (c) Hence the epoxy bridges aligning on the CNT structure is energetically favourable. The schematics were adapted from Li et al. (2006) [118].

reactive carbons on opposite sites of the hexagons on the f-CNT walls form epoxy bridges in an oxidising environment. The carbon atoms inside a graphite structure are arranged in a hexagonal lattice with each carbon atom being attached in a flat bond to three other atoms held together via VdW interactions. The so-called "epoxy bridge" forms through a single oxygen atom attaching itself to two adjacent carbon atoms. The severe strain resulting from this bridge is a result of the different 3D geometry of the bonds with the oxygen, acting like a wedge pushing the two carbon atoms further apart, increasing the bond lengths from 1.42 Å to 1.58 Å [118]. This mechanism is shown in figure 4.3.2a, where the oxygen atom moves down between the carbon atoms as maintaining the flat bonds of the graphite structure is energetically favourable. Further, Li et al. suggested that the epoxy group can move onto different sites in the graphite structure. They found that an epoxy group, moving to the site opposite another epoxy group on the same hexagonal ring (path 2 in figure 4.3.2b), had a lower energetic barrier to overcome (path 2 in figure 4.3.2c) and a more stable configuration than an epoxy group, moving to a site on the same side of the hexagonal ring (path 1 in figures 4.3.2b and 4.3.2c). Hence, a structure where the epoxy groups are aligned in the manner shown in figure 4.3.2d was found to be energetically favourable. The strain generated by the cooperative alignment of these epoxy bridges could lead to break initiation and propagation along the f-CNT walls resulting in nanotube “unzipping”.

Using scanning probe microscopy, Pandey, Reifenberger and Piner [119] studied the oxidation of graphene sheets and observed oxygen atoms arranged along zigzag edges and not in a line as suggested by Li et al. [118]. Further, the lattice constants and C-C bond lengths were found to be (0.273 ± 0.008) nm and (0.406 ± 0.013) nm (figure 4.3.3b). This is contrary to previous predictions [118].
Controversy around Long Term Fate of Carbon Nanotubes

Figure 4.3.3: Using scanning probe microscopy (image (a)), Pandey et al. (2008) [119] showed the oxygen atoms aligned along zigzag edges as shown in the schematic (b). The figure was adapted from [119].

More recently, Xu and Xue [120] also investigated how oxygen atoms line up on a pristine graphene sheet. Using first-principle calculations, they were able to distinguish between two possible states, a metastable clamped state and an unzipped state (figure 4.3.4a). In the clamped state, the oxygen absorbs onto the sp² bond without breaking it, hence the overall lattice structure of the graphene remains intact (figure 4.3.4b). The epoxy group forms an almost equilateral triangle with an angle between the C-O bonds close to 150°. However, this metastable clamped state is only possible for high oxidation densities. Below a certain density threshold, this configuration ceases to exist and the structure relaxes into the unzipped configuration, resulting in the creation of line defects on the graphene structure.

In general, the graphene structure is chemically inert and hence the carbon interacts only very weakly with the oxygen atoms. Unless there is an impurity in the form of a defect in the structure. In f-CNTs, defects can be introduced during the functionalisation process, especially since an oxidation step using strong acids often precedes the addition of the functional groups to the f-CNTs surface. The role of this functionalisation process in the biostability of f-CNTs has been studied in an ex vivo enzymatic degradation study, using f-SWNTs and mimicking the oxidative environment of the phagolysosomes. Liu et al. [41] found that the biostability of f-SWNTs depends strongly on the prior functionalisation process of f-SWNTs. Acid carboxylation of the f-SWNTs appeared to reduce their biostability, compared with p-SWNTs and f-SWNTs that underwent other surface modifications. A reduction in their overall lengths as well as total disintegration of the oxidised f-SWNTs were observed in some cases. The authors suggested that the carboxylate group leaves only one backbone bond intact, as opposed to the epoxide group which leaves three backbone bonds intact, leading to the creation of points of attack for further oxidative degradation. The carboxylate group would therefore result in a less biostable f-SWNTs than other functional groups, including the epoxide group. This suggests that it is not through the epoxy bridges that the degradation is initiated, but through the presence of the carboxylate groups.

If not controlled then this oxidation compromises the strength of the structures. However, the oxidation mechanism has also been used in a controlled manner, during the production of
Controversy around Long Term Fate of Carbon Nanotubes

Using first-principles calculations, Xu and Xue (2010) [120] studied how oxygen atoms line up on a pristine graphene sheet. (a) Two states were observed, the clamped and the unzipped state. (b) The different configurations lead to different C-C bond lengths. The figure was adapted from [120].

Single sheets of functionalised graphene through thermal exfoliation [121] and when producing nanoribbons by exfoliation of CNTs through oxidation [122].

In summary, there is a need to correlate the *in situ* work with animal studies and use HR TEM imaging to determine the mechanisms by which the CNTs degrade.
Part II

Instrumentation
Chapter 5

Instrumentation: Molecular Dynamics Simulations

In this chapter, the coarse-grained molecular dynamics (CG MD) techniques employed in this work will be introduced. For further reading, the manual for Gromacs users [123] provides a good overview of the methodology.

CG MD was used as part of the work presented here to complement the electron microscopy study on cellular uptake of carbon nanotubes (CNTs). The aim of the CG MD was to study the effect of CNT surface charge and surface charge distribution on the translocation of CNTs across the lipid bilayer (LB).

5.1 Molecular Dynamics Basics

Fundamentally, molecular dynamics (MD) simulations are based on solving Newton’s equations of motion for a system of N interacting atoms:

\[ m_i \frac{\partial^2 r_i}{\partial t^2} = F_i, \quad i = 1, \ldots, N \]  

(5.1.1)

where \( F_i \) is the force exerted on particle \( i \) of mass \( m_i \) at position \( r_i \). The forces \( F_i \) are related to the gradient of the potential function \( V(r_1, \ldots, r_N) \) by,

\[ F_i = -\frac{\partial V}{\partial r_i} \]  

(5.1.2)

Given initial conditions and velocities, these equations are solved simultaneously at each time step to generate the successive particle positions and velocities as a function of time. The coordinates of the particles as a function of time represent the trajectory of the system. The interactions of components are described by a suitable molecular mechanics force field (the term force field refers to the form and parameter set that describes the potential energy of the particles within the system).
However, there are some limitations to the MD simulations approach that need to be considered.

Firstly, since the motion of the atoms are described by classical mechanics, the simulations are classical and therefore ignore quantum mechanical effects. This approximation is a good description for motion at room temperature for most systems when no bonds are broken or formed. However, MD simulations cannot be used wherever quantum mechanical effects may become significant.

Secondly, a conservative force field is used, which is dependent on the position of the particle only. The motion of the electrons is considered to adjust to the motion of the nuclei instantaneously, which is called the ‘Born-Oppenheimer approximation’ [124], and remain in the ground state. The response of electrons to the nuclear motion, excited states of systems and electron transfer processes cannot be simulated. As a result, it is not possible to simulate chemical reactions accurately.

5.2 Coarse-grained Molecular Dynamics Model

The CG MD work presented as part of this thesis was based on the model developed by Marrink et al. [98,125], whereby 1 bead represents approximately 4 (non hydrogen) atoms. This coarse-graining approach results in a 100 fold increase in speed compared with the atomistic simulations, crucial for simulating large biological systems over longer time scales. However, as with all CG MD models, the coarse-graining is a balance between the efficiency of the model and the reduced accuracy introduced through the ‘graining’.

Initially, Marrink et al. used the model to study the LB [98] and then extended it by developing the MARTINI force field [125]. The MARTINI force field was parameterised systematically against experimental data to reproduce the partitioning free energies of compounds. In addition, more interaction energy levels and particle types were included in the MARTINI force field to more closely match experiments. To date, the model has proven to be a good tool for simulating biological systems. Amongst others, it has been used extensively for studying peptide and protein interactions with the LB [126–129] as well as for studying the translocation of fullerenes [130] and CNTs [97, 99] across the LB.

The basic parameters of the Marrink model, which was used for some of the work presented in this thesis, are described below. More details about the model can be found in a paper by Marrink et al. [125].

Interaction Sites As mentioned above, heavy non hydrogen atoms are mapped 4-to-1, which means that 4 heavy atoms are represented by 1 bead in the simulation. Different mappings can be chosen if found to be more suitable. For each bead one distinguishes between four types of interaction sites: polar (P), neutral (N), apolar (C), charged (Q). In addition, each bead can be described by a number of subtypes. For particles of type N and Q, the beads can be classed
Coarse-grained Molecular Dynamics Model

depending on their hydrogen-binding capabilities: acceptor (a), donor (d), both (da), none (0); or depending on their polarity (ranging from 1 for a low polarity to 5 for a high polarity).

Non-bonded Interactions  The interactions between non-bonded beads are described by the Lennard-Jones (LJ) potential energy function [131],

$$U_{LJ}(r) = 4\varepsilon_{ij} \left[ \left( \frac{\sigma_{ij}}{r} \right)^{12} - \left( \frac{\sigma_{ij}}{r} \right)^{6} \right]$$  \hspace{1cm} (5.2.1)

where $\sigma_{ij}$ represents the effective minimum distance of approach between two beads and $\varepsilon_{ij}$ represents the interaction strength. In the Martini force field model, the interactive size is assumed to be $\sigma = 0.47\text{nm}$ for most beads and the interaction strength $\varepsilon_{ij}$ is subdivided into 10 levels, ranging from 5.6 kJ/mol to 2.0 kJ/mol [125]. The interaction strength is chosen depending on the bead type. The LJ potential energy function smoothly shifts to 0 between the distance $r_{\text{shift}} = 0.9\text{nm}$ and $r_{\text{cut}} = 1.2\text{nm}$ [125].

In addition to the LJ interactions, the interactions between charged (Q) bead types of charges $q_i$ and $q_j$ are also governed by a Coulombic potential energy function,

$$U_{el}(r) = \frac{q_i q_j}{4\pi\varepsilon_0 \varepsilon_r r}$$  \hspace{1cm} (5.2.2)

where the dielectric constant for screening, $\varepsilon_r = 15$. The potential is shifted smoothly from $r_{\text{shift}} = 0\text{nm}$ to $r_{\text{cut}} = 1.2\text{nm}$ [125].

Bonded Interactions  The interactions between chemically connected sites is not described by the LJ potential energy function, but by a weak harmonic potential,

$$V_{\text{bond}}(R) = \frac{1}{2} K_{\text{bond}} (R - R_{\text{bond}})^2$$  \hspace{1cm} (5.2.3)

where $R_{\text{bond}} = \sigma = 0.47\text{nm}$ and the force constant $K_{\text{bond}} = 1250 \text{kJ mol}^{-1}\text{nm}^{-2}$ [98, 125].

Time Scale  The interpretation of the time scale of CG MD simulations is not straightforward as the dynamics are sped up compared with atomistic models. This is due to the larger particle sizes, which smooth out interactions (smoothing of the free energy landscape) and thereby speed up dynamics. The water permeation rate across the LB was found to be sped up by a factor of 4 [125]. Therefore, a conversion factor of 4 needs to be used when discussing ‘effective time’ compared with the ‘simulation time’.

Topologies  The CG parameters for the dipalmitoylphosphatidylcholine (DPPC) were taken from the CG model and force field developed by Marrink et al. [125]. As seen in figure 5.2.1a, the DPPC headgroup is composed of two hydrophilic groups: the choline (blue bead, type:
Coarse-grained Molecular Dynamics Model

Figure 5.2.1: Coarse-grained representations of: (a) a DPPC molecule, (b) CNT, (c) water and (d) ions. The beads in (a), (c) and (d) are coloured as follows: dark blue/olive=positive/negative charged particle, pink=mixed polar/nonpolar particle, light blue= polar particle, turquoise= hydrophobic particle. In schematic (a) the DPPC molecule is composed of 1 $Q_0$, 1 $Q_a$, 2 $N_a$, and 8 $C_1$ (4 on each tail of the molecule) beads. (b) The CNT is composed of hydrophobic particles, but is shown in red for better visualisation. (c) The $P_4$ water beads were used in combination with anti-freeze $BP_4$ beads to compensate for the high freezing temperature of the water, as previously introduced by Marrink et al. [125]. (d) $Na$ and $Cl$ ions were added to the water to balance the overall charge of the system containing CNTs with non-zero charges.

$Q_0$, which is positively charged, and the phosphate group (olive bead, type: $Q_a$), which is negatively charged. The glycerol ester moieties are represented by the pink beads (type $N_a$). Both lipid tails are described by 4 turquoise beads (type $C_1$) each, which are representing the 16 methylene/methyl components. The interactions within the DPPCs are governed by standard bonded interactions as described above. However, the glycerol bond length has been reduced to $R_{bond} = 0.37\text{nm}$. In water, the DPPC lipids self-assemble to form the LB.

The CG parameters for the CNT were based on the work by Sansom et al. [97]. A 4-to-1 mapping was used to conserve the hexagonal structure of the CNTs (figure 5.2.1b). Apolar beads were used to model the CNT carbon atoms. The diameter of the CNT was 1.4 nm and the length was 10 nm.

In order to mimic the functional groups present on the surface of f-CNTs, surface charges were added to the CNT structure. To simulate 4+ or 12+ positively charged CNT, 4 or 12 beads in the CNT structure were changed to $Q_0$ type beads respectively. To simulate 4- or 12- negatively charged CNT, 4 or 12 atoms in the CNT structure were changed to $Q_a$ type beads respectively. This added charge needed to be compensated for to preserve the overall neutral charge of the system. This balancing of charges was achieved by the addition of charged ions to the water (figure 5.2.1d). Here, hydrated sodium ions and hydrated chloride ions were used. The $Na$ ions are type $Q_{da}$ and have a charge of +0.7e. The $Cl$ ions are type $Q_{da}$ and have a charge of -0.5e.

The CG parameters for the water and antifreeze particles were taken from the model developed by Marrink et al. [125]. Again, 4 water molecules were represented as 1 $P_4$ interaction site (figure 5.2.1c). In addition, anti-freeze particles, the $BP_4$, were used to lower the freezing temperature of water as it was found to be too high compared with real water [125].
Simulation Protocol

5.3 Simulation Protocol

All simulations were performed with the Gromacs simulation package version 4.5.4 at constant temperature of 323 K and at constant pressure of 1 bar. Periodic boundary conditions were used in all directions. In addition, the number of particles was kept constant throughout each simulation. The LB contained 576 CG DPPC molecules (figure 5.2.1a). Overall there were over 37260 water molecules and 1 CNT in the box. The box dimensions were set to be $15 \times 15 \times 30$ nm. The timestep used in all simulations was 10 fs.

5.3.1 Energy Minimisation and Relaxation

Initially, the CNT, as shown in figure 5.2.1b, was placed along the $z$ axis approximately 10 nm away from the LB. However, the CNT was constrained in the $x$, $y$, and $z$ directions during the energy minimisation (EM) and relaxation phase. In addition, the center of mass (COM) position of the LB was constrained during the EM and the relaxation phase.

The EM used here was based on the steepest descent method. In the MD simulations the partial derivative of the potential energy with respect to all the coordinates is known. Hence, the system can take a step in the direction of the negative gradient (the direction of the force) to find a local energy minimum. In the steepest descent method, the system always moves into the direction of the negative gradient without considering any of the previous steps. Care was taken that, after the EM phase, the water molecules appeared to be distributed homogeneously throughout the space available in the simulation box.

The second phase was for the system to undergo a MD simulation for 2000 steps with the COM position of the CNT and the LB constrained. The aim of this non-steered MD phase was to achieve relaxation of the system.

5.3.2 Steered MD Simulation

The next step was to perform the steered MD (SMD) simulation, which was achieved by using the so-called ‘constrained pulling’ from Gromacs 4.5.4. The DPPC molecules were used as a ‘reference group’ and the CNT was used as a ‘pull group’. The CNT was then pulled along the vector parallel to $z$ through the LB at a constant velocity of 0.5 nm/ns relative to the reference group. The pulling velocity was chosen as it was found to be lower than the spontaneous insertion rate of the CNT into the LB by Wallace and Sansom et al. [97]. The SMD was run for 5.5 M steps.

In order to study the effect of charge and charge distribution on the translocation process, this simulation was repeated for the CNTs with the following charges: neutral (0), 4 positive charges at the tip (4+ tip), 4 negative charges at the tip (4- tip), 12 positive charges at the tip (12+ tip), 12 negative charges at the tip (12- tip), 12 positive charges randomly distributed along the CNT (12+ random) and 12 negative charges randomly distributed along the CNT (12- random).
Once the simulations were run, the number of contacts between CNT and lipids and the potential energy term were extracted using the Gromacs functions g_dist and g_energy respectively.

5.3.3 Position Constraint Simulation

In order to assess the potential energy of the system for various positions of the CNT with respect to the LB, a second set of MD simulations were run. The initial frames used in this second set of simulations were extracted from the previous SMD simulations for various CNT-LB COM distances. This time, the distance $d$ between LB and CNT for various CNT positions was kept fixed during the whole simulation which was run for 2 M steps. Then, the PE was calculated and extracted using the Gromacs inbuilt function g_energy, averaged and plotted for all positions. This simulation was repeated for the CNTs with the following charges: neutral (0), 12 positive charges at the tip (12+ tip), 12 negative charges at the tip (12- tip), 12 positive charges randomly distributed along the CNT (12+ random) and 12 negative charges randomly distributed along the CNT (12- random).

The potential of mean force (PMF) is the potential energy, as a function of some chosen coordinate, whose derivative gives the average force for that value of the coordinate. In practice, the PMF can be used to study the changes in the free energy of the system as a function of a particular coordinate of the system. In the systems presented in this thesis, the variations in the free energy were calculated as a function of the various positions of the CNT relative to the LB.

5.3.4 Visualisation

All visualisations of the CG MD simulations were achieved using VMD (Visual Molecular Dynamics) software support. VMD was developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign. The water was omitted in most figures in chapter 12 to achieve a better visualisation. However, it was included in all of the simulations.
This chapter describes the nanomaterials that were used to carry out the experiments presented in this thesis.

### 6.1 Multi-walled Carbon Nanotubes

All of the MWNTs used in the work presented in this thesis were grown using catalytic chemical vapour deposition (CCD). Chemicals and solvents were obtained from Sigma-Aldrich (USA) and they were used as received, unless specified otherwise.

#### 6.1.1 NH$_3$+ f-MWNTs

Pristine MWNTs were purchased from Nanostructured & Amorphous Materials Inc. (Houston) at 94 % purity (stock no. 1240XH). The composition of the material was analysed using Energy Dispersive X-ray spectroscopy (EDX) and was stated to be 98.39 % C, 0.45 % Cl, 0.23 % Fe, 0.93 % Ni. The outer diameter and the length of the pristine MWNTs were stated to be 20-30 nm and between 0.5 and 2 μm respectively.

NH$_3$+ f-MWNTs were prepared following a two-step chemical treatment, which consisted of oxidation and subsequent introduction of NH$_3$+ groups by amidation reaction. The procedure was as follows: Oxidised MWNTs were obtained following the procedure reported in reference [132], (200 mg) were heated in 10 mL of neat oxalyl chloride at 62°C for 24 hours. After evaporation in vacuo, the resulting nanotubes were dispersed in a solution of Boc$^1$-monoprotected diamino-triethylene glycol (TEG) (530 mg) in distilled tetrahydrofuran (THF) (15 mL) and heated at reflux for 48 hours [23]. The nanotubes were re-precipitated several times from methanol/diethyl ether by successive sonication and centrifugation. The Boc protecting groups were removed overnight using 4 M HCl in dioxane (20 mL) to afford ammonium functionalised MWNTs (185 mg) following evaporation of the acid solution and re-precipitation in diethyl ether. The Kaiser test [105] afforded a loading of 320 μmol/g of amino groups [132]. It was previously shown,

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$^1$Tertiary-Butyloxy carbonyl Group
using zeta potential measurements, that other such f-MWNTs, prepared following the same protocol, hold positive surface charges at neutral pH [133].

Studies by Ali-Boucetta et al. [134] found that this exact type of NH₃⁺ f-MWNTs did not show significant levels of cytotoxicity for NH₃⁺ f-MWNT concentrations up to 125 μg/mL. In addition, studies by Gaillard et al. [31] found that the presence of this class of f-MWNTs did not affect the viability of several different cell types. This specific type of NH₃⁺ f-MWNTs was developed for targeted gene delivery of plasmid DNA [19, 21].

### 6.1.2 COO⁻ f-MWNTs

COOH f-MWNT were purchased from Nanostructured & Amorphous Materials Inc. (Houston) at >95% purity (stock no: 1237YJS), and were used without any further functionalisation. The composition of the material was analysed using EDX and was stated to be 98.39 % C, 0.45 % Cl, 0.23 % Fe, 0.93 % Ni. Their outer diameter and their length were stated to be 20-30nm and 0.5-2μm, respectively. The sample was stated to contain 1.17-1.29wt% of the COOH functional group. The COOH functional groups on the f-MWNTs are expected to dissolve into H⁺ ions and RCOO⁻ anions in neutral aqueous solution. Using zeta potential measurements, it was shown that such carboxylated f-MWNTs, prepared following the same protocol, hold negative surface charges at neutral pH [133]. Therefore, this type of f-MWNTs will be referred to as COO⁻ f-MWNTs throughout the rest of this thesis.

### 6.1.3 Pristine MWNTs

The pristine MWNTs (p-MWNTs) were purchased from Nanocyl (Nanocyl 3100 Series) at >95% purity. The composition of the material was analysed using thermal gravimetric analysis (TGA) and was stated to be >95% C and <5% metal oxide. The diameter of the p-MWNTs was stated to be 5-10 nm with an average diameter of 10 nm and their average length was stated to be 1.5 μm.

The Pluronic-coated p-MWNT were prepared by dispersion in Pluronic F127 as previously described in [135]. The Pluronic-coated MWNTs were sterilized for 3 hours at 160 °C before they were suspended in 1 % Pluronic F127 to a final stock suspension of 1mg/ml. The CNTs were sonicated in an ultra sound bath for 2 × 15 min and were abandoned at room temperature overnight. After a 5 min centrifugation step at 2500 × g for an optimal wetting of the material, the CNTs raw material was sonicated again as described. Samples were centrifuged at 10,000 × g for 10 min using an Eppendorf mini spin centrifuge to separate the suspended carbon nanotubes (CNT bundles) from mainly non-tubes carbon fraction (CNT-pellet). The concentration of the CNT suspension was gravimetrically measured.

The p-MWNTs were coated in the surfactant to improved their solubility and to reduce aggregation. The p-MWNTs did not undergo any oxidising treatment. For the rest of the thesis they will be referred to as ‘p-MWNTs’.
This particular type of p-MWNTs was developed to deliver anticancer agent doxorubicin into cells for cancer therapy applications as described by Ali-Boucetta et al. [135].

6.2 Highly Ordered Pyrolytic Graphite (HOPG)

Highly Ordered Pyrolytic Graphite (HOPG) material was obtained from SPI (Structure Probe Inc). HOPG was used as it has large defect free regions of sp² carbon (more defect free than any other form of bulk carbon) and can therefore be used as a reference material for electron energy loss spectroscopy (EELS) quantification of the sp²/sp³ content of MWNTs.

Graphite and graphene sheets were obtained from the HOPG sample using the 'scotch tape' method, which was developed by Novoselov et al. [136] to separate large sp²-rich sites by mechanical cleavage. The HOPG sample was prepared for TEM by first cleaving the HOPG surface with adhesive tape. The freshly cleaved HOPG substrate was then suspended in ultra pure water. Holey carbon TEM grids were dipped into the HOPG-water solution and left to dry for 24 hours prior to imaging.
Chapter 7

Cell Culture and Sample Preparation

This chapter describes the cell culturing and sample preparation techniques that were used to carry out the experiments presented in this thesis.

7.1 Cell Cultures

The cells used in the work presented in this thesis were human monocyte-derived macrophages (HMMs) and A549 epithelial cell line.

The HMM cell isolation, cell culture, fixation and embedding procedures were performed by Dr Karin H. Muller in the Multi-Imaging Centre, Department of Physiology, Development of Neuroscience, University of Cambridge. The cell culture and fixation procedures using A549 cells and COO− f-MWNTs were carried out by Dr Andrew Thorley at the Royal Brompton Hospital. The embedding of A549 cells exposed to COO− f-MWNTs was carried out by the author of this thesis.

F12 Ham media, Macrophage Serum-Free Medium (Mø-SFM), fetal bovine serum (FBS), penicillin/streptomycin, and phosphate buffered saline (PBS) were purchased from Gibco, Invitrogen (UK). LymphoPrep was obtained from Axis-Shields (Oslo, Norway).

7.1.1 Isolation and Culture of Human Monocyte-derived Macrophages

Human monocyte-derived macrophages (HMMs) were obtained by in vitro culture of human monocytes isolated from human buffy coat residues (National Blood Service, Brentwood, UK).

Buffy coat residue was washed once with Phosphate-buffered saline (PBS) and the resulting cell sediment was mixed with an equal volume of fresh PBS. 30 mL of diluted buffy coat residue was layered onto 15 mL of LymphoPrep. After centrifugation at 20 °C for 30 min at 700 × g, the opaque interphase of mononuclear cells was removed and washed 3 × with PBS containing 4 mg/mL Bovine Serum Albumin (BSA) to remove platelets. Then, monocytes were enriched by an additional centrifugation step in a Percoll gradient (Denholm and Wolber, 1991). Mononuclear cells were resuspended in 4 mL of PBS and mixed with 8 mL of Percoll : Hanks’ Balanced
Salt Solution (10X concentrate; 6 : 1, at pH 7.0). After centrifugation at 20°C for 30 min at 450 \( \times \) g, the monocytes were collected from the top of the gradient, washed in PBS/BSA and seeded on ethanol-sterilized glass coverslips (ø 13 mm) in 50 μL culture medium at a density of about 0.25 – 0.5 \( \times \) 10^6 cells/coverslip. After incubation for 1 hour at 37°C, any remaining non-adherent cells were removed by washing twice with PBS. Adherent monocytes were cultured at 37°C in 5 % CO2 using Mo-SFM, supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin, for at least 6-7 days prior to experiments unless stated otherwise, renewing the culture medium twice a week.

### 7.1.2 A549 Epithelial Cell Cultures

Cell studies were performed using A549 - a cancerous human epithelial cell line. It originates from the human lung carcinoma and has been linked to alveolar epithelial type 2 cells.

Epithelial lung carcinoma cells (A549; ATCC®, CCL-185™) were maintained and passaged in F12 Ham media, supplemented with 10 % FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 1 % L-glutamine and 1 % non-essential amino acids, at 37°C in 5 % CO2. Cells were passaged twice a week using TrypLE Express at 80 % confluency.

### 7.1.3 Incubations of Cells with Multi-walled Nanotubes

The MWNTs were used as sterile stock solutions: the p-MWNTs were suspended in surfactant as described in section 6.1.3; the NH3+ f-MWNTs and the COO- f-MWNTs were dispersed at a concentration of 0.5 mg/ml in 5 % dextrose/water. Stock solutions were sonicated in a water bath for 15 min before they were dispersed in cell culture medium. These procedures increased the accuracy of the concentration measurements and also improved the dispersion of the MWNTs. The MWNTs were added to the cell culture drop by drop to minimise reaggregation.

The cells were exposed to the MWNTs using three different experimental protocols.

**Low Temperature Study** A549 cells were incubated with 50 μg/mL of NH3+ f-MWNTs for 4 hours, at 37°C and at 4°C (on ice). The aim of this experiment was to determine whether cellular uptake of the MWNTs occurs via active or passive entry mechanisms. The rationale for this is that culturing the cells at low temperature inhibits the active uptake mechanisms [32–34].

**24 Hours Study** A549 cells were incubated with 50 μg/mL of NH3+ f-MWNTs at 37°C for 24 hours. This study aimed to determine the pathway by which f-MWNTs entered this non-phagocytic cell type. The study was repeated using the COO- f-MWNTs to investigate the effect of charge on cellular uptake.
14 Days Pulse-chase Study  HMMs were exposed to NH$_3^+$ f-MWNTs at a concentration of 50 μg/mL for 4 hours (4 hours pulse). The cells were then washed and cultured in cell culture medium, in the absence of MWNTs, for 30 min, 24 hours, 3 days, 7 days and 14 days (the chase) prior to processing for TEM. The aim of this pulse-chase experiment was to establish the long-term fate of MWNTs inside the HMMs, i.e. how they are processed by the cells. The study was repeated using the p-MWNTs to investigate the effect of the oxidative functionalisation treatment on the biostability of MWNTs.

7.1.4 Sample Preparation for TEM

Following the incubations, cells were rinsed briefly in saline (0.9 % NaCl) to remove any non-ingested particles and were then fixed in 4 % glutaraldehyde in 0.1 % PIPES buffer, pH 7.2 for 1 hour at 4°C. The gluteraldehyde is a fixative which cross-links proteins in the cell and preserves the cell ultrastructure. After several rinses in PIPES, the cells were removed using cell scrapers and transferred to 15 mL pellets which were then centrifuged for 5 min at 1500 × g.

After several rinses in deionised water (DIW), selected samples were osmicated (1 % OsO$_4$, 0.15 % potassium ferricyanide; 2 mM CaCl$_2$ in DIW) for 1 hour at room temperature. The samples were then washed several times with DIW. The osmium tetroxide is a strong oxidant that cross-links lipids, acting as a second fixative. It also serves to enhance contrast for TEM imaging, due to the high atomic number of osmium. The osmication procedure was omitted for cells prepared for the low temperature study and the pulse-chase incubations.

Then, all the samples were bulk stained for 1 hour at room temperature in the dark, using uranyl acetate. Following 2 washes in DIW, the sample was dehydrated in graded solutions of ethanol (70 %, 95 %, 100 %), 3x in each for 5 min. After additional two washes in 100 % acetonitrile, the sample was infiltrated with Quetol 651 resin (ethylene glycol diglycidyl ether monomer; purchased from Agar Scientific, Dorset, UK) over 4 days using fresh resin each day. The resin was then cured at 60 °C for 24 hours.

Sections (50-150 nm) were cut onto distilled water using a 35° wedge angle diamond knife using a Power Tome XL ultramicrotome (RMC Products by Boeckeler, Tuscon, Arizona, USA). The microtomed sections were collected on unsupported 400 mesh bare copper grids and on unsupported finder grids (G2764C F4 Finder grids copper 3.05 mm; purchased from Agar Scientific, Dorset, UK).

7.2 In Vivo Study

The animal exposures were executed by Antonio Nunes in the Nanomedicine Laboratory, Centre for Drug Delivery Research, The School of Pharmacy, University of London.
7.2.1 Animal Handling and Experimentation

All animal experiments were performed in compliance with the UK Home Office (1989) Code of Practice for the housing and care of animals used in scientific procedures. Six to eight-weeks old female C57BL6\(^1\) were caged in groups of 4-7 with free access to food and water. A temperature of 19-22°C was maintained, with a relative humidity of 45-65 %, and a 12 hours light/dark cycle. Stereotactic administration of f-CNT: C57BL6 mice were anesthetized with Avertin\(^\oplus\) (0.5 mL/ 100 g) and mounted on a stereotactic apparatus. Injections of up to 1 μL volume were made at specific stereotactic locations (Bregma +0.5 mm and +1.5 mm from sagittal line) in the motor cortex by means of a glass pipette (30 μm tip diameter) mounted on a motorised (0.1 μm step) three-axis micromanipulator connected to an injector (Sutter Instruments, Novato, CA, USA). A total of 1 μL of CNT dispersion was released at 700 μm below the cortical surface. During injections, the animals were oxygenated and heated using a blanket with a thermostat to ensure a 37°C rectal temperature. At the end of all surgical procedures, the scalp incision was closed with Mersilk sutures (Ethicon, UK), and the antibiotic gentamicin was topically administered to prevent infections. In these conditions the whole procedure requires about 20 min, and recovery from anaesthesia occurs after 60-90 min. After recovery, the animals were returned to their home cages. Mice were culled at 2 days and 14 days.

7.2.2 Sample Preparation for TEM

Mice were transcardially perfused under terminal anaesthesia (Euthatal\(^\oplus\)) with 3 % glutaraldehyde in 0.5 M cacodylate buffer (pH 7.4). After perfusion, brains were removed, immersed in the perfusate for 2-4 days at 4°C, allowing adequate penetration of the primary fixative. The brains were then transferred to 1M cacodylate buffer until ready to be processed. Specimens of 1 mm were cut using brain matrices (Zivic Instruments\(^\oplus\), USA). Each specimen was washed several times with deionised water, submitted to a second fixation with osmium tetroxide (OsO\(_4\)) for 90 min, rinsed with deionised water, then dehydrated by a series of ethanol grades: 70 %, 90 %, 100 %. Specimens were infiltrated with propylene oxide, followed by 1:1 (v/v) propylene oxide:araldite resin and finally were left in neat resin overnight at room temperature. Specimens were then placed at the desired orientation in the embedding moulds and placed in 60°C oven to allow for resin polymerisation for 2-3 days.

\(^1\)C57BL6 are a common strain of laboratory mice.
Chapter 8

Electron Microscopy

The theory of transmission electron microscopy (TEM) is introduced in the following chapter. Particular attention is paid to TEM of biological samples, electron tomography and electron energy loss spectroscopy (EELS).

In the following chapter, all equations, if not referenced otherwise, were taken from the book ‘Transmission Electron Microscopy’ by Williams and Carter [137].

8.1 Imaging with Electrons

In 1925 de Broglie theorised that electrons possess wave-like properties with a wavelength smaller than the wavelength of visible light [138]. De Broglie showed that the wavelength $\lambda$ of an electron is inversely proportional to its momentum. From de Broglie’s relation, it follows that the wavelength of electrons accelerated over a potential $V$ decreases with increasing acceleration voltage and vice versa. However, for particles travelling at speeds close to the speed of light in vacuum $c$, relativistic effects have to be accounted for. Then, the wavelength $\lambda$ of an electron of rest mass $m_0$, accelerated by a potential $V$, can be described by the following relation,

$$\lambda = \frac{h}{\sqrt{2m_0eV \left(1 + \frac{eV}{2m_0c^2}\right)}} \quad (8.1.1)$$

In the case where $eV \ll m_0c^2$, then $\frac{eV}{2m_0c^2} \to 0$ and the treatment becomes non-relativistic.

In the microscope, electrons accelerated over a potential of 300 kV are travelling at high speeds and the relativistic wavelength becomes $\approx 0.002\text{nm}$. For particles accelerated over a potential of 80 kV however, the relativistic effects become less important.

The wavelength of electrons travelling in a TEM are between 0.001 and 0.01 nm. This finite size of the wavelength limits the resolution of a TEM. However, in reality, other factors, originating from the TEM set-up, are more limiting. These limiting factors will be discussed in section 8.4.
8.2 Beam Specimen Interaction

When the fast electrons forming the beam pass through a thin, electron-transparent specimen, a range of secondary signals are generated which can be used for imaging and analysis purposes. A selection of these are shown in figure 8.2.1.

For the purpose of TEM imaging of biological specimens, the specimens generally needs to be less than 100 nm thick in order to achieve electron transparency. Most fast electrons pass through thin specimens without scattering, resulting in the ‘direct beam’, shown in figure 8.2.1. Only very few electrons are scattered, some losing energy (inelastic scattering) and some not (elastic scattering). It is these electron scattering events that form the basis of all imaging and spectroscopy analysis.

8.2.1 Interaction Cross-section

For every sample exposed to electrons of a known energy, it is possible to calculate the probability of a particular electron interacting with the specimen, the ‘interaction cross-section’ ($\sigma$).

To simplify the concepts it is possible to consider the scattering in 2D first. Then, the total cross-section for scattering can be described by,

$$Q_{\text{total}} = N\sigma_{\text{total}} = \frac{N_A\sigma_{\text{total}}/\rho}{A}$$

(8.2.1)
Beam Specimen Interaction

where \( N \) is the number of atoms per unit volume, \( \sigma_{\text{total}} \) is the total scattering cross-section for an isolated atom \( (\sigma_{\text{total}} = \sigma_{\text{elastic}} + \sigma_{\text{inelastic}}) \), \( N_A \) is the Avogadro number, \( A \) the atomic weight of the atoms in the specimen and \( \rho \) the specimen density. The units of measurement for the cross-section are ‘barns’ \( (1 \text{ barn} = 10^{-24} \text{cm}^2) \).

For a specimen of ‘small’ thickness \( t \), the probability of an interaction is,

\[
p = Q_{\text{total}} t = \frac{N_A \sigma_{\text{total}} (\rho t)}{A} \quad (8.2.2)
\]

Since the units of measurement for the total cross section, as defined in equation 8.2.1, are units of length\(^{-1}\), relation 8.2.1 can be used to predict the distance an electron has to travel on average through the specimen before interacting with the atoms in the specimen. This distance covered by the electron is called the mean free path \( (\lambda_m) \) and is defined by,

\[
\lambda_m = \frac{1}{Q_{\text{total}}} = \frac{A}{N_A \sigma_{\text{total}} \rho} \quad (8.2.3)
\]

Although most electrons travel through thin samples without interacting, some incident electron can undergo several scattering events. These are called ‘plural scattering’ events. Since the probability of a scattering event occurring is related to the sample thickness (equation 8.2.2), the use of samples of thickness \(< \lambda_m\) minimises the occurrence of plural scattering events, resulting in better imaging and spectroscopy conditions overall.

So far the scattering events have been treated in 2D only, whereas in reality, scattering events need to be described in 3D. The relation between the scattering angle \( \theta \) and the solid angle is \( \Omega = 2\pi (1 - \cos \theta) \) and therefore, \( d\Omega = 2\pi \sin \theta d\theta \).

This will give the differential cross-section:

\[
\frac{d\sigma}{d\Omega} = \frac{1}{2\pi \sin \theta} \frac{d\sigma}{d\theta} \quad (8.2.4)
\]

\[
\sigma_\theta = \int d\sigma = 2\pi \int_0^\theta \frac{d\sigma}{d\Omega} \sin \theta d\theta \quad (8.2.5)
\]

From equation 8.2.5, the cross-section for scattering into all solid angles up to \( \theta \) can be calculated.

8.2.2 Elastic Scattering

Elastically scattered electrons do not undergo a detectable loss of energy during interaction with the specimen. Elastically scattered electrons are scattered through an angle \( \theta \), and hence do not form part of the direct beam. We can distinguish between two different cases of elastic scattering: electrons that are weakly scattered though interaction with the electron cloud and
electrons that are strongly scattered through interaction with an atomic nuclei. The latter is referred to as Rutherford high-angle scattering.

The high-angle scattering is analogous to the Rutherford experiment where $\alpha$ particles, back-scattered from a metal foil, were used to deduce the existence of the atomic nucleus. The following expression was derived for the Rutherford experiment,

$$\frac{d\sigma(\theta)}{d\Omega} = \frac{e^4 Z^2}{16(E_0)^2 \sin^4 \frac{\theta}{2}}$$  \hspace{0.5cm} (8.2.6)$$

where $e$ is the electron charge, $Z$ is the atomic number, $E_0$ is the energy of the incident electrons and $\theta$ is the scattering angle.

By integrating equation 8.2.6 from 0 to $\pi$, we obtain the total elastic nuclear scattering cross-section:

$$\sigma_{\text{nucleus}} = 1.62 \times 10^{-24} \left( \frac{Z}{E_0} \right)^2 \cot^2 \frac{\theta}{2}$$  \hspace{0.5cm} (8.2.7)$$

From equation 8.2.7, $\sigma_{\text{nucleus}} \propto Z^2$. Hence, heavier elements have a larger scattering cross-section and thus scatter more strongly than lighter elements. This equation represents the scattering of a single nucleus. If we consider a sample of thickness $t$, using equation 8.2.2, the scattering cross-section of the atoms in the sample becomes,

$$Q_{\text{nucleus}} t = 1.62 \times 10^{-24} \left( \frac{N_A \rho t}{A} \right) \left( \frac{Z}{E_0} \right)^2 \cot^2 \frac{\theta}{2}$$  \hspace{0.5cm} (8.2.8)$$

The scattering depends more strongly on $Z$ than on the thickness of the sample, since it is proportional to $Z^2$, but only proportional to $\rho t$. This treatment ignores the screening effects from the nuclear electron cloud as well as the wave-like nature of electrons. These approximations are fine for electrons scattered at high angles. However, for low-angle scattering events, the nuclear scattering is no longer the dominant scattering contribution and a different description is required.

For low-scattering angles, the use of the so-called atomic form factor, $f(\theta)$, and the related quantity in a solid, the structure factor, $F(hkl)$, is more appropriate. This approach is based on the wave-like motion of the electrons and is dependent on the scattering angle $\theta$. $F(hkl)$ describes the electron scattering from a unit cell. It is obtained by summing all $f(\theta)$ for a unit cell and multiplying it by a phase factor, which accounts for the phase differences of the electrons scattered from different atomic planes. Since variations in atomic species are not investigated here, it is not necessary to give detailed explanations about the origin of these factors. More detail can be found in Williams and Carter [137].

Furthermore, in the case of coherent elastic scattering processes, diffraction patterns arise from the interference of the scattered electrons. The constructive and destructive interference between the waves of the electrons deflected by the atom planes, leading to the diffraction pattern, can be explained using Bragg’s law.
The condition for constructive interference is described by Bragg’s law,

\[ n\lambda = 2d \cdot \sin \theta_{\text{Bragg}} \]  

(8.2.9)

where \( n \) is an integer, \( d \) is the distance between the atomic planes and \( \theta_{\text{Bragg}} \) is the angle between the scattered plane wave and the atomic plane.

Hence, the diffraction pattern contains information about the crystallographic structure and about the orientation of the atomic planes.

### 8.2.3 Inelastic Scattering

Inelastic scattering events include any scattering events during which the fast electron undergoes a detectable loss of energy. For conventional TEM, we only require the transmitted electron beam and information can be impaired by the presence of inelastically scattered electrons. However, these inelastically scattered fast electrons contain valuable information about the specimen which can be extracted directly from the fast electrons and from subsequent emissions from the specimen. These subsequent scattering emissions include secondary electrons, Auger electrons, photons and X-rays (figure 8.2.1). Furthermore, a continuous spectrum of X-rays is emitted from the fast electrons as they are slowed down through interactions with the electron cloud or the presence of the Coulomb field of the atomic nuclei of the specimen. This signal is known as the Bremsstrahlung.

An incident electron can lose a characteristic amount of energy in three distinct ways when interacting with the specimen. It may ionise an individual atom by exciting an electron from an inner shell to a higher energy state. Or, it can create plasmon oscillations through the collective excitation of the outer shell electrons. Lastly, an incident electron may excite phonons, which are collective lattice vibrations.

Many of these emissions are visible in the features of the electron energy loss spectrum (EELS). This technique is based on analysis of the energy lost by the electrons during interactions with the specimen and is described in more detail in section 8.9.

Further, many of the scattering events result in the emission of x-rays which are characteristic of the elements present in the specimen. These can be probed using energy dispersive X-ray spectroscopy (EDX), a technique described in section 8.10.
8.3 The Transmission Electron Microscope

In this section, the basic set-up of a TEM microscope column, as seen in the schematic in figure 8.3.1, will be explained. The column of the microscope is held under high vacuum, during and between operation, to minimise the interactions between the electrons and the gas molecules in the column and to avoid damaging the electron source.

![Ray diagram of the TEM set-up in imaging and diffraction mode. (Figure adapted from [137])]
The electrons are emitted at the electron source at the top of the microscope column. The emitted electrons are then accelerated through application of a so-called ‘accelerating voltage’. The accelerated electron beam then passes through a series of two condenser lenses for a conventional TEM set-up (for simplicity only one condenser lens is shown in figure 8.3.1), or three condenser lenses for the FEI Titan microscope, as described in section 8.5. Condenser apertures are placed below the condenser lenses. Subsequently, the objective lens focuses the electrons emerging from the condenser lens system onto the back focal plane. An objective aperture can be inserted into the back focal plane to select the desired angular range of electrons only. How the chosen angular range affects the contrast in the final image will be discussed in section 8.6. Depending on the strength of the intermediate lens, the second intermediate image is formed (in imaging mode) or a diffraction pattern is formed (in diffraction mode). The purpose of the projector lens is to vary the magnification of the image or diffraction pattern.

8.4 The Electron Lens System and Aberrations

The lenses in a conventional TEM are an arrangement of electromagnets, composed of a soft iron pole piece with a coil of copper wire surrounding it. The strength of the magnetic field controls the path of the electrons travelling through the lens.

Since the lenses in the TEM focus and direct the electron beam down the column, imperfections in the lenses as well as the geometry of the lenses can reduce the resolution. There are three factors from the lenses that limit the microscope resolution: spherical aberration, chromatic aberration and astigmatism.

Spherical Aberrations

The lenses act inhomogenously on the electron rays that travel off the axis, resulting in spherical aberrations. When passing through a lens, the further an electron is away from the lens axis, the stronger it is deflected back by the lens. Where a perfect lens forms a point in an image, the lens suffering from spherical aberration forms a disc instead. The spherical aberration of the objective lens is most noticeable during TEM imaging, whereas the spherical aberration of the condenser lens is most noticeable in scanning TEM (STEM) imaging (due to the beam being focused most strongly by the condenser lens in STEM imaging). The spherical aberration corrector of the FEI Titan used in this study is described in section 8.5.4.

Chromatic Aberrations

The electron beam is not perfectly monochromated on exiting the source of the microscope as the electrons vary slightly in energy and thus in wavelength. In addition, electrons lose different amounts of energy when passing through the sample. Both factors result in chromatic aberrations since electrons of lower energies are deflected more strongly by the objective lens than the higher energy electrons. Electrons emitted from a point in a sample form a disc of finite size in the image. The spread in energy of the electrons exiting the specimen depends strongly on the thickness of the sample, thus thicker samples are more affected by chromatic aberrations.
**Astigmatism**  Imperfectly symmetrical magnetic fields acting on the electrons can lead to astigmatism. Inhomogenous magnetic fields can arise from imperfectly cylindrical pole pieces, and from the soft iron core of the pole piece containing imperfections and inhomogeneities. Other factors include apertures that are not properly centred on the axis, or the presence of contaminants on the apertures. Astigmatism can be counteracted by use of stigmators (small octupoles) that introduce compensating fields to balance out the inhomogeneities.

These factors originating from lens defects and from the geometry of the lenses limit the resolution of a TEM microscope significantly, usually more than the inherent limitations due to the wavelengths of the electrons that were described at the beginning of this chapter.
8.5 FEI TITAN

The FEI Titan is fitted with a three condenser lens system, an electron monochromator and a double hexapole aberration corrector (CEOS, GMbh). A schematic of the FEI Titan set-up is shown in figure 8.5.1.

Figure 8.5.1: Schematic of the FEI Titan 80/300 (not to scale).
8.5.1 Electron Source

Electron sources rely on the principle that solids emit electrons if raised to high enough temperatures (thermionic sources) or if under high electric field (field emission gun).

In thermionic sources, the material needs to be heated to high enough temperatures for the electrons to gain sufficient energy to overcome the work function of the material. Hence, the materials suitable for use as thermionic emission electron sources must have a low work function, such as lanthanum hexaboride (LaB₆), or they must have a high melting point, e.g. tungsten.

Characteristics such as the brightness, cross-over size, coherence and energy spread of the electron beam are determined by the source. The brightness of an electron beam is measured by the electrons leaving the source, with the units of measurement being the current density per unit area per unit solid angle. A small energy spread results in greater coherence of the beam, as the emitted electrons have a narrower range of wavelengths. Field emission sources achieve better brightness, greater coherence and a smaller energy spread. Therefore they are more suited for analysis at the nanoscale than thermionic sources.

A field emission gun (FEG) emits electrons when an intense electric field is applied. All work presented in this thesis, unless specified otherwise, has been done on the FEI Titan which is equipped with a thermally assisted FEG, a so-called Schottky type emitter. The Titan FEG consists of a fine tip (diameter < 0.1μm) composed of tungsten which is coated with a thin layer of zirconia (ZrO₂) to lower the work function of the tip. As a result of the applied field, the electrons inside the tip can escape through tunnelling. The layer of zirconia increases the overall brightness of the source due to its lower work function. Aside from the application of an electric field, the tip in this FEG is also heated to temperatures of 1800°C in order to increase the number of electrons emitted and also to reduce any contamination sitting on the tip. The resulting electron beam has a small electron energy spread.

8.5.2 Pre-specimen Lens System

As seen in figure 8.5.2, the pre-specimen lens system of the FEI Titan contains one electrostatic lens and five electromagnetic lenses, including the electrostatic gun lens, the first condenser lens (C1), the second condenser lens (C2), the third condenser lens (C3), the minicondenser lens (MC) and the upper objective lens.

The first lens (starting at the top of the column), the gun lens, is placed after the field emitter and extractor. Increasing the strength of this lens increases the demagnification of the source and hence decreases the beam current. The lenses are arranged in such a way that the object plane of one lens is the image plane of the following lens. Thus the image plane of the gun forms the object plane of the first condenser lens.

A zoom system is composed of two neighbouring lenses, where one can move the intermediate image up and down between them, without changing the position of the object of the first lens or the image of the second lens. This results in a varying convergence angle at the image of
the second lens. The lens C3 is fixed and can only be changed with the mode of operation by switching between TEM and STEM mode (also called ‘probe’ mode). Thus, Titan has two zoom condenser systems: from C1 to C2 and from C2 to C3. The zoom condenser system C1-C2 affects both the beam current and the spot number of the microscope. In contrast, the zoom condenser system C2-C3 affects the beam differently in TEM than in probe mode. In TEM, the C2-C3 defines the illumination area, providing true parallel illumination. In probe mode, the C2-C3 determines the convergence zoom and the convergence angle ($\alpha$). Hence, the C2 aperture is the beam-defining aperture in both, TEM and probe mode.

The specimen is positioned between the upper and lower objective lens, so the magnetic field of the objective aperture acts from both sides of the specimen. In TEM mode, the minicondenser (MC) lens focuses the beam onto the sample as a parallel beam, as seen in figure 8.5.2. In probe mode, the MC lens ensures parallelity of the beam and the upper condenser lens focuses the beam down to a spot onto the specimen.

### 8.5.3 Post-specimen Lens System

Forward-scattered electrons are focused by the lower objective lens to form an image or diffraction pattern at the first image plane or the back focal plane respectively as seen in figure 8.5.3. By adjusting the intermediate lens strength one can switch between imaging and diffraction mode. An objective aperture can be inserted in the back focal plane to improve contrast when imaging, as described in section 8.6.
8.5.4 Aberration Corrections

Spherical aberrations, as described in section 8.4, were explained for the first time by Scherzer [140] in 1936, who also suggested techniques to correct them. However, it was not until much later that the technological challenges were overcome and aberration correctors could be fitted to microscopes.

The Titan microscope is fitted with such a spherical aberration corrector (Cs). Generally Cs correctors comprise non-round lenses which do not suffer from spherical aberrations [141, 142]. In the case of the Titan, the Cs is composed of two electromagnetic hexapoles and four additional lenses, as seen in figure 8.5.4. Any non-rotational symmetric aberrations introduced by the first hexapole lens are compensated for by the second hexapole lens [143]. Furthermore,
any additional rotationally symmetric spherical aberrations introduced by the hexapoles, compensate for the spherical aberrations introduced by the objective lens. By varying the strength of the hexapole, one can compensate for any spherical aberrations from the objective lens accordingly.

Figure 8.5.4: Schematic of the Titan Cs corrector, where OL = objective lens, HX1 = hexapole lens 1 and HX2 = hexapole lens 2. (Figure adapted from [144])

8.5.5 Image Formation

The FEI Titan is equipped with two charge-coupled device (CCD) cameras - a Gatan multiscan CCD (for TEM imaging and diffraction analysis) and a Gatan Imaging Filter (GIF) CCD (for EELS analysis). A CCD camera is a metal oxide semiconductor, comprising the pixels. These pixels store a charge proportional to the intensity of the electron beam hitting the CCD camera. This charge is then read out serially (pixel row by pixel row).

Both cameras used for the work presented here were 2048 x 2048 detectors. The GIF CCD has a shorter readout time because it is subdivided into four quadrants. Generally one can reduce the readout time by ‘binning’, moving more than one row at a time into the readout array. This introduces an averaging effect. The binning into x and y direction must be the same when imaging, but can be different when acquiring EELS. The EEL spectra acquired for the work presented here were binned in the y direction only to improve signal-to-noise ratios.

8.6 TEM Imaging and Contrast

Contrast arises when a specimen scatters electrons inhomogenously. Image contrast is not to be confused with image resolution. Contrast is the difference in intensities between two adjacent features in an image. Whereas image resolution is the ability to resolve these features.

The scattering cross-section from equation 8.2.2 depends on the atomic number, Z, the density and the thickness of the sample. And since scattering of the electrons can occur via different processes, different contrast mechanisms can contribute to the overall contrast in an image. In