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**Characterisation of nanoparticle distribution, integrity and
toxicity in vivo and in vitro using advanced spectroscopic and
microscopic approaches**

A thesis submitted for the degree of Doctor of Philosophy (PhD)

by

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at

University of Dublin, Trinity College

2011

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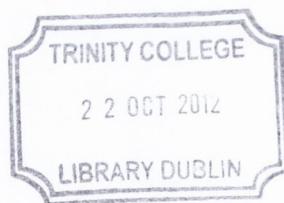
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Summary

Much uncertainty surrounds the potential toxicity of new engineered nanomaterials to human health and the environment, either during manufacture, use or at the end of their life cycle. The current generalised methodologies used to assess the cytotoxicity of nanoparticle (NP) are inconclusive. A systematic approach, investigating NP distribution, integrity and cellular responses to NP exposure need to be applied. With such a broad range of NP available with different physical and chemical properties it is important to recognise that techniques tailored for investigating the interaction of particular NP with cells are required.

The ultimate fate of NP in humans following exposure is determined by how our immune system deals with these foreign bodies. Phagocytic cells represent the first line of defense in the body against foreign invaders and therefore their interactions with different nanomaterials are investigated in this thesis. Using novel adaptive approaches to well established microscopic and spectroscopic techniques, a broad range of new protocols tailored to identify underlying cellular responses to different NP exposure scenarios, imaging their distribution and measuring the integrity of NP were developed and optimized.

Evaluation of the autophagic response induced by different NP exposure scenarios has been carried out thus providing a fundamental insight into the consequences of NP exposure during different stages of their lifecycle. It was found that single walled carbon nanotubes, silver nanowires and their composite thin films induced an elevated autophagic response in phagocytic cells.

In this study Raman spectroscopy has been established as an objective, robust, non-invasive, non-destructive technique for identifying, imaging and assessing the integrity of single walled carbon nanotubes (SWCNT) within cells and tissues. This technique revealed a time dependant degradation of the SWCNT driven primarily, but not exclusively, by myeloperoxidase.

Fluorescent lifetime imaging microscopy technology for the first time revealed that the localization of negatively charged quantum dots (QD) to their ultimate nuclear and nucleolar location affects the photoluminescent lifetime of the QD and thereby provides a sensitive readout for the physical interactions between QD and these biological domains.

Observations have been made through the course of this study that once again highlight how little we yet understand about nanoparticles and how human cells and tissues interact with them. Studies like this demonstrate the subtle dangers and potential pitfalls of further and more intensive use of NP and highlight the need for further research. Awareness that NP compounds are not biologically inert and that more concerted steps need to be taken before we should further implement their use.

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Associated publications

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- Conroy, J. Investigation of carbon nanotube biodegradation in isolated neutrophils and lung tissue using Raman spectroscopy. SPEC 2010 – Shedding Light on Disease: Optical Diagnosis for the new Millennium, Manchester, June 2010
- Conroy, J.* Investigation of carbon nanotube biodegradation in isolated neutrophils and lung tissue using Raman spectroscopy – Chemometrics Ireland 2010, Dublin Institute of Technology, Dublin, November 2010
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An *in vitro* integrated ultrasensitive approach to biocompatibility analysis of silver nanowires. NanoImpactNet, Lausanne, February 2011

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Peer reviewed Publications

Nonfunctionalised nanocrystals can exploit a cell's active transport machinery delivering them to specific nuclear and cytoplasmic compartments.

Igor Nabiev, Siobhan Mitchell, Anthony Davies, Yvonne Williams, Dermot Kelleher, Richard Moore, Yurii Gun'ko, Stephen Byrne, Yury P. Rakovich, John F. Donegan, Alyona Sukhanova, **Jennifer Conroy**, David Cottell, Nikolai Gaponik, Andrey Rogach, and Yuri Volkov.

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Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation.

Valerian E. Kagan, Nagarjun V. Konduru, Weihong Feng, Brett L. Allen, **Jennifer Conroy**, Yuri Volkov, Irina I. Vlasova, Natalia A. Belikova, Naveena Yanamala, Alexander Kapralov, Yulia Y. Tyurina, Jingwen Shi, Elena R. Kisin, Ashley R. Murray, Jonathan Franks, Donna Stolz, Pingping Gou, Judith Klein-Seetharaman, Bengt Fadeel, Alexander Star and Anna A. Shvedova

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A.A. Shvedova, O. Kapralova, W. Feng, E.R. Kisin, A. Murray, R. Mercer, C. St Croix, M. Lang, S. Watkins, N. Konduru, B. Allenh, **J. Conroy**, B. M. Mohamed. A. D. Meade, Y. Volkov, A. Star, B. Fadeel, V.E. Kagan

Abbreviations

AgTF	Silver thin film
AFM	Atomic force microscopy
AgNW	Silver nanowire(s)
BF	Bright field
BMDM	Bone marrow derived macrophage(s)
BSA	Bovine serum albumin
C	Control
Chloro	Chloroquine
CNT	Carbon nanotube(s)
DNA	Deoxyribonucleic acid
e-waste	Electronic waste
FCS	Foetal calf serum
FLIM	Fluorescent lifetime imaging microscopy
FTIR	Fourier transfer infrared
GFP	Green fluorescent protein
HCS	High content screening
HiPco	High pressure CO disproportionation process
hMPO	Human myeloperoxidase
HRP	Horseradish peroxidase
ITO	Indium tin oxide
k/o	Knock out
LC3	Light chain protein
LCD	Liquid crystal display
LED	Light emitting diode

MPO	Myeloperoxidase
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MWCNT	Multi walled carbon nanotube(s)
nanoFET	nanoscale field-effect transistors
NC	Nanocrystal(s)
NP	Nanoparticle(s)
PBMC	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffer saline
PCA	Prinicipal component analysis
PL	Photo luminescence
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocyte(s)
PSF	Phagolysosomal stimulant fluid
PVDF	Polyvinylidene fluoride membrane
QD	Quantum dot(s)
RNA	Ribonucleic acid
SWCNT	Single walled carbon nanotube(s)
TE	Transverse electric
TEM	Transmission electron microscopy
TF	Thin Film(s)
TGA	Thioglycolic acid
TM	Transverse magnetic
w/t	Wild type

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Chapter 1

1. Introduction

In western society electronic devices such as mobile phones and computers become rapidly obsolete leading to an alarming increase in electronic waste (e-waste) way beyond the capacity of disposal and recycling centres. Illegal dumping of e-waste from developed countries is being exported predominantly to India, China, Ghana and Nigeria where there is no control or regulation for the disposal of proven toxic substances, let alone the new emerging and less characterised materials. These people and their environment are suffering as a consequence.

Future implications on human health and the environment following exposure to new engineered nanomaterials, either during manufacture or at the end of their life cycle, is an issue of grave concern. Much scientific uncertainty surrounds the toxicity of new engineered nanomaterials that are already in use in many existing consumer products. There are three fundamental issues that need to be addressed to unravel this uncertainty. Firstly, it is the development of minimally invasive and non-destructive methods of nanoparticle detection within living cells and tissue, secondly establishing the ultimate fate and localisation of nanoparticles within these biological systems and finally uncovering the cellular mechanisms induced following the exposure of nano-objects. The range and diversity of nanoparticle properties makes this a complex task requiring innovative approaches to existing techniques.

This thesis adapts a range of microscopic and spectroscopic techniques to explore the distribution, integrity and toxicity of three nanoparticle types within cells and tissues namely quantum dots, single walled carbon nanotubes and silver nanowires. These nanoparticles have been specifically selected for their diverse properties and widespread use in established and emerging technologies such as liquid crystal displays, light emitting diodes, and conducting thin films which has resulted in a constant increase in production volumes.

1.1. Current cutting edge applications of nanoparticles

The rapid expansion of nanotechnology has opened up an immense array of nanoparticle application ranging from nanowire circuits (Yan, Choe et al. 2011) to the delivery of small molecules into cells by femtosecond activated carbon black nanoparticles (Chakravarty, Qian et al. 2010). Vollmer et al in 2002 presented an optical biosensor capable of detecting BSA and streptavidin concentrations (Vollmer, Braun et al. 2002). The addition of these proteins caused an overall shift in the optical resonance wavelength in a microcavity proportional to changes in the protein concentrations. In 2011 Loncar and his group presented a combination of fiber taper and resonant scattering spectroscopy, where they showed high Q-factor, dual polarized TE (transverse electric) – TM (transverse magnetic) photonic crystal nanobeam cavities in silicon (McCutcheon, Deotare et al. 2011). The authors anticipate that such a phenomenon could be exploited for biosensing. Lieber and his group perfected a technique which permitted the introduction of kinks at defined positions during growth along semiconductor nanowires (Tian, Xie et al. 2009). They can grow kinked or zig-zag nanowires in which straight sections are separated by triangular jolts. Using this technology they were able to develop 3D flexible nanoscale field-effect transistors (nanoFET) as localized bioprobes in single living cells. These 3D nanoFET probes coated in phospholipids bilayers were able to enter the cell and record electrical signals from beating cardiomyocytes (Tian, Cohen-Karni et al. 2010). Park *et al* used surface-modified vertical silicon nanowires as a method for delivering biomolecules into cells. This technique allows for the delivery of these biomolecules without compromising the cell, without chemical modification or viral packaging (Shalek, Robinson et al. 2010).

Biomarker measurements are often made at single time points and can therefore often miss a transient change that may have occurred between measurements. For example following myocardial infarction levels of serum cardiac troponin I, creatine kinase and myoglobin elevate and then return to baseline levels. Taking a measurement at the wrong time could result in such an elevation being missed. Cima and his group presented an implantable magnetic relaxation sensor that can take readings over 72 hours. This device can non-invasively monitor changes in the relaxation properties of antibody coated

superparamagnetic iron oxide nanoparticles following exposure to the biomarker of interest (Ling, Pong et al. 2011). Using a combination of micrometer-sized silver flakes and multiwall carbon nanotubes decorated with self-assembled silver nanoparticles Baik *et al* have developed highly conductive, printable and stretchable thin films (Chun, Oh et al. 2011). Such films have applications in electronic devices, sensors, actuators and speakers.

The beneficial aspects of using many different nanoparticles for a broad range of applications in industry, medicine and everyday life are plentiful. The long term health implications to the environment and human health remain very much under-explored. Traditional methods of cytotoxicity analysis may no longer be appropriate for nanoscale objects and many reports have indicated the potential for misleading readouts. Therefore, novel methods of toxicity assessment in the nano-field must be explored and optimised.

1.2. Exposure routes and immune response to nanoparticles

The risk to human health is increasing exponentially with nanoparticle uses and applications, one of the key routes of exposure being inhalation. Representing the largest epithelial surface area in the body, the lungs are continuously exposed to foreign airborne particles and invading organisms during respiration (Zhang, Summer et al. 2000). The lungs, as with all parts of the body, have a defense mechanism made up of innate (non-specific) and acquired (specific) immune responses. Alveolar macrophages, which make up part of the innate immune response, constitute the first line of phagocytic defense against foreign bodies in the airways (Sibille and Reynolds 1990). These cells play a prominent role in immunity by initiating inflammatory and immune responses and have important phagocytic, microbicidal and secretory functions. Macrophages have been shown to readily phagocytose and kill invading bacteria. Following phagocytosis, the phagosomes will then fuse with intracellular granules to form a phagolysosome. Killing of the microbes is achieved by a combination of oxidative and non-oxidative mechanisms (Pluddemann, Mukhopadhyay et al. 2011). The potent oxidative killing mechanism also known as “respiratory burst” involves the generation of antimicrobial reactive oxygen species (ROS) via the membrane bound NADPH oxidase enzyme complex, whereas the non-oxidative include antimicrobial peptides (AMPs) and degenerative proteases. This

phagocytic function is particularly important following exposure to nanoparticles and will be discussed and investigated in the coming chapters. Other cells which participate in the phagocytosis and breakdown of foreign materials are the polymorphonuclear leukocytes (PMNs or neutrophils). PMNs represent the largest population of intravascular phagocytes in blood vessels and they migrate to infected tissue sites when signaled by various inflammatory stimuli. Macrophages are capable of generating various mediators which signal the recruitment of large numbers of PMNs to the required site (Zhang, Summer et al. 2000). PMNs generate toxic proteases for killing microorganisms making their phagocytic potential much stronger than that of macrophages (Sibille and Reynolds 1990).

Macrophages represent the first line of defense in the body against foreign invaders followed by PMNs and therefore the interaction of these cells with different nanomaterials are investigated in this thesis.

1.3. Nanoparticle Induced Toxicity

The toxicology of nanoparticles (NP) is an essential subject of research and interest in light of such a broad range of novel applications and uses of these exciting NP. An interdisciplinary approach needs to be taken to appropriately assess NP risks (Oberdorster, Oberdorster et al. 2005). Evidence in the literature is still very much conflicting as to whether or not certain particles are in fact toxic or what the predominant mechanisms driving cytotoxicity are. For example, with regards to CNT research some groups found that acid treatment (Porter, Gass et al. 2009) or functionalising the surface of the CNT (Dumotier, Lacotte et al. 2006; Lee, Park et al. 2011) rendered them less toxic. Another group found that the damage caused by intravenous injection of MWCNT into the testes of mice was reversible and did not affect fertility (Bai, Zhang et al. 2010). On the other hand in many reports there exists a strong link, following exposure to a range of NP, between oxidative stress and NP toxicity. Oxidative stress occurs when an imbalance between damaging oxidants (hydrogen peroxide and hydroxyl radicals) and protective antioxidants (Vitamin C and Glutathione) are disrupted (Stone and Donaldson 2006). Following exposure to various CdTe/CdSe QDs oxidative stress was induced in macrophages (Clift,

Boyles et al. 2010). Many reports on silver nano-particles suggest that oxidative stress might mediate the cytotoxicity of the particles (Chen and Schluesener 2008). The exposure of human bronchial epithelial cells to titanium dioxide led to reactive oxygen species and the induction of oxidative stress related genes (Park, Yi et al. 2008). The delivery of CNT by inhalation or aspiration to the lungs of mice has been shown extensively to induce oxidative stress (Shvedova, Kisin et al. 2007; Shvedova, Kisin et al. 2008; Inoue, Yanagisawa et al. 2010). This mechanism typically leads to cell death by necrosis or apoptosis. However there is a major gap in our understanding of one of the three major cell death mechanism/homeostatic processes known as autophagy. Further investigation of this could improve our understanding in situations where toxicity following NP exposure is observed but the driving mechanism is not.

However, one question that remains to be addressed is what happens to the NP following uptake in cells and tissue. A non-destructive, non-invasive technique is required to image and assess the integrity of these NP following exposure.

1.3.1. Autophagy

The reported toxicity induced by nanoparticles typically results in an increase in oxidative stress resulting in acute toxicity via apoptosis or the cell death by necrosis. However, very few studies have investigated a further cell death mechanism, know as autophagy (Seleverstov, Zahirnyk et al. 2006). Autophagy is a homeostatic process involving the lysosomal degradation of damaged cytoplasmic organelles or mis-folded proteins (Levine, Mizushima et al. 2011). It comes from the Greek words “phagy” and “auto” meaning “eat” and “self” respectively (Yang and Klionsky 2010). Autophagy involves the formation of a double membrane vesicle called an autophagosome which encompasses the cytoplasmic organelles (or proteins, bacteria, nanoparticles) and then fuses with lysosomes to degrade its contents (Sanjuan, Dillon et al. 2007). Figure 1 shows the steps involved in the formation, maturation and degradation of autophagic vesicles (Rubinsztain 2006). The manner in which cargo is selected for degradation by the autophagosome still remains to be answered (Baehrecke 2005). Autophagy is involved in

the cellular survival mechanism in times of stress, allowing cells to recycle nutrients during conditions of starvation, and it also has roles in the inflammatory and immune systems of the body (Baehrecke 2005). As a result of these functions autophagy or autophagic dysfunction has been implicated in a number of neurodegenerative diseases such as Parkinson's or Huntington's (Rubinsztein 2006), which result in an unnatural increase or decrease in autophagic activity within the cell.

Nanomaterials such as quantum dots (Seleverstov, Zahirnyk et al. 2006; Stern, Zolnik et al. 2008), gold (Li, Hartono et al. 2010), nano neodymium oxide (Chen, Yang et al. 2005), fullerenes (Yamawaki and Iwai 2006), functionalised SWCNT (Liu, Zhang et al. 2011) and PAMAM nanoparticles (Li, Liu et al. 2009) have been shown to induce autophagy. The most reliable methods for the detection of autophagy are electron microscopy, fluorescence microscopy and western blotting (Kaminsky, Abdi et al. 2011). A common marker of autophagosomes in fluorescent microscopy or western blotting is the light chain associated protein LC3. In mammalian cells, LC3 is cleaved at the C-terminal by Atg4 homologues to become LC3-I. Via an ubiquitin-like enzymatic reaction, this LC3-I protein conjugates to phosphatidylethanolamine to become LC3-II, which then becomes associated with the autophagosome inner and outer membranes (Figure 1) (Kaminsky, Abdi et al. 2011). Using an antibody to this LC3-II or GFP-tagged LC3 it is then possible to visualize the LC3 puncta by fluorescent microscopy and quantify them. There is a direct correlation between the amount of LC3 and autophagosome formation (Kabeya, Mizushima et al. 2000). A common drug used to treat cells and cause the accumulation of autophagosomes is called chloroquine (Sanjuan, Dillon et al. 2007) and it was adopted as a positive control in this study.

There is plenty of scope for further investigation into this autophagic process in association with NP exposure. It could prove to be one of the missing links in understanding the cellular mechanisms involved in a cells stress response to NP.

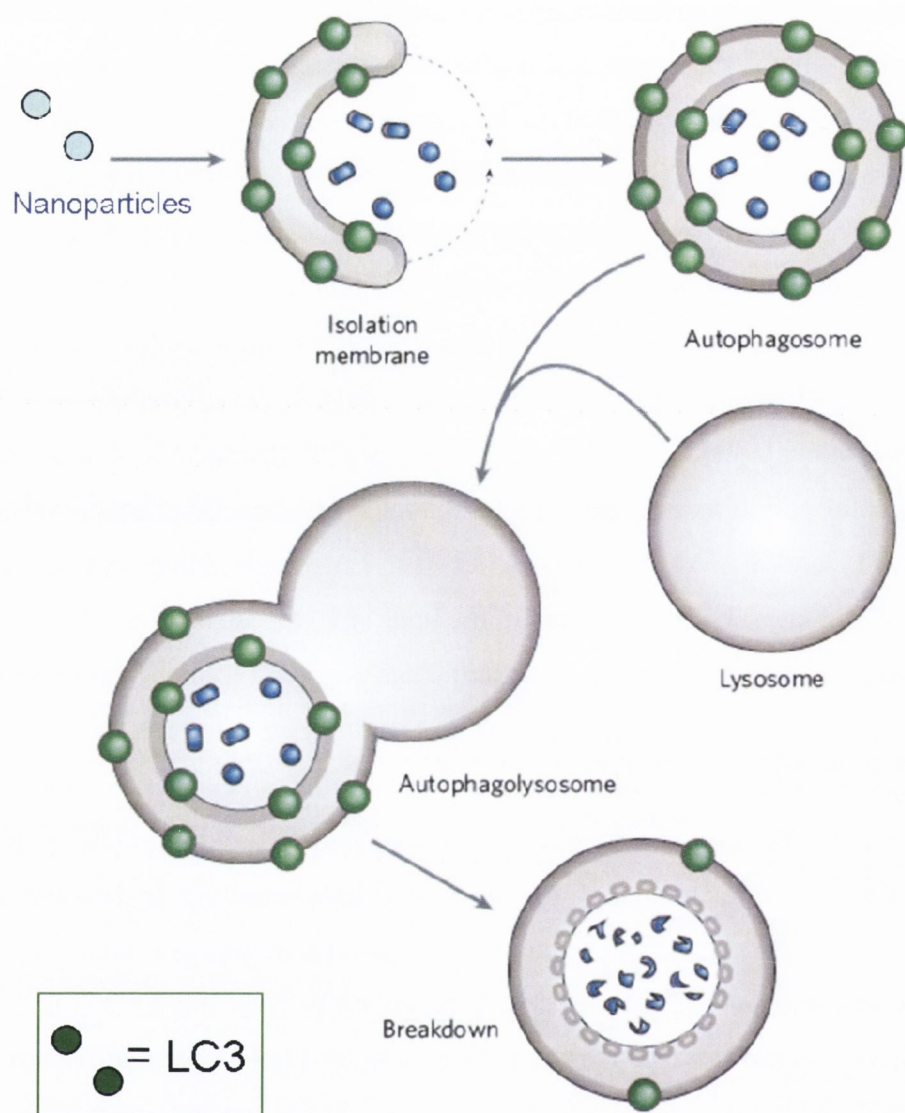


Figure 1 Schematic of events during autophagic process. It is thought that nanoparticles induce the formation of a double-membrane-bound-autophagosome. The NP are encapsulated into this autophagosome which then fuses with lysosomes to form an autophagolysosome. The contents of this autophagolysosome is then degraded by the acidic lysosomal hydrolases. (Adapted from Nature; 443, 780-786, 2006)

1.4. Nanoparticle detection and monitoring

1.4.1. Raman spectroscopy

Raman spectroscopy is based on the inelastic scattering of light. When monochromatic light illuminates a sample, most of the light is elastically scattered at the same wavelength as the incident light (Rayleigh scattering). However a very small portion of this incident light is in-elastically scattered at a different wavelength to the incident light. This is known as Raman scattered light and is due to the interaction of the light with the vibrations and rotations of the molecules in the sample (Smith and Dent 2005). The exact energy required to excite these vibrations and rotations depends on the masses of the atoms involved and the types of bonds between them. The vibrations and rotations may also be influenced by the molecular structure, molecular interactions and the chemical microenvironment of the molecule. Therefore the positions, relative intensities and shapes of the bands in the Raman spectrum carry detailed information about the molecular composition of the sample. This makes it very effective as a tool for correlating spectral changes to the chemical or biochemical composition of the sample in question.

The Raman spectral signal from SWCNT is strong and possesses discrete bands (radial breathing mode (RBM) at $\sim 200 \text{ cm}^{-1}$, the D-band at $\sim 1350 \text{ cm}^{-1}$, the G-band at $\sim 1590 \text{ cm}^{-1}$ and the 2-D band at $\sim 2600 \text{ cm}^{-1}$). The frequency of the radial breathing mode $\sim 200 \text{ cm}^{-1}$ according to theoretical prediction, is inversely proportional to the tube diameter, which does not depend on the chirality of the tube (Pimenta, Marucci et al. 1998). The disordered D-line $\sim 1350 \text{ cm}^{-1}$ is generally attributed to defects or impurities of the nanotube sidewall (Duesberg, Blau et al. 1999). The G-band at $\sim 1590 \text{ cm}^{-1}$ is typically the strongest peak in the SWCNT Raman spectrum and can be attributed to C-C bond motions which only depend weakly on diameter and helicity of the tubes. When interpreting the peaks in a Raman spectrum it is not the actual intensity of individual peaks that is of interest but rather the relative intensity of these peaks to one another. For example an increase in the relative intensity of the D band to the G band indicates damage or oxidation to the sidewall of carbon nanotubes and is a well documented method for assessing CNT integrity (Giordani, Bergin et al. 2006), (Spudat, Meyer et al. 2008).

Unlike FTIR spectroscopy, Raman spectroscopy is a very weak water scatterer and has proven to be a very useful tool suited to *in vivo* studies due to the minimal interference from water, such as cervical cancer diagnosis (Lyng, Faoláin et al. 2007), mapping of bronchial tissue (Kolijenic, Bakker Shut et al. 2004), live cell analysis (Bonnier, Meade et al. 2010), lung cancer cell analysis (Oshima, Shinzawa et al. 2010), oesophageal cancer diagnosis (Shetty, Kendall et al. 2006), and analysis of normal, benign and malignant breast tissue (Chowdary, Kumar et al. 2009). Raman spectroscopy is an ideal technique to assess the integrity of SWCNT, whose Raman spectra are very well documented and understood, especially when applied to biological cells and tissues which will pose minimal background signal.

Raman spectroscopy also has an advantage over conventional tissue analysis techniques which suffer from processing associated artifacts such as fixatives, contrasting agents and not fully biocompatible or marginally toxic fluorescent probes and markers. Harsh conditions imposed by the physical interaction of an electron beam or light (particularly UV) can be avoided and kept to a minimum using Raman spectroscopy approach.

1.4.2. High content screening

High content screening (HCS) is a cutting edge rapidly evolving technique implementing automated inverted fluorescent microscopy principles. The light source is typically a mercury or xenon lamp with capabilities of imaging in the visible spectrum (~400 nm– 700 nm). It incorporates an automated stage, auto focus, can image biological samples in a range of micro-plates (eg 96 well plate, 48 well, 24 well etc.) and glass slides with automated image capture and analysis. HCS has revolutionized traditional fluorescent microscopy enabling rapid image acquisition in an objective manner. It has been used by numerous pharmaceutical and research laboratories to carry out screening of drugs.

HCS has been used to assess acute toxicity of numerous NP (Jan, Byrne et al. 2007), however much controversy and inconsistency has been reported on the reliability of traditional cytotoxicity methods for measuring NP toxicity (Hirsch, Roesslein et al. 2011).

It is an extremely powerful tool for assessing acute toxicity in a rapid manner however it does not provide any insight into the cellular mechanisms ensuing following NP exposure and leaves many biochemical questions unanswered.

1.4.3. Atomic force microscopy

Atomic force microscope (AFM) is a member of the scanning probe microscopes (SPM) family which was developed in 1986 by Binnig and his colleagues, with the first commercial AFM made available in the early 1990's (Morris, Kirby et al. 1999). Unlike traditional microscopes which use light to image a sample, AFM rather uses a probe to touch and "feel" the surface of the sample in question. The best resolution obtainable using a light microscope is determined by the diffraction limit, therefore the best achievable resolution using light of 400 nm is ~ 200 nm. However, AFM is not limited by light but rather the diameter of the tip used to probe the surface of the sample which can go down as small as ~ 1 nm in diameter using CNT at the end of the tip. Typically a silicon nitride tip is used with a diameter of ~ 20 nm. Therefore the probe cannot "see" anything below that limiting size.

AFM opens up new opportunities for studying single cells and molecules on the nanoscale and has a major advantage over electron microscopy in that imaging can be carried out in buffer solution. It offers a means to interrogate the spatial organization, assembly, conformational properties and interactions of individual components on the cells membrane (Dufrene 2008). Another intriguing AFM application is the possibility to measure the interaction of the probing tip with the cell surface, which provides data on the mechanical properties of the sample. Using AFM Lekka *et al* were able to compare the elasticity of normal and cancerous human bladder cells, by determining the Young's modulus values (Lekka, Laidler et al. 1999). They found that the normal cells had a Young's modulus one order of magnitude higher than the cancerous providing information on the cell cytoskeleton. A similar study to measure live cell stiffness was carried out by Cross *et al* (Cross, Jin et al. 2007). Previously a study by our group optimized a novel AFM method to measure the mass and volume of platelets on the nanoscale deposited on a gold chip to improve the modeling of shear stress experienced by the platelets (Santos-Martinez, Medina et al. 2010). AFM can also be used to measure the surface roughness by

calculated the route-mean square roughness (Camesano, Natan et al. 2000; Revzin, Tompkins et al. 2003). This technique could be used to measure the roughness of different substrates made up of nanomaterials providing further insight into the surface mechanics encountered by living cells for example however such measurements were beyond the scope of this thesis and could be considered in future work. An intriguing study by Perna *et al* showed that using traditional cell viability assays revealed that cells exposed to HgCl_2 at a concentration of 10^{-6} M had no cytotoxic effect. However, using a combination of Raman spectroscopy and AFM they revealed the contrary and found structural and biochemical modifications as a result of chemical stress to the cells. This highlights the need for suitable techniques to explore beyond the scope of traditional cytotoxicity tests, which is of particular importance when probing the interaction of cells and NP.

1.4.4. Fluorescent lifetime imaging microscopy (FLIM)

A fluorescent lifetime image is generated based on the lifetime of the fluorophore signal and not the intensity. A pulsed laser source excites the fluorophores, the nanosecond decay kinetics of the electronic excited-state fluorophores, known as the fluorescence lifetime (τ), are then mapped spatially using a microscope with a high-frequency modulation detector (Bastiaens and Squire 1999). FLIM can be measured in either the time-domain or the frequency domain. Time domain measurements involve excitation of the sample with a short pulse of light (ideally faster than the lifetime), resulting in fluorescence intensity that decreases exponentially with time. The frequency domain involves the excitation of the sample with sinusoidally modulated light, resulting in emission of light that is phase shifted, and has a reduced modulation depth, which are then used to calculate the phase and modulation lifetimes. In cells the factors affecting the fluorescence lifetime include; ion intensity, hydrophobic properties, oxygen concentration, molecular binding and molecular interaction by energy transfer when two proteins approach each other. Lifetime is independent of dye concentration, photobleaching, light scattering and excitation light intensity.

A study by Zhang *et al* found that using gold nanorods as labels in kidney cells for FLIM imaging provided a better contrast ratio and more detailed features than traditional fluorescence intensity imaging (Zhang, Yu et al. 2010). FLIM has also proven to be a

sensitive read out technique for measuring subtle differences in cell pH (Lin, Herman et al. 2003). A novel FLIM application was achieved by Sud *et al* whereby they used FLIM as an intracellular oxygen detector (Sud and Mycek 2009). FLIM is unique technique which can be used for cellular and NP readout applications, providing information beyond the scope of traditional fluorescence microscopy.

1.5. Nanomaterials

Nanomaterials have been defined by the European Commission, Brussels as of 18th October 2011 as follow: ““Nanomaterial” means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm”. This highlights the necessity to explore exposure scenarios that include unbound nanomaterials and also their composite naomaterials. Such interest in the cytotoxicity of these different exposure routes was anticipated and has been addressed in this thesis where cells have been exposed to unbound nanoparticles and their composite thin films.

Naomaterials can range from spherical quantum dots (QD) with core diameters of 2-10nm to silver nanowires that are ~50nm in diameter and micrometers in length. There are many benefits from using nanoparticles in place of the bulk material; for example in electronics they allow for devices which are smaller in size, they are generally less expensive, tend to require less power and afford greater sensitivity. However these advantages are solely a function of scale. The most exciting use of nanoparticles is that in comparison to bulk materials they have new and potentially advantageous properties. For example semiconductor QD have superior photostability (Boldt, Byrnes et al. 2006), their optical properties are size dependant (Kapitonov, Stupak et al. 1999) and they have a high luminescence quantum yield (Gaponik, Talapin et al. 2002) making them attractive alternatives to fluorescent markers in biolabeling.

Carbon nanotubes have generated a great deal of interest since their discovery in 1991 (Iijima 1991) due to their exceptional electrical and physical properties (Dresselhaus, Dresselhaus et al. 1995). They have a high aspect ratio and they are hollow making them

attractive for bio-applications such as drug delivery and their rich electronic properties have been explored for nanoscale biosensors (Paradise and Goswami 2007).

In the field of nanotechnology both gold and silver have emerged as powerful tools due to their electrical, thermal and optical properties. Silver, however, has many advantages over gold. In fact it has the highest electrical conductivity ($6.3 \times 10^7 \text{ S.m}^{-1}$) and thermal conductivity ($429 \text{ W.m}^{-1}.\text{K}^{-1}$) of all metals (Sun 2010). Silver has a higher optical extinction coefficient, sharper extinction bands, higher ratio of scattering extinction and extremely high field enhancements when compared to gold.

1.5.1. Carbon nanotubes

Carbon is one of the most important elements in existence. It exists in a number of different allotropes, with diamond being the most well known. Figure 2 illustrates how graphene, a flat monolayer of carbon atoms, is the basic building block for other graphitic materials such as fullerenes, graphite and carbon nanotubes (Geim and Novoselov 2007). The most energetically favourable is graphite. Here, sheets of strongly bound atoms are weakly held together in layers by van der Waals forces. Carbon nanotubes are sheets of graphene rolled up to make hollow cylinders, usually in the range of $\sim \text{nm}$ in diameter and $\sim \mu\text{m}$ in length (S. Reich, C. Thomsen et al. 2004). Single walled carbon nanotubes (SWCNT) are made up of a single layer of graphite whereas multiwalled carbon nanotubes (MWCNT) comprise a number of concentrically arranged cylinders of graphene. They can be either metallic or semiconducting, which is determined by the chiral vector direction and magnitude (Pimenta, Marucci et al. 1998).

Since their discovery in 1991 (Iijima 1991), an extensive amount of research has been carried out on carbon nanotubes (CNT), their unique, mechanical, electrical and magnetic properties lending to a range of commercial applications. Nanotubes have also shown to be able to withstand high bending radii and kinks (Iijima, Brabec et al. 1995), making them attractive along with their electronic properties for flexible electronics. They have a large surface area to volume ratio which is attractive for bio-applications such as drug delivery (Chaudhuri, Soni et al. 2010) and their rich electronic properties have been

explored for nanoscale biosensors (Paradise and Goswami 2007; Lu, Gu et al. 2009). Recently Gogotsi and his group successfully developed CNT based cellular endoscopes, permitting the maneuvering of attolitre volumes within single cells and probing the intracellular environment with a spatial resolution of $\sim 100\text{nm}$ (Singhal, Orynbayeva et al. 2011).

However, these attractive characteristics make the biological interactions of CNT with biological interfaces largely unpredictable. They have been reported to induce toxicity in cells (Cheng, Muller et al. 2009) and frustrated phagocytosis (Brown, Kinloch et al. 2007). Conflicting reports on the toxicity induced in tissue following CNT exposure have been reported (Shvedova, Kisin et al. 2008) (Schipper, Nakayama-Ratchford et al. 2008). Therefore this study will investigate cell viability and the autophagic response of phagocytic cells to SWCNT.

It is of fundamental importance to look at the ultimate fate of CNT and whether it is possible that they may be degraded at any stage following use in industry or the laboratory, perhaps by bacteria in the air or soil or by cells in humans following exposure. Some studies have already been carried out *ex vivo* to investigate possible degradation routes. The degradation of carboxylated and pristine SWCNT in horseradish peroxidase (HRP) was carried out by Star *et al* where they found the carboxylated degraded to a much higher degree (Allen, Kotchey et al. 2009). They suggest that a strong adsorption of the HRP facilitated such degradation whereas the hydrophobic nature of the pristine tubes repels the HRP thus preventing the enzyme's oxidative effects. Bianco and his group investigated the degradation of oxidized SWCNT and oxidized MWCNT in the presence of either phagolysosomal stimulant fluid (PSF) or HRP (Russier, Menard-Moyon et al. 2010). Using TEM and Raman they found that the SWCNT were degraded to a much higher degree than the MWCNT and that the "more perfect" nanotubes degraded to a lesser extent, indicating that the surface functionalisation (carboxyl) plays a critical role in the degradation process.

Carbon nanotubes have a very elegant Raman spectrum made up discrete bands. The radial breathing mode (RBM) at $\sim 200\text{ cm}^{-1}$, the D-band at $\sim 1350\text{ cm}^{-1}$, the G-band at $\sim 1590\text{ cm}^{-1}$ and the 2-D band at $\sim 2600\text{ cm}^{-1}$. All of these bands along with Raman

spectroscopy will be discussed and explained later in this chapter. Raman spectroscopy is used in this study to investigate the integrity of SWCNT within cells and tissue.

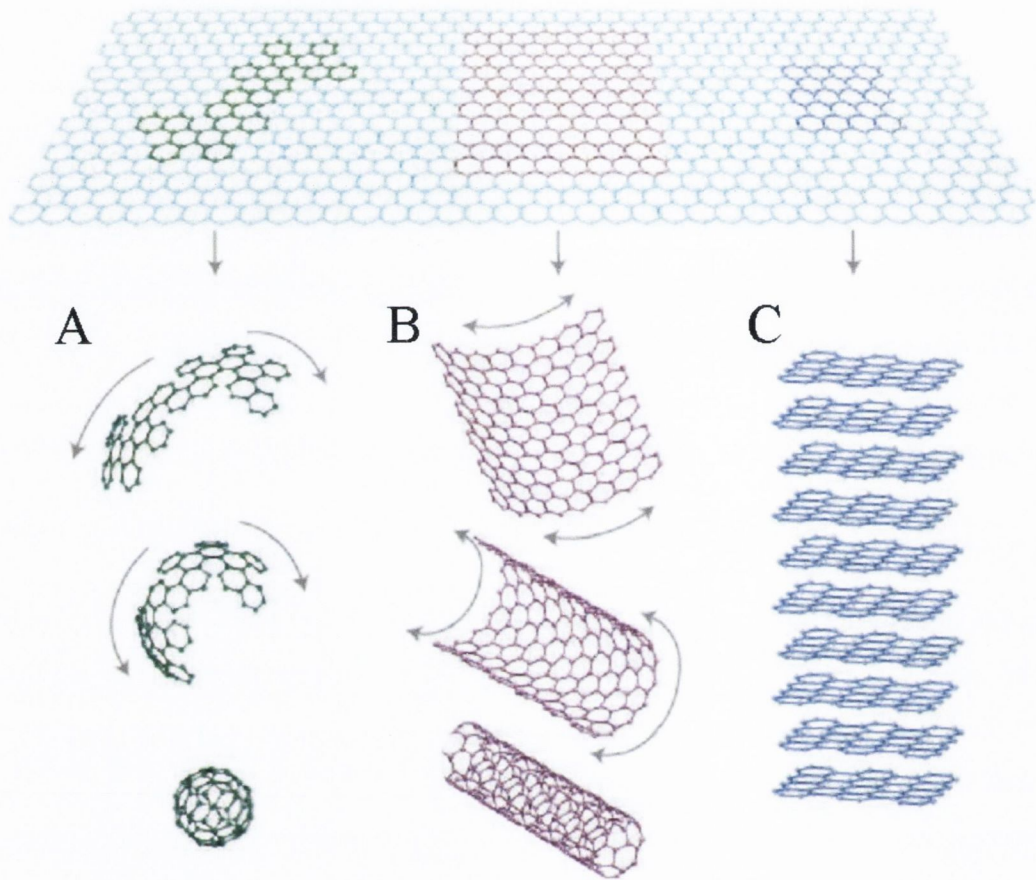


Figure 2. Different allotropes of graphene. Illustrates how graphene, a flat monolayer of carbon atoms, is the basic building block for other graphitic materials, (A) wrapped into a ball to form fullerenes, (B) rolled up into a hollow cylinder to form carbon nanotubes or (C) multiple stacked layers forming graphite. (Image reproduced from Nature Materials; 6, 183-191, 2007)

1.5.2. Silver nanowires

Silver has the highest electrical conductivity ($6.3 \times 10^7 \text{ S.m}^{-1}$) and thermal conductivity ($429 \text{ W.m}^{-1}.\text{K}^{-1}$) among all metals (Sun 2010). It is malleable in nature and has high optical reflectivity. All of these properties make it a promising material in the development of nanowires. For example the role of nanowires in applications such as

nanoscale electronic devices (Yanson, Bollinger et al. 1998; Kim, Cha et al. 2011) and in forming flexible thin electrodes (De, Higgins et al. 2009) has been explored.

Silver nanoparticles have been found to possess anti-bacterial properties which have been exploited in many bioapplications (Eby, Luckarift et al. 2009; Lv, Su et al. 2010) and antimicrobial paints (Kumar, Vemula et al. 2008). Silver nanowires (AgNW) are solid crystalline structures and their lengths can range from hundreds of nanometers up to tens of microns and their diameters in the nanorange. Figure 3 shows two examples of electron microscopy (EM) images of the AgNws used in this study. The typical average length was $\sim 6\mu\text{m}$ and the diameter of the wires was 50nm. Note that the ends of the AgNW has a “triangular” appearance and this is where a fivefold symmetrical silver nanoparticle will have acted as a nucleation site, the addition of material to this end-face allows for the growth of the nanowire by Oswald ripening.

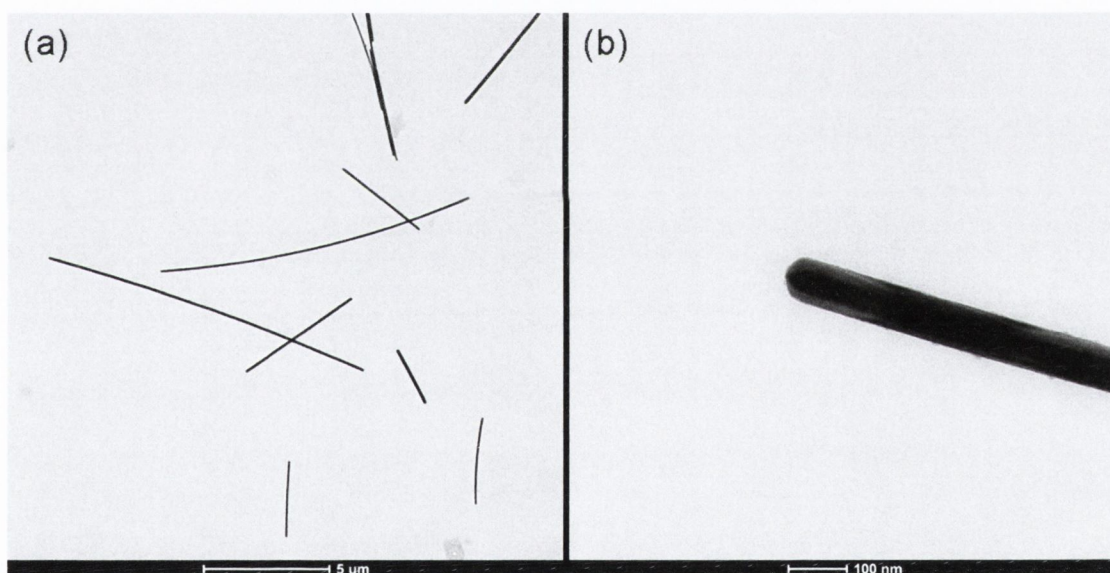


Figure 3. Electron microscope images of silver nanowires used in this study.

However there is much uncertainty surrounding the toxicity of AgNW. As with many nanomaterials the long term effects following use, exposure, and disposal requires novel and more rigorous human and environment risk assessment. Yang *et al* reported on increased mutation frequency during DNA replication caused by silver nanoparticles (Yang, Shen et al. 2009). Their entry into the environment as a result of their current use in

a vast number of applications has been reported (Nowack 2010). This study will explore cell viability and autophagic response of phagocytic cells following AgNW exposure.

As mentioned earlier moving from the bulk material to the nanoscale brings with it some fascinating and exciting properties. AgNW exhibit transverse and longitudinal plasmon resonance which results from the coherent coupling of the surface electronic states with the incident electromagnetic waves that have wavelengths greater than the wire dimension (Luu, Doorn et al. 2011). This phenomenon will become very apparent and will be illustrated further in chapter two in which imaging of the AgNW (50 nm diameter) with 488 nm laser light is demonstrated. This is of particular benefit when looking at the interaction of the AgNW in living cells using confocal microscopy.

1.5.3. Quantum dots

Inorganic semiconductor nanocrystal (NC) or quantum dots (QD) are quantum-confined objects (Klimov, McBranch et al. 1999) possessing discrete energy levels and exciton radii that are smaller than the bulk exciton Bohr radius (Bawendi, Carroll et al. 1991). They therefore represent a class of material intermediate between single molecules and bulk solid-state materials (Wang, Qu et al. 2003). The carrier energies of these spherical QDs increases as the radius of the QD decreases (Wang and Herron 1991; Klimov, McBranch et al. 1999). Although the focus here is on one type of QD there are a number of different QDs, CdS, CdSe, (Rogach, Kornowski et al. 1999) CdTe, (Rogach, Franzl et al. 2007) CdHgTe, (Harrison, Kershaw et al. 2000) HgTe, (Rogach, Kershaw et al. 1999) and ZnSe, (Shavel, Gaponik et al. 2004) in use, with a broad number of applications such as in light emitting diodes (LED), (Gao, Lesser et al. 2000; Gaponik, Talapin et al. 2000; Minot, Kelkensberg et al. 2006; Bertoni, Gallardo et al. 2007; Dai, Ji et al. 2011; Qian, Zheng et al. 2011) QD-bead conjugates for use in biotechnology, (Wang, Rogach et al. 2002) microcavity lasing (Cha, Bartl et al. 2003) and photosensitive films (Guldi, Zilbermann et al. 2005). They are also an attractive alternative to fluorescent markers in bio-labeling as they have high photostability (Boldt, Byrnes et al. 2006), their optical properties are size dependent (Kapitonov, Stupak et al. 1999) and they have a high luminescence quantum yield (Gaponik, Talapin et al. 2002). Another potential advantage

of using QDs for biological purposes is the possibility of their specific functionalisation and directed specific targeting within a cell. However, the behaviour of “naked” non-functionalised QDs in biological environments is a field less well studied and understood but of great importance to the safety of the populace.

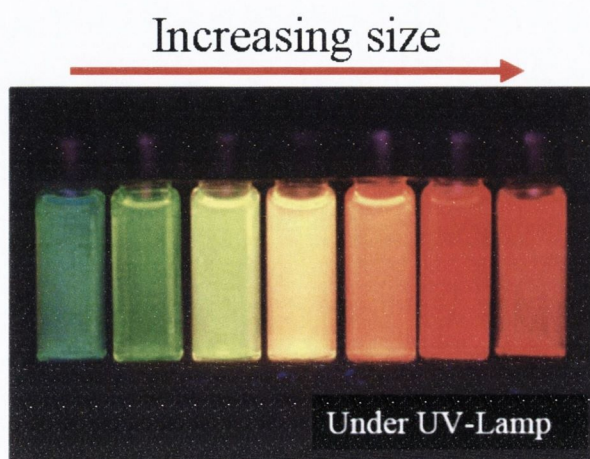


Figure 4. QD emission under UV lamp. QD emission wavelength is dependent on size. The optical properties of QDs are size dependent with the smaller emitting towards the blue end of the spectrum whereas the larger emit towards the red.

Given the constant increase in their uses in nanotechnology there are rising possibilities of human exposure to such particles e.g. research laboratory or in an industrial manufacturing environment. The mechanism of uptake of nanoparticles of different natures by live cells has been well documented and the focus of a number of research studies worldwide (Alivisatos 2004; Alivisatos, Gu et al. 2005; Lovric, Cho et al. 2005; Parak, Pellegrino et al. 2005; Smith, Ruan et al. 2006). Due to their extremely small size such particles have the capacity to enter airway passages, cross epithelial barriers or directly enter the vascular system rendering them potentially harmful to living organisms (Maysinger, Lovric et al. 2007). In this study, the exposure of whole cells to QDs will be examined with a specific focus on their interactions with the most commonly encountered proteins and macromolecules (Cedervall, Lynch et al. 2007) - ribonucleic acid (RNA), deoxyribonucleic acid (DNA), core histones and serum proteins. Albumin type protein constitutes over 50% of the total blood protein pool. The core histones (H2A, H2B, H3 and H4) are proteins found in abundance in the nuclei of eukaryote cells which are actively involved in cell cycle and regulation. They are involved in the structural organisation of

chromosomal DNA, through interactions between the negative phosphate backbone of the DNA and the positive lysine and arginine amino acids side chains of the core histones (Luger, Mäder et al. 1997). The nano-particles used in this study were negatively charged thioglycolic acid (TGA) capped Cadmium Telluride (CdTe) QDs with the negative charge attributed to the TGA capping and the presence of carboxylic groups (Gaponik, Talapin et al. 2002). In this study, the potential mechanisms underlying the QDs tropism to nuclei and nucleoli were studied and their highly luminescent properties were exploited to localise them in the intracellular environment.

1.6. Aims and Objectives of this Thesis

The risk of human and environmental exposure to NP during fabrication, use or disposal as e-waste, is on the rise with their increased use and applications in electronic devices. This biological interaction can alter the properties and influence the behaviour of NP. It is therefore imperative to explore and understand what happens to both the cells and the NP following their interaction. Existing microscopic and spectroscopic techniques were adapted and optimized here to address the toxicity, integrity and distribution of NP following exposure to cells and tissue.

The aims of this thesis are to:

- Investigate acute toxicity and autophagic responses of phagocytic cells following exposure to carbon nanotubes, quantum dots, silver nanowires and their enabled thin films.
- Adapt and optimize Raman spectroscopy to locate, map and assess the integrity of carbon nanotubes within phagocytic cells and mouse lungs following their exposure to SWCNT
- Development and optimization of appropriate chemometric techniques for Raman spectral analysis.
- Establish the ultimate target compartments of quantum dots within phagocytic cells and investigate their interaction and integrity within these biological domains using fluorescent lifetime imaging.

Chapter 2

2. Characterisation of autophagic response in phagocytic cells following their exposure to SWCNT, AgNW and their enabled thin films

2.1. Introduction

Nanomaterials including single walled carbon nanotubes (SWCNT), silver nanowires (AgNW) and quantum dots (QD) are widely used in the field of electronic devices due to their unique electronic and mechanical properties and are viable choices in the pursuit of conductive, transparent and flexible electrodes. One of the main areas of interest is the use of transparent conductors in flat panel displays, which require a conducting, transparent material. The current industry standard for such a material is indium tin oxide (ITO). ITO has good electrical conductivity, transmittance in the visible region of greater than 80% and infrared reflectance (Suzuki, Matsushita et al. 2002). However, ITO is polycrystalline and thin films can crack under tension or compression (Chen, Cotterell et al. 2002). Therefore, ITO is unsuitable for the next generation of flexible displays. Another quest lies with the need for low cost, easily producible light emitting devices with QD as one of the leading candidates in this field. With their tunable band gap, and efficient photoluminescence they are ideal for future displays to replace light emitting diodes (LED) and a significant amount of research has gone into such displays (Bendall, Paderi et al. 2010).

However, much uncertainty surrounds these nanomaterials from a human health and environmental point of view, as the potential toxicity of these materials and their enabled products, either during manufacture or at the end of their life cycle, has not been adequately assessed.

QD, CNT and AgNW, have all been shown to induce toxicity (Lovric, Bazzi et al. 2005; Lovric, Cho et al. 2005; Davoren, Herzog et al. 2007; Navarro, Piccapietra et al. 2008; Cheng, Muller et al. 2009). However, the main focus of research into cellular toxicity which is induced by NP has focused on the apoptotic and necrotic cascades which are the principle pathways of cell death. We are now in a new era of toxicology where

subtle changes in different cellular mechanisms are understood to have significant long term impacts on the health of the organism. These subtle changes are now considered to be putative markers or flags indicating toxicity. A better understanding of the subtle changes which occur post-exposure to NP and appropriate monitoring could indicate the risk of subsequent deleterious effects.

The homeostatic process known as autophagy involves the lysosomal degradation of damaged cytoplasmic organelles or mis-folded proteins and is involved in cellular survival in times of stress. Autophagy is an area that is largely unexplored and only a limited number of studies have shown it is induced following exposure of cells to NP (Chen, Yang et al. 2005; Seleverstov, Zabirnyk et al. 2006; Stern, Zolnik et al. 2008; Johnson-Lyles, Peifly et al. 2010; Li, Hartono et al. 2010; Liu, Zhang et al. 2011).

Linking between the underlying cellular responses and intracellular location of NP will lead to a more comprehensive understanding of their implications on human health and the environment.

2.2. Aims and Objectives

Since phagocytic cells are one of the first lines of defense in the body against invading pathogens and foreign bodies, they were used to study the potential toxic effects of QD, SWCNT and AgNW on human cells. The interaction of these cells with SWCNT and AgNW and their enabled thin films were investigated by a multi-platform approach incorporating high content screening, atomic force microscopy, electron microscopy, western blotting, confocal microscopy and fluorescence microscopy. Firstly, the phagocytic cells were grown on thin films incorporating either AgNW or SWCNT. Secondly cells were exposed to loose, unbound, non-embedded AgNW or SWCNT, enabling a comparison between two different nanoparticle exposures.

The aims of this part of the study are:

- To analyse cytotoxicity of AgNW, SWCNT and QDs using conventional high throughput techniques.
- To evaluate phagocytic cell viability following exposure to AgNW and SWCNT and their enabled thin films using atomic force microscopy and confocal microscopy.
- To investigate the level of autophagic expression in phagocytic cells following exposure to AgNW, SWCNT and their enabled thin films using various microscopic techniques and autophagocytic markers.

2.3. Materials and methods

2.3.1. Synthesis of SWCNT and SWCNT based thin films

The carbon nanotube suspensions and carbon nanotube based thin films were provided by Dr. Philip E. Lyons from Prof. Jonathan N. Coleman's group in Trinity College Dublin as follows.

Aqueous solutions of surfactants were prepared at a concentration of 5 mg/mL by overnight stirring. These solutions were then used to make stock dispersions of Iijin arc discharge SWCNTs (Iijin Nanotechnology) such that the mass ratio was 5:1. The dispersion was subjected to 5 minutes of high-power sonication (Vibra Cell CVX; 750W, 20%, 60kHz), then placed in a sonic bath (Model Ney Ultrasonic) for 1 hour, and then subjected to another 5 min of high power sonication. It was then allowed to rest overnight before being centrifuged at 5500 rpm for 90 min. The supernatant was carefully decanted and saved. The post-centrifuge nanotube concentration was determined from absorbance measurements (Cary 6000i).

To be able to prepare films of controllable thickness a metric of some sort must be determined. If we assume that the porosity of nanotube films doesn't deviate with reduced thickness then we can control the thickness by simply controlling the mass deposited. The film thickness, t , was calculated from the deposited mass per unit area, M/A , using $M/A = \rho t$, where ρ is the film density. While the density is not known for these thin films, it was shown in the previous chapter that the vast majority of thick films made from a range of nanotube types had densities between 450 and 700 kg/m³ (nanotube networks tend to be very porous). Thus the density is taken as 570 ± 150 kg/m³ accepting this will result in a ~25 % error in the nominal thickness and any dependant quantities such as DC or optical conductivity. Initially, HiPCO SWNT films with a thickness of ~100nm and 200nm were prepared by diluting the stock dispersions down to concentrations ranging between 10⁻¹ mg/ml and 10⁻³ mg/ml in order to determine the concentration that resulted in the highest conductivity. This dilution was accomplished by addition of surfactant stock solution and 1 minute tip sonication. Electrical testing of the films showed that although there was only a

small variation, a maximum DC conductivity was found for films prepared from dispersions with CNT = 0.005 mg/ml.

Transferring the films deposited from the cellulose ester membranes entails washing the film after filtration with Millipore water and then rolling the film onto a pre-heated ($\sim 90^{\circ}\text{C}$) Polyethylene terephthalate (PET) sheet. Pressure is then applied for a set amount of time before dissolving the membrane away using acetone and methanol.

Problems with the film lifting off the PET during processing were addressed by varying the pressure applied during drying (290 gcm⁻² down to 2.9 gcm⁻²) and the drying times (2hrs @ 900C on a hot plate, 6hrs Room T in vacuum). The conductivity of the HiPCO SWNT films increased with decreasing pressure, but this was countered by a low adhesion of the film to PET resulting in the film being destroyed during the dissolving process. A value of 175 gcm⁻² was chosen as it gave slightly lower conductivities but better adhesion and hence a better chance of a fully transferred film. While different setting times and temperatures affected the conductivity slightly, an optimum level of conductivity and film stability was seen with the 2hrs at 900C on a hot plate.

The membranes were initially dissolved by simply placing the film in four sequential baths of acetone followed by a final wash of methanol and then drying in air. The turbulence of the liquid bath caused damage to the fragile films, resulting in the film lifting of the PET. An initial exposure to acetone vapour was used before using liquid baths. The exposure to acetone stiffened the membrane and allowed liquid baths to be used with little damage to the film. The effect of the level of removal of MCE from the film surface on the conductivity was investigated by varying the exposure time to the solvent, the volume of solvent and the number of baths used. The conductivities of the films were similar, allowing us the use four 15min exposures to 500ml acetone followed by 15mins exposure to 500ml methanol.

As films of arc-discharge Ijii SWNTs produced the highest network conductivities they were used for the remainder of work presented here. Films with a thickness of ~ 50 nm were prepared using the optimised technique discussed above with dispersions of aqueous solutions of sodium dodecyl sulphate (SDS), sodium dodecylbenzene sulfonate (SDBS),

lithium dodecyl sulphate (LDS), Sodium Cholate (SC) and Triton® X-100. We found the conductivities of these films to be (2.3×10^5 , 2.1×10^5 , 2.2×10^5 , 1.9×10^5 , 3.8×10^4 Sm⁻¹) respectively. We suggest that the deviation of the Triton results is due to lower dispersion quality and hence a poorer quality film. Due to their popularity, we chose to focus on SDBS and SDS for the remainder of this work.

A range of films were then fabricated with thicknesses ranging between 10 – 100 nm. Transmission scans were made using an Epson Perfection V700 photo flat-bed transmission scanner with a bit depth of 48 bits per pixel and a spatial resolution of 6400 dpi. The numerical output of the scanner was calibrated by scanning a range of neutral density filters. The resultant calibration curve was used to transform the output to represent transmittance. This results in a transmittance map with a transmittance value for every pixel. Transmission maps were transformed into absorbance maps by applying $A = -\log T$ to each pixel. The mean and standard deviations of the transmittance or absorbance were calculated from the entire data set (i.e., from the entire set of pixel values).

In order to fit the thin films into 96 well plates, to enable cell culture on them, small round films ~ 6 mm in diameter were cut from larger films.

2.3.2. Synthesis of AgNW and AgNW based thin films

Silver nanowires were purchased from Seashell Technologies (www.seashelltech.com) as suspensions in isopropylalcohol ($C=12.5\text{mg/mL}$ as measured by thermogravimetric analysis, Perkin-Elmer Pyris). A small volume of the dispersion was diluted down to 0.1 mg/mL with Millipore water. This was subjected to half an hour low power sonication in a sonic bath (Model Ney Ultrasonic). The dispersion was further diluted down to a concentration $C = 0.002\text{ mg/mL}$ with Millipore water and sonicated for a further 30 minutes. Silver nanowire films were prepared by vacuum filtration of the above dispersions using porous mixed cellulose ester filter membranes (MF-Millipore membrane, mixed cellulose esters, hydrophilic, $0.2\text{ }\mu\text{m}$, 47 mm). A range of films with varying thickness was prepared by filtering different dispersion volumes, giving different deposited masses. These films were characterised by their deposited mass per unit area, M/A . The

deposited films were transferred to a polyethylene terephthalate (PET) substrate using heat and pressure.⁵ The PET was placed on a hot plate at 100 °C. The AgNW film/membrane was placed on the PET with the AgNWs in contact with the PET. A 3kg weight was then placed on top for 2 hours. The cellulose filter membrane was then removed by treatment with acetone vapour and subsequent acetone liquid baths followed by a methanol bath.⁵

The film area was 36mm in diameter. Thin silver films were prepared using an Edwards S Auto 306 evaporator. Commercially available ITO was purchased from UQG Optics Ltd. Transmission scans were made using an Epson Perfection V700 photo flat-bed transmission scanner with a bit depth of 48bits per pixel and a spatial resolution of 6400 dpi. The numerical output of the scanner was calibrated by scanning a range of neutral density filters. The resultant calibration curve was used to transform the output to represent transmittance. This results in a transmittance map with a transmittance value for every pixel. Transmission maps were transformed into absorbance maps by applying $A = -\log T$ to each pixel. The mean and standard deviations of the transmittance or absorbance were calculated from the entire data set (i.e., from the entire set of pixel values).

For the purposes of this study thin films ~ 6mm in diameter (which were cut from larger films), 20 nm thick with a transmittance of 93.2 % were used.

2.3.3. QD synthesis

Quantum dots (QD) used here were provided by Dr. Stephen Byrne from Prof. Yury G'unko's group in the Chemistry dept., Trinity College Dublin. Thioglycolic acid (TGA) capped CdTe QDs were prepared according to published procedures (Byrne, Corr et al. 2006; Rogach, Franzl et al. 2007). These QDs were stored at 4 °C in the dark and used as received but before incubation with the cells, they were diluted in the appropriate warm medium (37 °C) to reach the desired final concentration of 1×10^{-6} M. Using the polynomial fit described by Yu *et al.*, (Yu, Qu et al. 2003) the calculated diameter of the TGA capped QDs was 3.2 nm.

2.3.4. Cell culture

2.3.4.1. *Phagocytic – THP-1 cells*

Human monocytic leukemia THP-1 cells (American Type Culture Collection, USA) were maintained in RPMI 1640 (with Glutamax) (Gibco/BRL, Invitrogen, Carlsbad, CA) containing 10% FCS (Gibco/BRL) with 100 µg of penicillin/mL and 100 mg of streptomycin/mL (Sigma, P4333). Prior to treatment, cells were plated onto either SWCNT or AgNW thin films in 96 well plates (Nunc), at a density of 0.5×10^5 cells/ml and differentiated with 100nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 72hrs.

2.3.4.2. *Bone marrow derived macrophages – LC3 GFP cells*

Immortalised mouse splenic macrophages were stably transfected with EGFP-LC3 by Hardy Kornfeld's group, University of Massachusetts, MA. Creation of the GFP-LC3 vector and macrophages is described by Harris *et al* (Harris, Hartman et al. 2011). Cells were cultured and maintained in DMEM medium (Invitrogen) supplemented with 10% FCS (Gibco), 100 µg of penicillin/mL and 100 mg of streptomycin/mL (Sigma, P4333) and 5µg/mL puromycin (essential at all times to maintain selection of the plasmid). Once the cells reached 80% confluency, they were split and seeded at 1/400 dilution into 8 well chamber slides prior to treatment with either chlorquine, SWCNT or AgNW.

2.3.5. Cell staining

The cells were fixed with 2% paraformaldehyde for 30 minutes at room temperature and permeabilised with 0.1% Triton-X for 3 minutes. Cells were washed twice with PBS and the anti- α -tubulin (mouse) primary anti-body (Sigma), (1:500 dilution) was added for 60 minutes. Cells were washed twice with PBS and then a solution made up of (Nucleus stained with Hoechst (Sigma), 1:1000 dilution, secondary anti-body alexa 488nm

(Molecular probes) (1:1000 dilution), and actin stained with Rhodamine Phalloidin (Invitrogen) (1:250 dilution)) was added for 60 minutes. Two more final washes with PBS and a cover slip was then mounted on the thin films using mounting media (Dako) and left to dry overnight before imaging.

2.3.6. Confocal Microscopy

Confocal imaging was carried out on the fixed cells grown on the thin films using a Zeiss Meta LSM 510 system (Carl Zeiss). A 63 x oil immersion lens was used on the inverted confocal system. Excitation wavelengths used were 405 nm, 488 nm and 561 nm and emission filters were BP 420-480 nm, BP 505-530 nm and 572-754 nm respectively.

Live cell confocal imaging of the BMDM-LC3 cells was carried out using the same confocal system. However, the excitation wavelength used was 488 nm and the emission filter was BP 505-530 nm. The cells cultured and treated in the 8 well LabTeks were imaged every 30 minutes on a heated stage (37°C).

2.3.7. Atomic force microscopy

For AFM imaging, THP-1 cells were grown on either, glass coverslips, SWCNT thin films or AgNW thin films (same as section 1.4.3). For dry phase imaging, the cells were then fixed using 2.5% glutaraldehyde (Sigma) for 30 minutes at 37°C. The cells were then dehydrated through ascending grades of ethanol (60% for 20 minutes, 80% for 20 mins, 90% for 20 minutes and finally 100% for 30 minutes). The coverslips with the cells were then mounted onto glass slides using mounting media (Dako) with the cells facing upwards. The slide was then mounted onto the atomic force microscope (AFM) and clipped down to ensure no movement during acquisition. AFM images were recorded using an Ntegra spectra hybrid AFM/Raman system (Figure 5, NT-MDT, Russia).

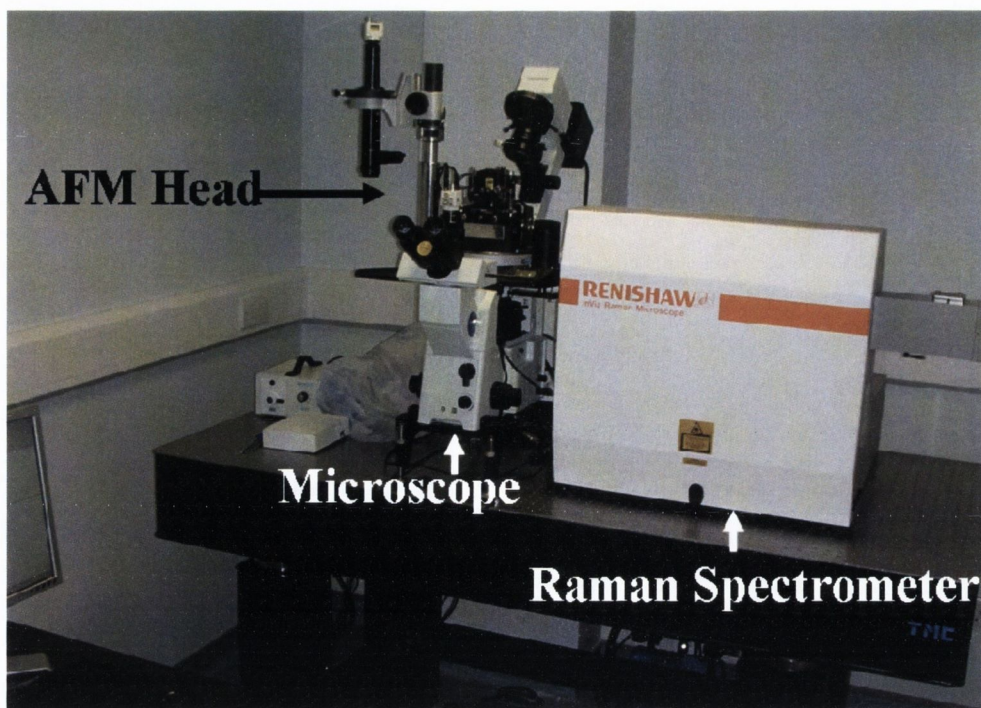


Figure 5 Hybrid system of Raman spectrometer and atomic force microscope (AFM) (Ntegra Spectra, NTMDT, Russia) used throughout this entire study.

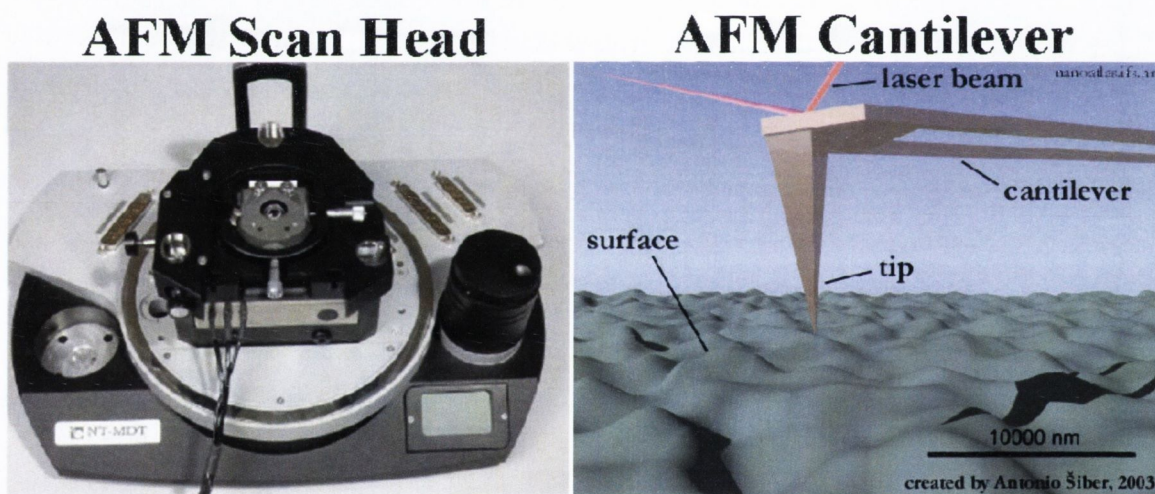


Figure 6 Close up of the AFM scan head which is mounted onto the microscope in Figure 5 and a cartoon of a magnified AFM cantilever scanning a non specified surface.

Dry phase imaging was carried out in semi-contact mode using a silicon nitride tip (NSG 10, Golden silicon probes). For dry phase imaging the tips typically have a force constant of between 5.5 Nm^{-1} and 22.5 Nm^{-1} . The resonance frequency of the tip was found to be $\sim 280 \text{ KHz}$. Height AFM images of varying cells were recorded at 0.55 Hz . This dry

phase technique was optimized by our group in a previous study on platelets (Santos-Martinez, Medina et al. 2010).

For liquid phase imaging a dedicated liquid phase head was used. The AFM tips used for liquid typically have a force constant of between 0.5 and 0.01 Nm^{-1} . Here Veeco MLCT silicon nitride tips were used with a force constant of $\sim 0.03 \text{ Nm}^{-1}$ at a speed of $\sim 0.55 \text{ Hz}$.

2.3.8. High content screening

High content analysis was carried out using the InCell 1000. THP-1 cells were seeded at 10,000 cells per well in 200 μl of medium. Once differentiated, the cells were treated with SWCNT (5, 2, 1, 0.2 and 0.1 $\mu\text{g/ml}$), AgNW (5, 2, 1, 0.2 and 0.1 $\mu\text{g/ml}$) or QD (3, 2, 1, 0.2, 0.1 μM) for 4, 8, 24, 48 and 72 hours. The cells were then fixed in 2% para-formaldehyde, rinsed twice in PBS and then stained the nucleus blue using Hoechst (1:1000) and the actin red using Rhodamine phalloidin (1:250) for 1 hour followed by two rinses with PBS. 200 μl of PBS was then left in each well prior to imaging.

2.3.9. MTT

Cell viability was measured by CellTier 96® AQueous One solution cell proliferation assay kit according to the manufacturer's instruction (Promega, Madison, WI) (Verma, Davies et al. 2010). THP-1 cells were seeded in 96 a well plate as described in section 2.3.4.1 and treated with QD, SWCNT, AgNW or seeded onto SWCNT thin films and AgnW thin films within the 96 well plate. 100 μl of cell culture was then incubated with 20 μl of MTS tetrazolium solution (provided by manufacturer) for 4 hours. The relative cell viability was calculated by determining the absorbance at 490 nm using a microplate reader (Tecan, Mannedorf, Switzerland).

2.3.10. Transmission electron microscopy

For transmission electron microscopy (EM), cells in culture were fixed in 2.5% glutaraldehyde in cell culture medium. The cells were then taken to the Electron Microscopy Core Facility, UCD Conway Institute, UCD, Belfield, Dublin 4 for staining and EM imaging. The EM imaging was carried out by Cormac O'Connell.

Cells were fixed in 2.5% glutaraldehyde over night, and processed into resin by routine methods (Principles and Techniques of Electron Microscopy: Biological Applications by M A Hayat (2000) Cambridge University Press; 4th edition). Briefly, after fixation, cells were washed in phosphate buffer pH 7.4, and placed in 1% osmium tetroxide for one hour at room temperature. They were then dehydrated in a series of alcohols [% Ethanol (time minutes), 30 (10), 50 (10), 70 (10), 90 (10), 100 (20), 100 (20), 100 (20)] and embedded in epoxy resin (Agar 100 (Agar Scientific Ltd)) (Luft, J H (1961) Improvements in epoxy embedding methods. J Biophys Cytol 9, 409). After polymerisation overnight at 60°C, small blocks were cut from the epoxy 'cake', the plastic (of the six-well plate) removed and the block orientated 'en face' in the tissue-block holder of a Reichert-Jung 'Ultracut' ultramicrotome. Ultrathin sections were cut using a diamond knife (Diatome Ltd). The thickness of the sections was between 60-90 nm. After collection, ultrathin sections were stained with lead and uranium. Sections were examined in a Tecnai 120 (FEI Ltd) transmission electron microscope (TEM) at an accelerating voltage of 120kV. Images were recorded with a MegaView III side-mounted TEM CCD camera (Olympus).

2.3.11. Preparation of cellular lysates of THP-1 cells

2.3.11.1. Cell lysis

Briefly, cells were washed with ice-cold PBS and lysed in lysis buffer (Appendix 1). Lysis was carried out at 4°C for 30 min. Cell lysates were centrifuged at 16,000×g for 15 min at 4°C and clear supernatants were collected.

2.3.11.2. Determination of protein concentration

The protein content in cell lysates was determined using Bradford method based Bio-Rad protein assay according to manufacturer's instructions (Bio-Rad Laboratories GmbH, Germany). Briefly, 5 µl of cell lysate were mixed with 1 ml of Bio-Rad staining solution (diluted 1:5) and incubated for 5 min at room temperature before its OD₅₉₅ (optical density at 595 nm) was measured. Protein content was calculated against a standard curve prepared using bovine serum albumin (BSA).

2.3.11.3. Sample preparation

Cell lysates were normalized for equal protein content as indicated for particular immuno-blotting or affinity precipitation experiment and final volumes were adjusted with lysis buffer. Protein samples were then boiled at 100°C for 5 min with Laemmli sample buffer (Appendix 2) and centrifuged briefly (1 min) to remove any insoluble solids.

2.3.11.4. Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE)

Protein samples were resolved by electrophoresis on SDS-PAGE gels to separate the proteins according to their size and molecular weight. The gel apparatus (ATTO Corporation, Japan) was assembled according to the manufacturer's instructions. Resolving acrylamide gels were prepared to 12% by adding components in the order indicated in Table A1.1 (Appendix 1). The gel was quickly poured in between the two glass plates until the acrylamide solution reached 1 cm below the plastic combs. The gel was overlaid with isobutanol to prevent oxidation, since oxidation can inhibit polymerisation. The gel was allowed to set for approximately 40 min at room temperature. Isobutanol was washed off using distilled water. The stacking gel was made by adding the components in the order indicated in Table A1.2 (Appendix 1). The stacking gel solution was then poured on top of the resolving gel. The plastic combs were inserted into the stacking gel to make protein sample loading wells and the gel was allowed to set for approximately 40 min at room temperature. The combs were removed from the gel and any gel lanes that were not straight were straightened using a gel-loading tip. The gels were placed into the electrophoresis box that was filled with 1X SDS-PAGE running buffer (Appendix 1). Equal amounts of protein samples and the protein molecular weight ladder

were loaded into the wells. Electrophoresis was carried out at 100 V / 20 mA per gel for approximately 1.5 h until the dye front had reached just above the gel base, at which stage electrophoresis was discontinued.

2.3.12. Western blotting

Western blotting was carried out for the transfer of electrophoresed proteins to polyvinylidene fluoride (PVDF) membrane using the semi-dry transfer technique according to the manufacturer's instructions (ATTO Corporation, Japan). The PVDF membrane (0.45 μm) was activated by soaking it in methanol for 1 min. The membrane was then immersed in transfer buffer (Appendix 1) for 10 min at room temperature. A gel sandwich was made by placing 4 sheets of Whatmann 3 mm filter paper (pre-soaked in transfer buffer) on the anode of the Western blotting apparatus. The PVDF membrane was then placed on top of the filter papers and kept moist by flooding it with transfer buffer. The gel was then placed on top of the PVDF membrane and any air bubbles between the gel and membrane were carefully removed. Another 4 sheets of Whatmann 3 mm filter (pre-soaked in transfer buffer) were placed on top of the gel. The cathode was lowered carefully onto the gel sandwich and any excess transfer buffer remaining on the anode was removed with paper towels to prevent the apparatus from short-circuiting. Western blotting was performed for 1 h at 100 mA / 200 V per gel at room temperature.

2.3.13. Immuno-detection and development of blots

Following Western blotting, non-protein bound sites on the PVDF membrane were blocked by incubating the membrane in freshly prepared 5% non-fat milk in PBST (Blocking solution, Appendix 1) for 1 hour at room temperature with constant gentle agitation. Blots were then washed three times with PBST and incubated with appropriate primary antibodies (diluted according to the manufacturers instructions in blocking buffer) overnight at 4°C with constant agitation. Following incubation with primary antibody,

blots were washed five times with 0.1% PBST to remove any unbound antibody. Blots were then incubated with the relevant horseradish peroxidase (HRP) or alkaline phosphatase-conjugated secondary antibody (diluted according to the manufacturers instructions in blocking buffer) for 1 h at room temperature with constant agitation. Unbound secondary antibody was then removed by washing the membrane five times with PBST for 10 minutes.

The immuno-blots were visualized using the Enhanced Chemiluminescence (ECL) method according to manufacturer's instruction (Amersham, Arlington Heights, IL, USA). ECL reagents were placed on the membrane and incubated for 1 minute. Membranes were then exposed to Kodak X-OMAT S film for the appropriate time period (range 15 sec to 10 min). Exposed films were developed using an automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Gevaert AG, Munich, Germany).

2.3.14. Densitometric analysis

Densitometric analyses of the western blots were performed by using GeneTools software (Syngene, Cambridge, UK). The relative values of the samples were determined by giving an arbitrary value of 1.0 to the respective control samples of each experiment.

2.3.15. Fluorescence microscopy

An Olympus fluorescent microscope, 100x oil immersion lens, was used to count the numer of puntca (autophagic vesicles) and thereby quantify the amount of autophagy induced in LC3 cells. Using GFP tagged to the LC3 in the BMDM cells it was possible to count the number of puncta present in each cell. Table 1 is an example of the type of data that was collected for each sample. On average about 300 cells per sample were counted and each experiment carried out 3 times.

Table 1 Table designed to collect data on the LC3 cells.

Sample name	Time point	Field of view	Number of LC3 +ve cells	Number puncta	Number cells with puncta
		1			
		2			
		3			
					
					

2.4. Results

SWCNT and AgNW are widely used in the field of electronic devices due to their unique electronic and mechanical properties and are viable choices in the pursuit of conductive, transparent and flexible electrodes. However, much uncertainty surrounds these nanomaterials from a human health and environmental point of view, as potential toxicity of these materials, either during manufacture or at the end of their life cycle, has not been adequately assessed.

The results here firstly measure the acute toxicity of SWCNT, AgNW and QD using conventional high throughput methods. Secondly the level of autophagic expression in phagocytic cells following their exposure to AgNW, SWCNT and their enabled thin films is evaluated. Following the nanomaterial definition in section 1.5, the unbound SWCNT, AgNW and their composite thin films can be regarded as nanomaterials. Note that QD were only used in the initial stages of this part of the study as thin films made from SWCNT and AgNW were only available, those made from QD were beyond the scope of this project. Chapter 4 addresses the interaction of loose unbound QD with phagocytes further.

2.4.1. Confocal microscopy of phagocytes on thin films

Confocal microscopy was used here to image the phagocytic cells on the thin film surfaces. Our group has previously used it to measure lambda scans of NP but is beyond the scope of this study (Nowostawska, Corr et al. 2011). In order to assess cell viability a comparison was made between the images of cells grown on glass coverslips and those grown on the thin films. This was the first step in investigating the biocompatibility of thin films made from SWCNT or AgNW. All of the cells were stained as follows; nucleus (blue), actin (red) and tubulin (green), which enabled the visualization of an unhealthy cell due to apoptosis or necrosis. Signs of apoptosis are; membrane blebbing, chromatin aggregatiuon, shrinking of cytoplasm and condensation of nucleus, fragmentation of cell into smaller bodies and the formation of membrane bound vesicles. Signs of necrosis are; loss of membrane integrity, swelling of cytoplasm and mitochondria, total cell lysis and no vesicle formation.

An example of the control cells grown on glass coverslips is shown in Figure 7. Overall the cells appear to have conventional morphology, the nuclei are round and of normal size with no signs of condensation or blebbing. The size of the cells, is within the limits of normal variation and the cytoplasm appear intact also with no signs of fragmentation or lysis. A number of similar images were taken of the phagocytic cells grown on the two types of thin films. Compared to the control cells (Figure 7), those grown on the SWCNT thin films (Figure 8) and on the AgNW thin films (Figure 9) also look to be of normal size and shape with no signs of toxicity. It proved to be difficult in both thin films to image the blue nuclear channel which could be due to the films absorbing at this wavelength. However it was still possible to establish that there were no signs of nuclear condensation or blebbing. The size and overall shape of the cytoplasm was also normal with no signs of membrane bound vesicles, shrinking of the cytoplasm or total cell lysis.

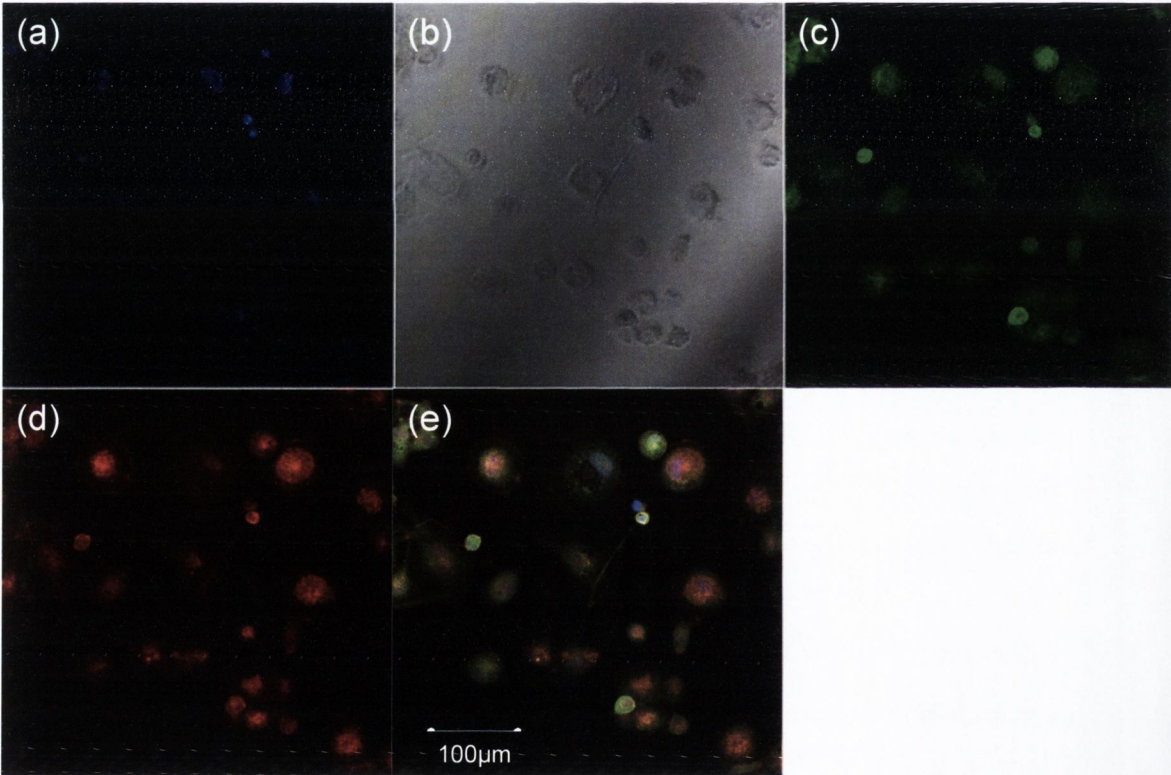


Figure 7 Control sample of a glass cover slip with THP-1 cells. (a) Nucleus, (b) brightfield, (c) tubulin, (d) actin and (e) composite image.

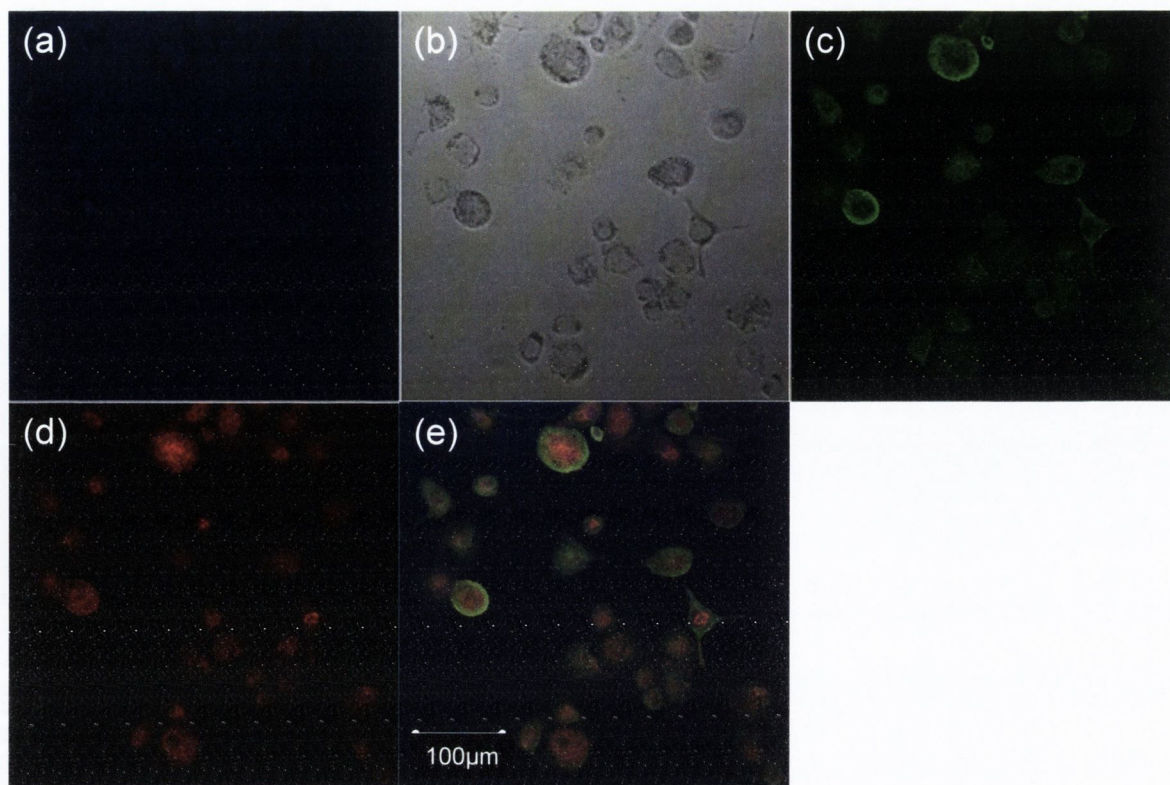


Figure 8 CNT thin film and THP-1 cells. (a) Nucleus, (b) brightfield, (c) tubulin, (d) actin and (e) composite image.

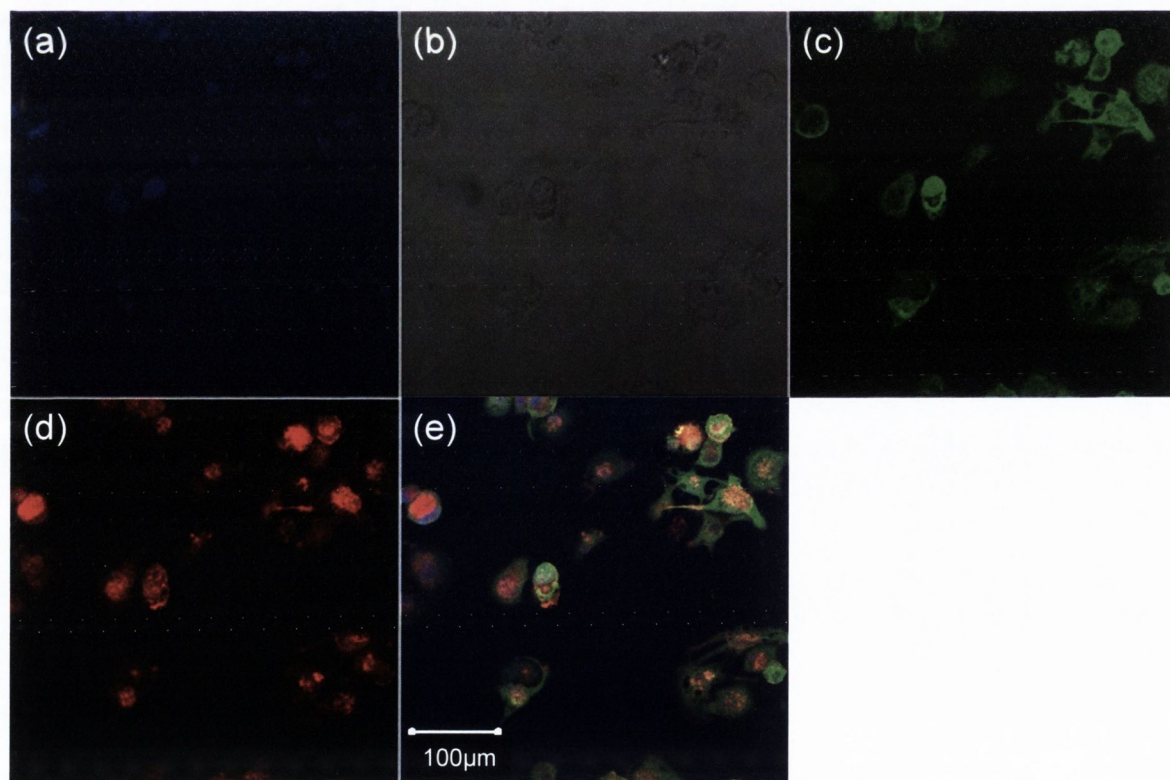


Figure 9 Ag thin film and THP-1 cells. (a) Nucleus, (b) brightfield, (c) tubulin, (d) actin and (e) composite image.

2.4.2. Investigation of cell morphology on thin films using atomic force microscopy

As a significant number of morphological features of the cell cannot be interrogated by conventional confocal and fluorescence techniques, AFM was implemented for further thorough investigation of the cell morphology and topography. The interaction of SWCNT, AgNW and their enabled thin films was assessed using AFM. It was thought that AFM could also provide some insight into the interaction of the cells with the unbound SWCNT and AgNW and also with whatever substrate they are growing on.

Imaging in both liquid and dry phase can be carried out using AFM. The fixing and dehydration techniques are outlined in section 2.3.7. To assess whether or not the fixing and dehydration process had any negative effect on the cell structure or topography both live and fixed THP-1 cells grown on glass coverslips were imaged and compared. An example of a height based AFM image of a live THP-1 cell in complete CO₂ independent medium at 37°C is shown in Figure 10. This cell is ~ 50 µm x 50 µm and ~ 3 µm in height, the outline of the cell and where it adheres to the glass substrate can be seen. A typical AFM image of a THP-1 taken using dry phase, following dehydration, is shown in Figure 11. The dimensions of this cell are ~ 25 µm x 25 µm in width and length and about 3 µm in height. By comparison there is no significant difference between the liquid and dry phase images. Both cells are semi-spheroid in shape, have lamellar features around the edges of the cells and the membrane appears intact. They are higher in the middle of the cell and “fried egg” like appearance owing to the large nucleus in the middle and lower surrounding cytoplasm. These cells are resting on the glass substrate with no signs of phylopodia emerging.

In conclusion the dehydration technique did not change the morphology of the cell dramatically, therefore dry-phase AFM imaging was used throughout the rest of this study.

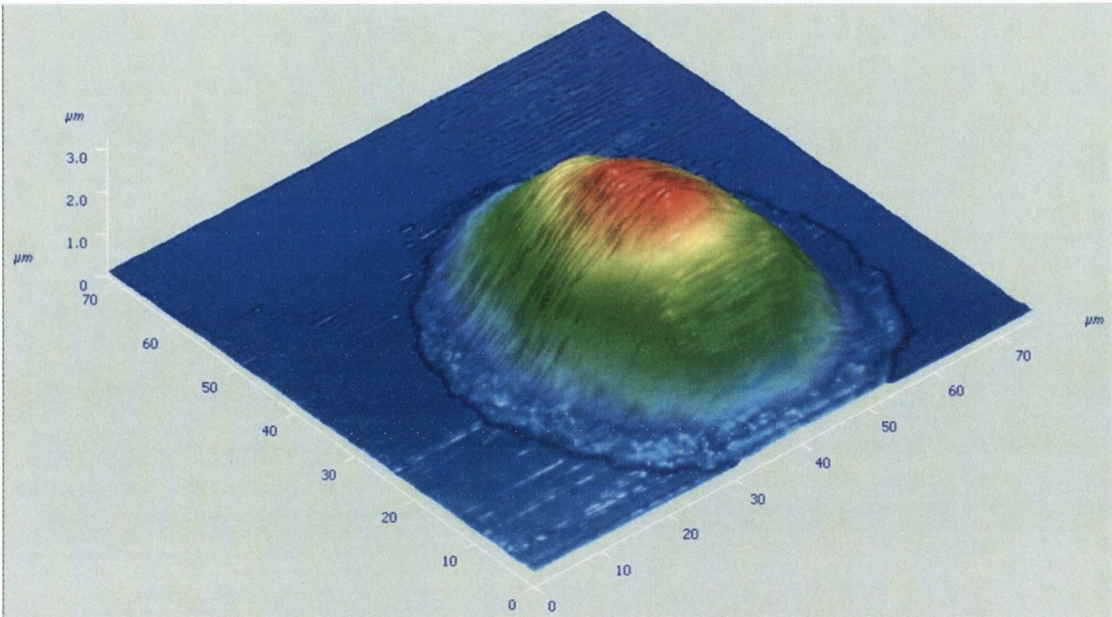


Figure 10 Liquid phase AFM image of a live THP-I phagocytic cell in media. The cell is about 50 μm x 50 μm in length and width and about 3 μm in height. This is an example of a control, untreated healthy cell growing on a glass substrate.

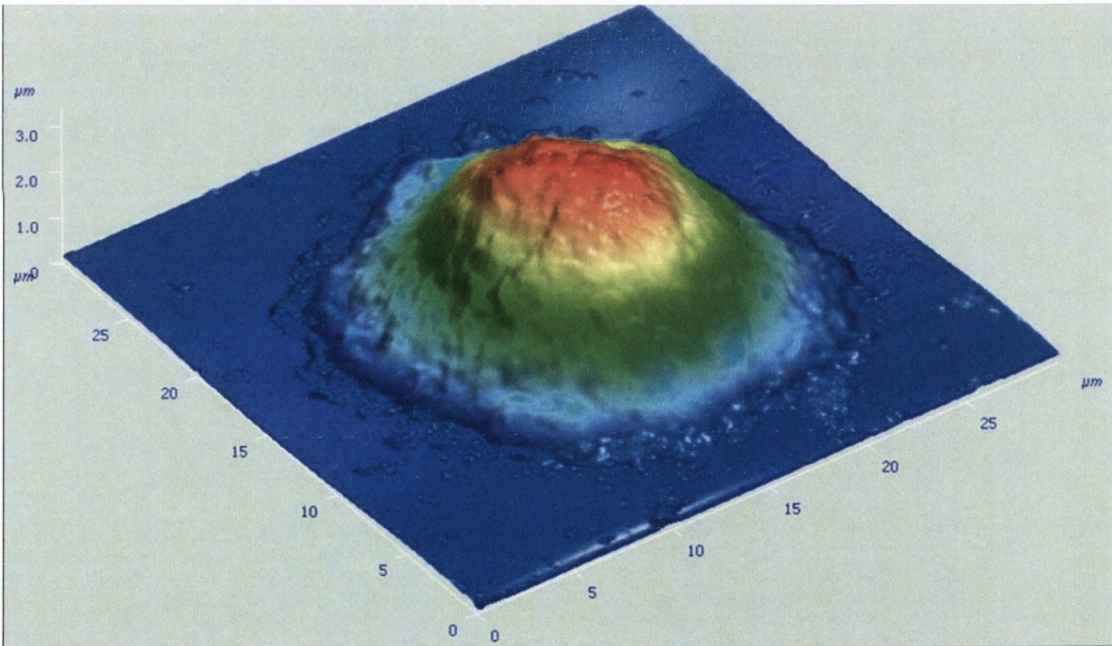


Figure 11 Dry-phase AFM image of a fixed, dehydrated THP-I cell. This cell is about 25 x 25 in width and length and about 3 in height. This is an example of a control, healthy cell grown on a glass coverslip and when compared to the live cell in Figure 10 it is clear to see that the fixing and dehydration technique used here does not change the morphology of the cell dramatically.

Figure 12 is an AFM image of a phagocytic cell grown on a SWCNT thin film. Similar to the control cell (Figure 11) this cell maintains a semi-spheroid shape and lamellar structure around the edge. However it displays a more ruffled cell membrane, suggesting an interaction or response to its surroundings but there are no signs of rupture to the membrane. The area around the cell remains high centre suggesting there is no condensing of the nucleus. The roughness of the SWCNT thin film substrate compared to the smooth glass can also be seen.

An example of a phagocytic cell grown on a glass substrate following exposure to “free” SWCNT can be seen in Figure 13. This cell also maintains the lamellar structure around the edge of the cell and a higher area in the middle of the cell owing to the nucleus. However it is more triangular in shape and appears to be spreading out in three directions. This would suggest the formation of filopodia due to the cell interacting with and phagocytosing the unbound SWCNT. There are no signs of any rupture to the cell membrane or rounding up of the cell, the shape and size indicate a cell of normal morphology. Evidence of small nanomaterials on the smooth glass substrate can be seen due to the SWCNT.

The cell grown on the AgNW thin film (Figure 14) is similar to Figure 12 in that it has a semi-spheroid shape, lamellar edges and a tall dome shaped centre due to the presence of the nucleus. The entire cell is also more ruffled, indicating active adhesive interactions with the underlying nanomaterial substrate. The roughness of the AgNW thin film surface can be visible down to $\sim 50 - 100$ nm which enabled visualization of individual nanowires. These AFM images prove that the AgNW are not flush with the polymer surface but rather form an uneven surface in which the cells can interact with.

Figure 15 is an example of a phagocytic cell grown on a glass substrate following exposure to unbound AgNW. Again this cell is semi-spheroid in shape, has a lamellar edge and a higher dome shaped centre due to the nucleus. It is ruffled entirely and the cell's edges appear to have some interaction with the unbound AgNW. It is possible to see the individual AgNW on the smooth glass substrate.

Even at such magnification, where individual cells are scanned by the AFM, a significantly higher resolution image of the interaction between the cells and the unbound SWCNT, AgNW and their composite thin films was possible. Further investigations of a significantly higher magnification are achievable with AFM, with the tip diameter (typically ~ 20 nm) the resolution limit. Scanning smaller regions of cell where an NP is interacting with the cell membrane could provide comprehensive insight into the details of the cell membrane-nanoparticle interaction. However, this is beyond the scope of this study.

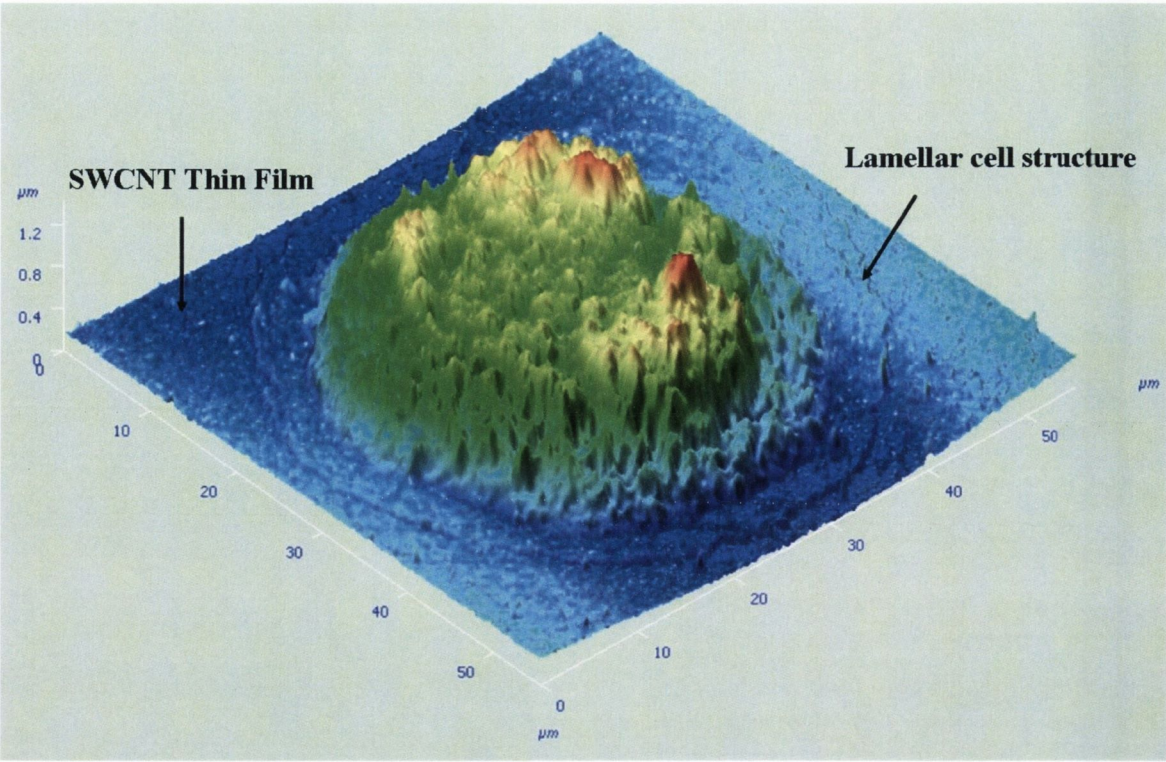


Figure 12 Dry phase AFM height based image of fixed THP-1 cell on SWCNT thin film.

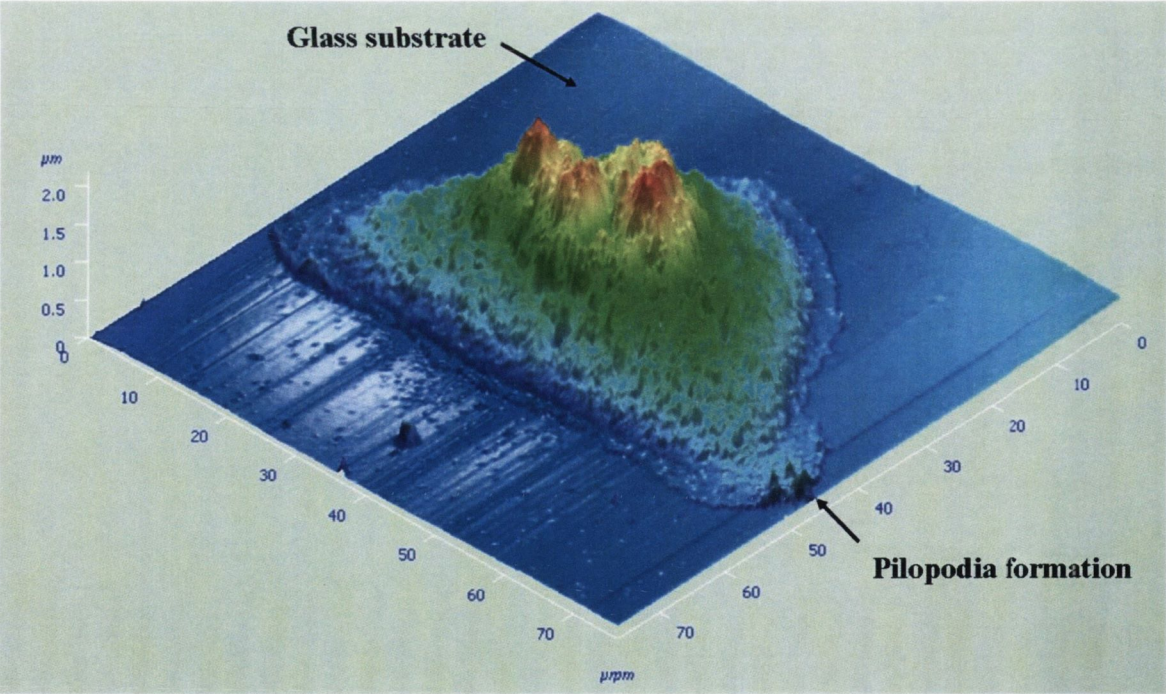


Figure 13 Dry phase AFM height based image of fixed THP-1 cell on glass coverslip following exposure to SWCNT.

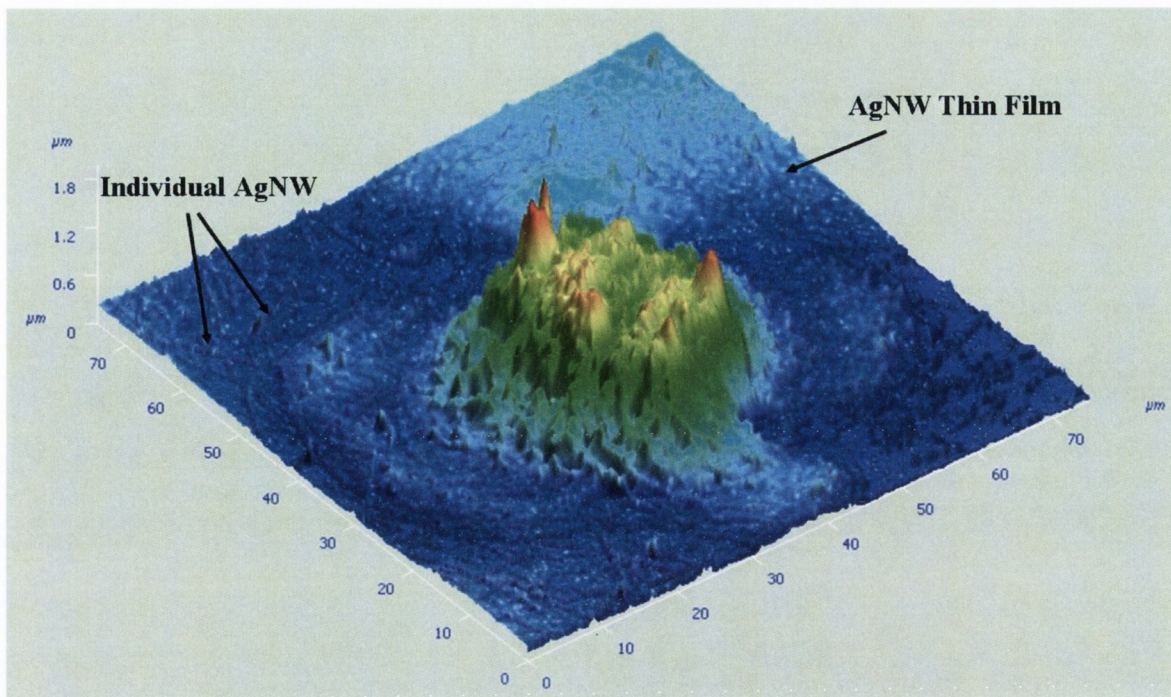


Figure 14 Dry phase AFM height image of THP-1 cell on AgNW thin film.

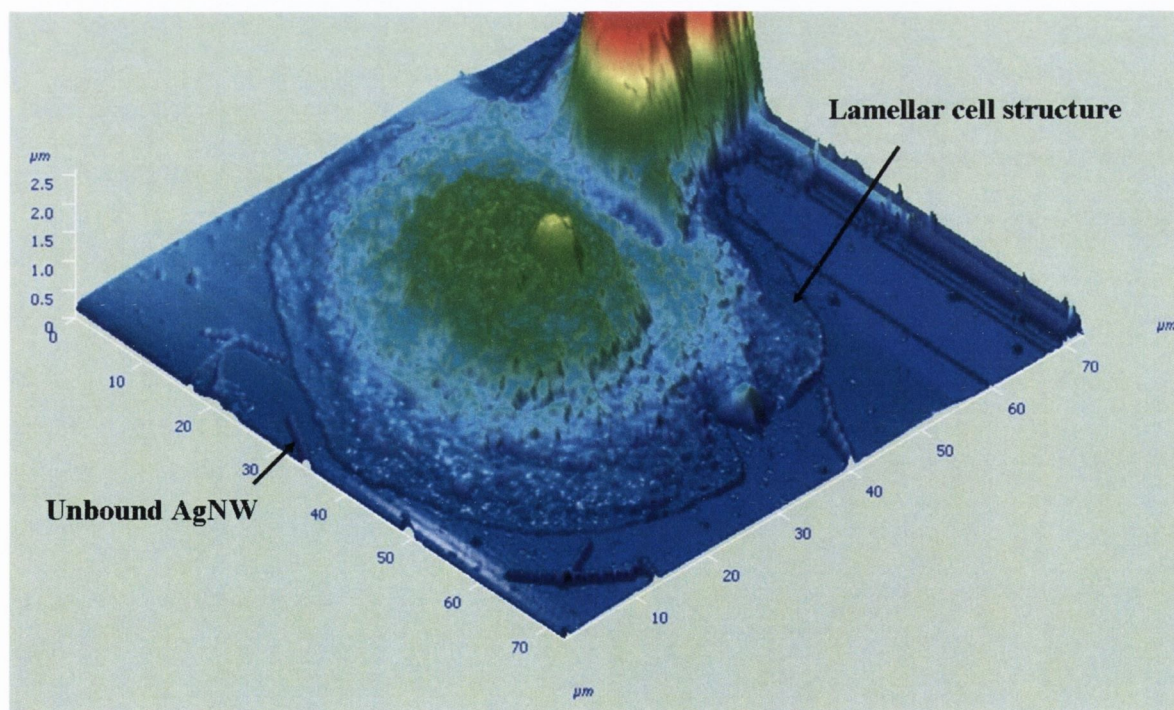


Figure 15 Dry phase AFM height image of THP-1 cell on a glass substrate following AgNW exposure. Note in this image a rounded up cell that is greater than $2.5\mu\text{m}$ can be seen and is not used for comparison purposes with the other AFM images.

2.4.3. Toxicity of thin films constituent nanoparticles addressed using HCS and MTT

The confocal and AFM images from the previous section indicate no signs of acute toxicity following exposure to the unbound SWCNT, AgNW and their enabled thin films. However, the effect of exposure to the constituent NP to the phagocytic cells had to be addressed in more depth.

Using High Content Screening, a high throughput automated fluorescent microscopy technique, the toxicity of suspensions of SWCNT, AgNW and QD was addressed. All of these materials have been reported to induce varying degrees of toxicity (Lovric, Bazzi et al. 2005; Lovric, Cho et al. 2005; Davoren, Herzog et al. 2007; Navarro, Piccapietra et al. 2008; Cheng, Muller et al. 2009). Using HCS (following staining of the nucleus (blue) and actin (red)) the nuclear channel was used to count the total cell number and therefore calculate fold change in cell number following the different NP exposures and time points. This enabled an assessment of the level of acute toxicity of the nanomaterials. The metabolic activity of the cells was also investigated using an MTT assay, which is an alternative conventional method of measuring NP toxicity to the cells.

Figure 16, Figure 17 and Figure 18 illustrate the calculated fold change in cell number following exposure of THP-1 cells to AgNW, SWCNT and QD respectively. In each of these experiments Ni nanowires were used as a positive control and the acute toxicity of these nanomaterials to the cells is observed as the cell number in each experiment drops to almost zero. Also for each experiment the cell number was normalized to the control well, which had no treatment. Following exposure to the AgNW (Figure 16) there is no significant difference between the cell number at the different time points or concentrations, indicating very low toxicity. As these THP-1 cells are differentiated monocytes they will not undergo any proliferation, therefore cell number is a measure of the number of cells that survive following treatment. Those that have been treated with SWCNT also indicate a small reduction in cell number but not significant, again indicating very low toxicity (Figure 17). The most striking reduction in cell number is observed

following QD exposure (Figure 18) after, 24, 48 and 72 hours. The number of cells after 72 hours plummets to about 25%, thus indicating acute toxicity.

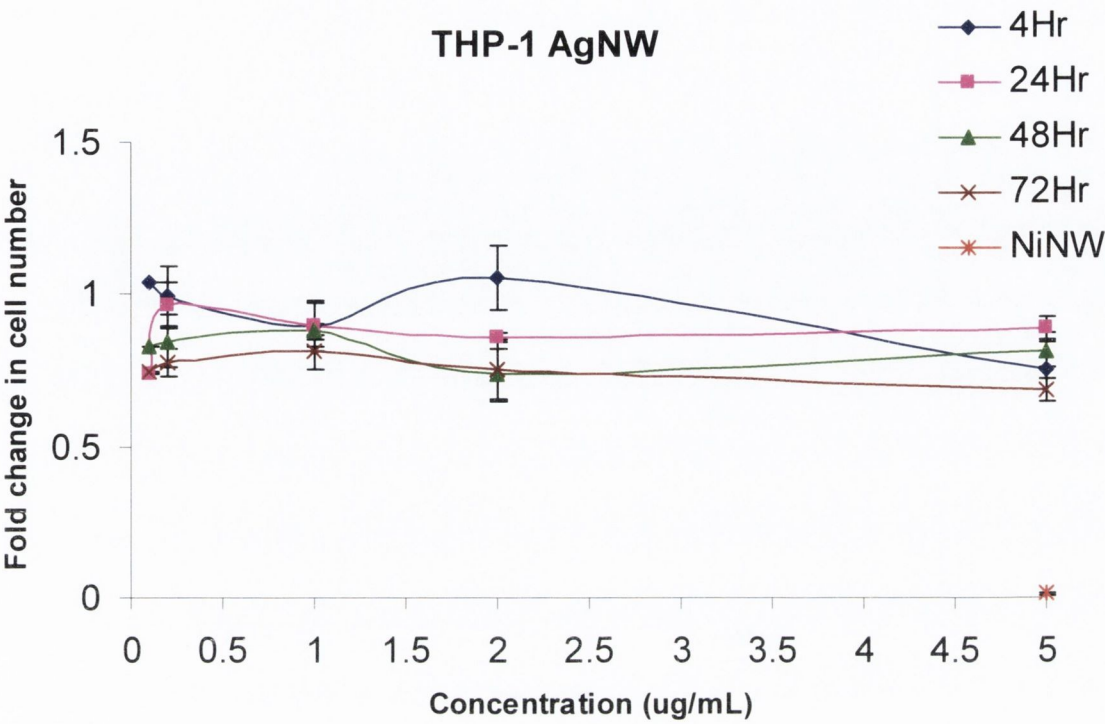


Figure 16 Dose response HCS analysis of cell growth following exposure to AgNW. The Y-axis represents fold change in cell number as a function of AgNW concentration (X-axis) and time (indicated in the legend).

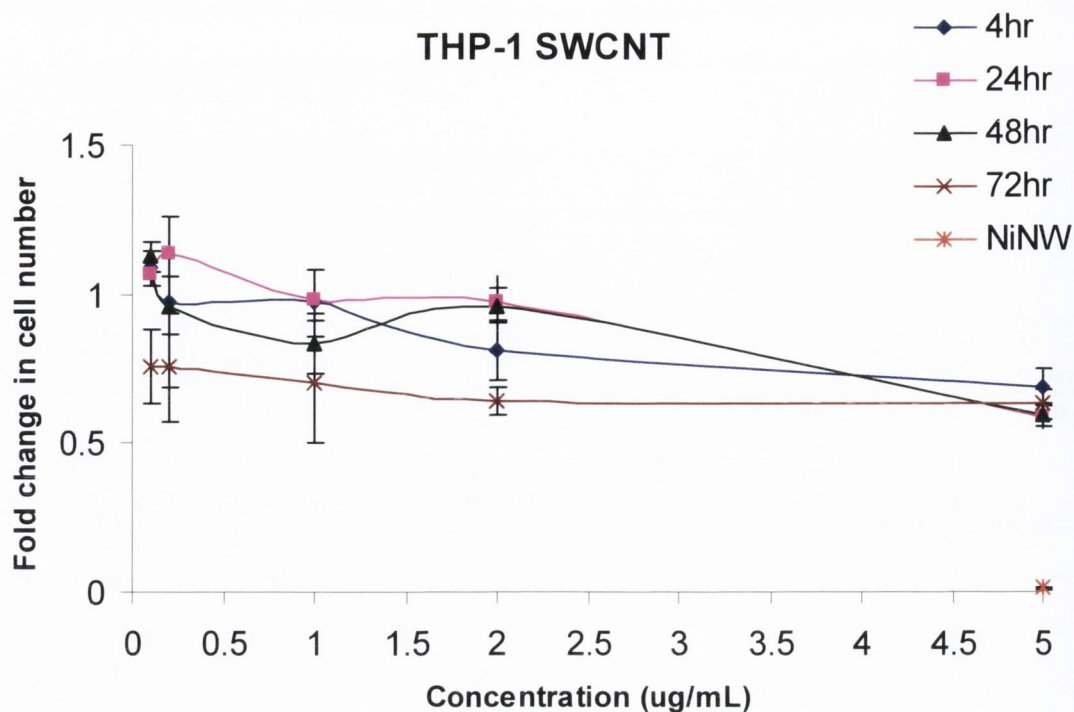


Figure 17 Dose response HCS analysis of cell growth following exposure to SWCNT. The Y-axis represents fold change in cell number as a function of SWCNT concentration (X-axis) and time (indicated in the legend).

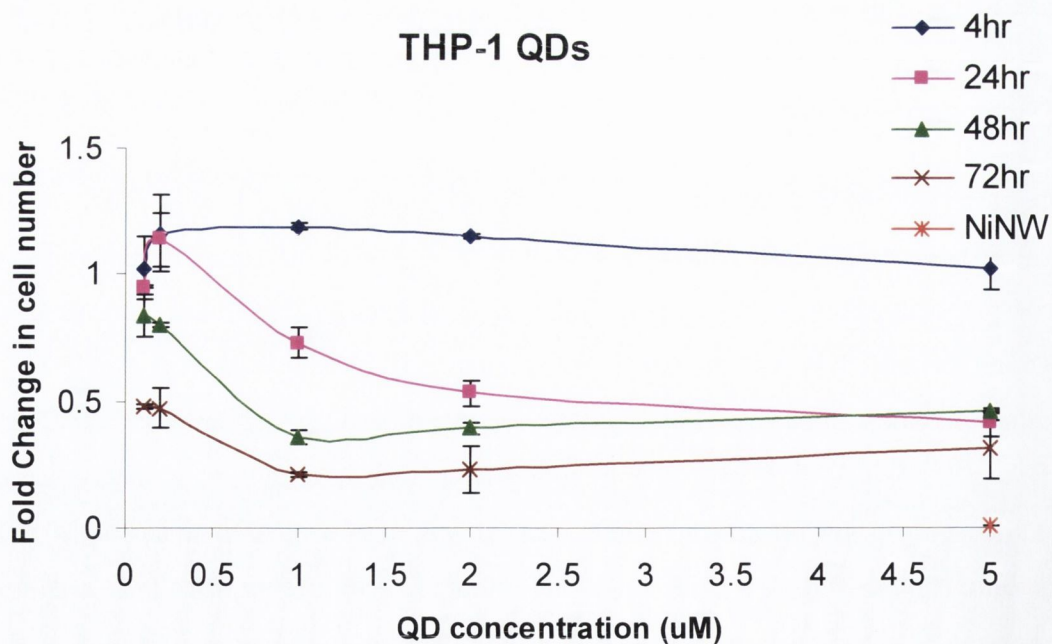


Figure 18 Dose response HCS analysis of cell growth following exposure to QD. The Y-axis represents fold change in cell number as a function of QD concentration (X-axis) and time (indicated in the legend).

MTT measures the cells metabolic rate giving an indication of cell viability. This compliments the HCS which was used to measure cell number as the control cells had no treatment and displayed 100% viability (Figure 19), all of the other results were normalized to the control data. Chloroquine which induces cell death and the QD which proved to induce acute toxicity, were both used as positive controls reducing the cells metabolic rate. The chlorquine and QDs reduced the metabolic activity to ~10% and ~50% respectively. Cells grown on both the SWCNT and AgNW thin films are of the same viability as the negative control, indicating no toxicity (Figure 19). Those cells following exposure to the loose, unbound CNT in solution (Figure 19) also showed the same metabolic rate as the negative control again indicating no toxicity. However those exposed to the AgNW constituent nanoparticles in solution had a reduced level of cell viability (between 85-90%), indicating a very low level of toxicity.

Experimental results from the HCS and MTT assays, which give a broad overview of cell health, indicate little or no toxicity to phagocytic cells following their exposure to AgNW, SWCNT or their enabled thin films. However, using these techniques it was not possible to establish what cellular mechanisms are underway in the cells. This required more powerful, close-up studies involving higher resolution techniques, which are presented in the coming sections.

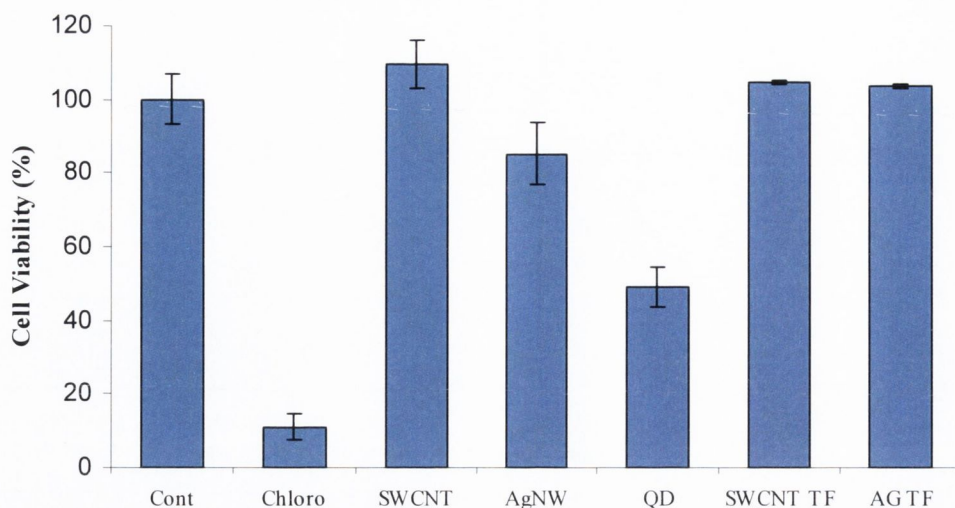


Figure 19 Results of MTT assay 24 hour exposure of THP-1 cells to chloroquin, SWCNT, AgNW, QD, SWCNT thin films and AgNW thin films.

2.4.4. TEM reveals an increase in autophagic vacuole formation following NP exposure

Transmission electron microscopy (TEM) allowed further insight at a much higher resolution to the possible cellular mechanism induced in the phagocytic cells following their treatment with unbound SWCNT or AgNW.

Figure 20 is a TEM image of a TH-1 cell alone. The nucleus (N) can be seen clearly along with many vesicles (V), as is normal for this type of cell. A number of philapodia (P) can also be seen. One of the most striking features observed following THP-1 exposure to SWCNT (Figure 21) and AgNW (Figure 22) is a dramatic increase in the vesicle size with a significant influx into these giant vesicles of many smaller vesicles. On closer inspection, many of these smaller vesicles are autophagosomes.

Autophagy involves the formation of a double membrane vesicle called an autophagosome which encompasses the cytoplasmic organelles (or proteins, bacteria, nanoparticles) and then fuses with lysosomes to degrade its contents (Sanjuan, Dillon et al.

2007). Autophagy is involved in the cellular survival mechanism in times of stress, allowing cells to recycle nutrients during conditions of starvation, and also has roles in the inflammatory and immune systems of the body (Baehrecke 2005).

Figure 23 (a and b) illustrates two examples of the formation of double membrane autophagic vesicles, following SWCNT or AgNW exposure respectively. Excessive or chronic autophagy has been observed in cells as a result of starvation. Excessive or defective autophagy can lead to cell death by self-destruction or the accumulation of misfolded proteins and old organelles respectively. Therefore the presence of these autophagic vesicles above the basal homeostatic level following exposure to SWCNT and AgNW in the phagocytic cells, is an indication of a stress response.

Higher resolution images of the influx of these smaller autophagic vesicles into the giant vacuoles can be seen in Figure 24 (a-f) and Figure 25 (a-f). The EM images in Figure 24 (a-f) are of THP-1 cells following 3 hour exposure to SWCNT. In each image (a-f) examples of the typical double membrane autophagosome vesicles can be seen. The presence of these vesicles in such abundance, above the basal homeostatic level, indicates a stress response from the the cells to the SWCNT.

Figure 25 (a-f) illustrates multiple examples of EM images obtained of THP-1 cells containing many autophagosomes following 3 hour exposure to AgNW. Similar to the response of the cells following SWCNT this increase in autophagic vesicles, following AgNW exposure is indicative of a stress response by the cell.

Despite extensive TEM imaging, within the time constraints of this study it was not possible to obtain definitive images of individual SWCNT or AgNW within the cells. However, using confocal Raman spectroscopy (chapter 3) and confocal microscopy (section 2.4.6) it was possible to confirm the internalisation of SWCNT and AgNW respectively.

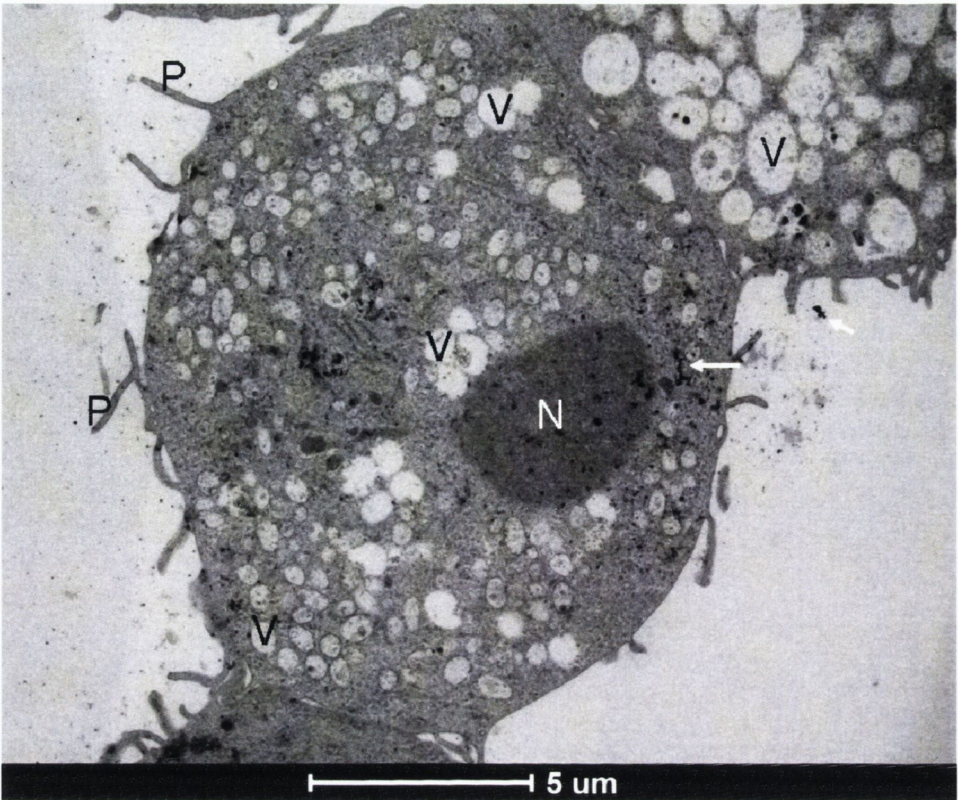


Figure 20 Electron microscope image of a THP-1 cell with no treatment. A large number of vacuoles are present which is expected for this phagocytic cell line (V). The nucleus (N), a number of philpodia (P) and some staining artefacts (white arrows) are also indicated.

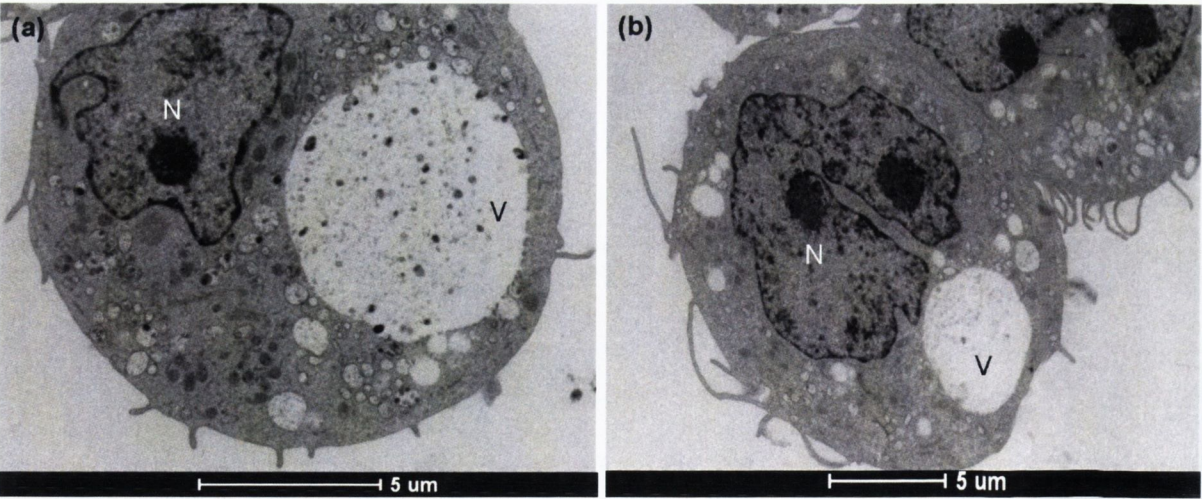


Figure 21 Typical TEM images of THP-1 cells (a & b) following 3 hours exposure to SWCNT. The most striking difference between these images compared to the control (Figure 20) is the dramatic increase in the size of the vacuoles (V). A number of small vesicles are filtering into and fusing with this large vacuole.

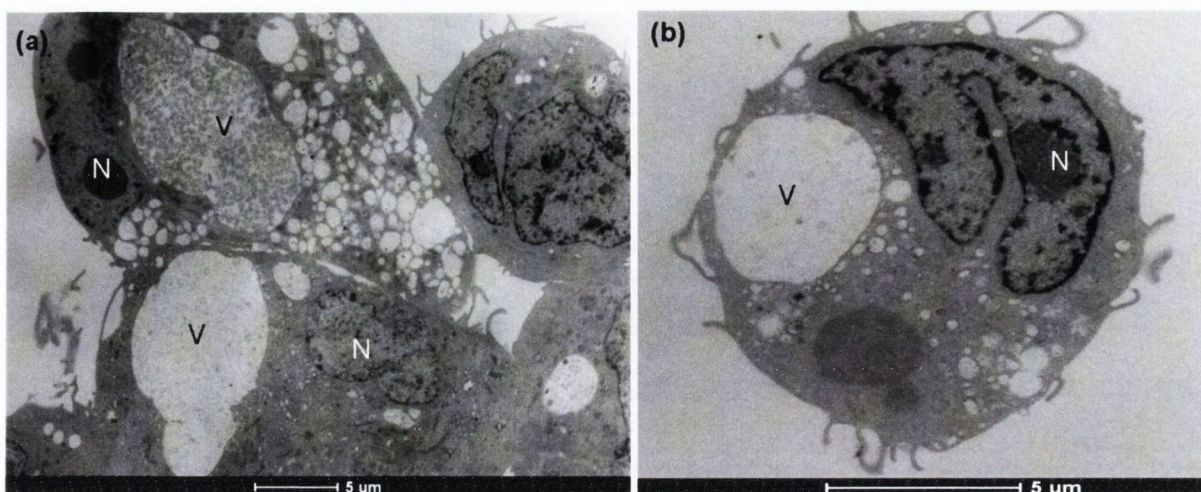


Figure 22 Typical TEM images of THP-1 cells (a & b) following 3 hours exposure to AgNW. Similar to Figure 21 the most striking feature of these images compared to the control (Figure 20) is the dramatic increase in the size of the vacuoles (V). A number of small vesicles are filtering into and fusing with these large vacuoles.

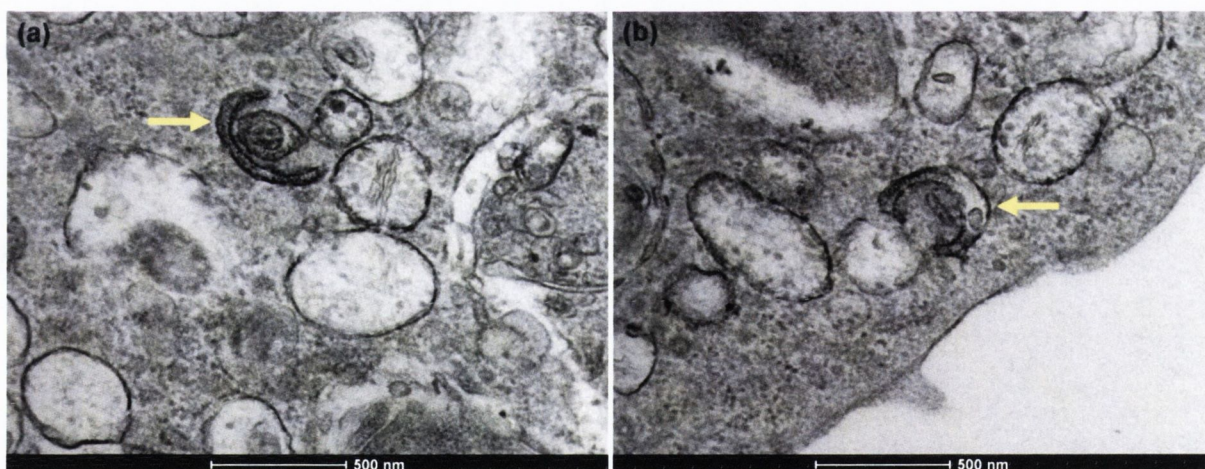


Figure 23 Autophagosome formation in THP-1 cells following treatment with (a) SWCNT and (b) AgNW. Formation of typical double membrane autophagic vesicle structure is indicated (yellow arrows).

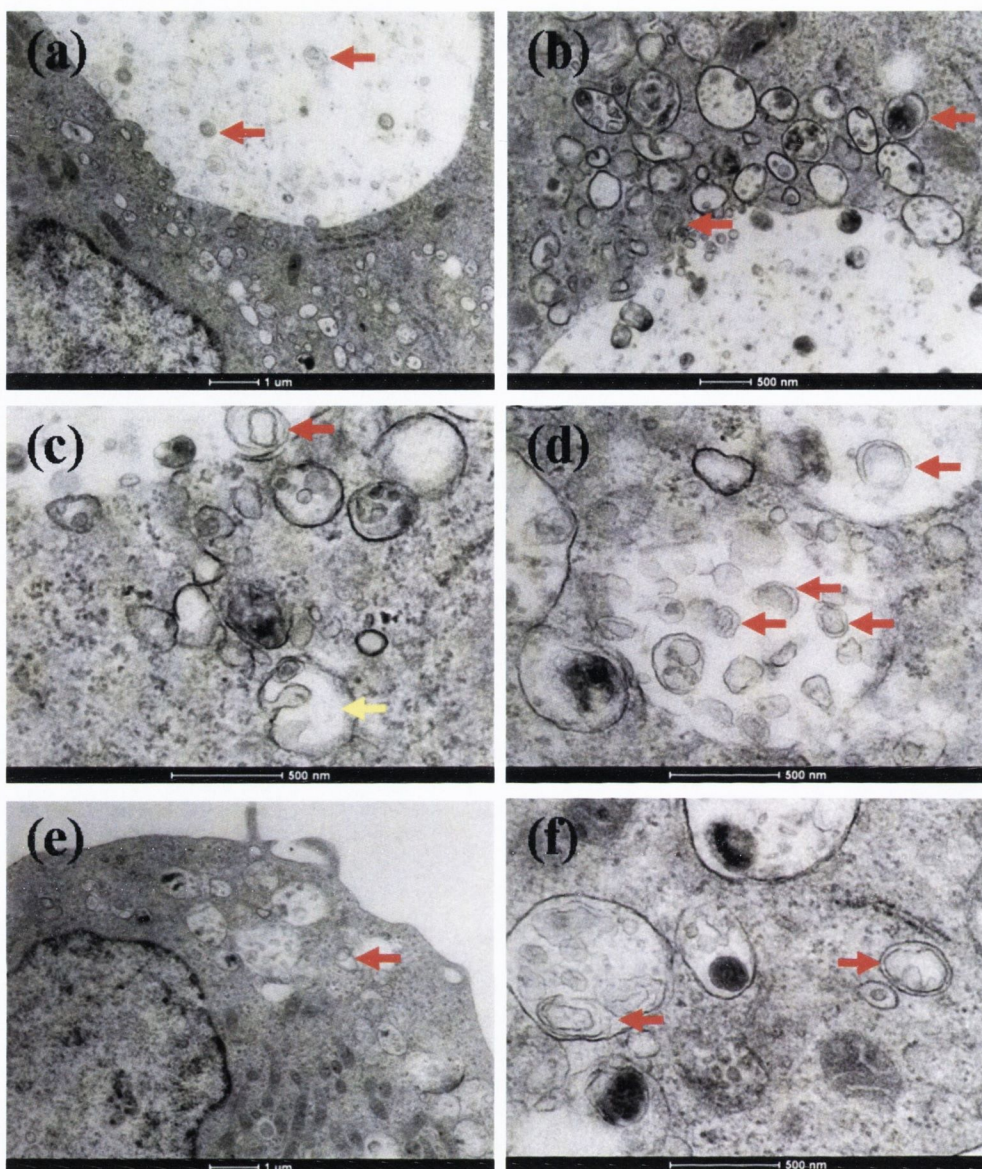


Figure 24 TEM images of THP-1 cells following exposure to SWCNT for 3 hours. Many examples of the typical autophagic double membrane are illustrated (red arrows) and formation of double membrane autophagic vesicle.

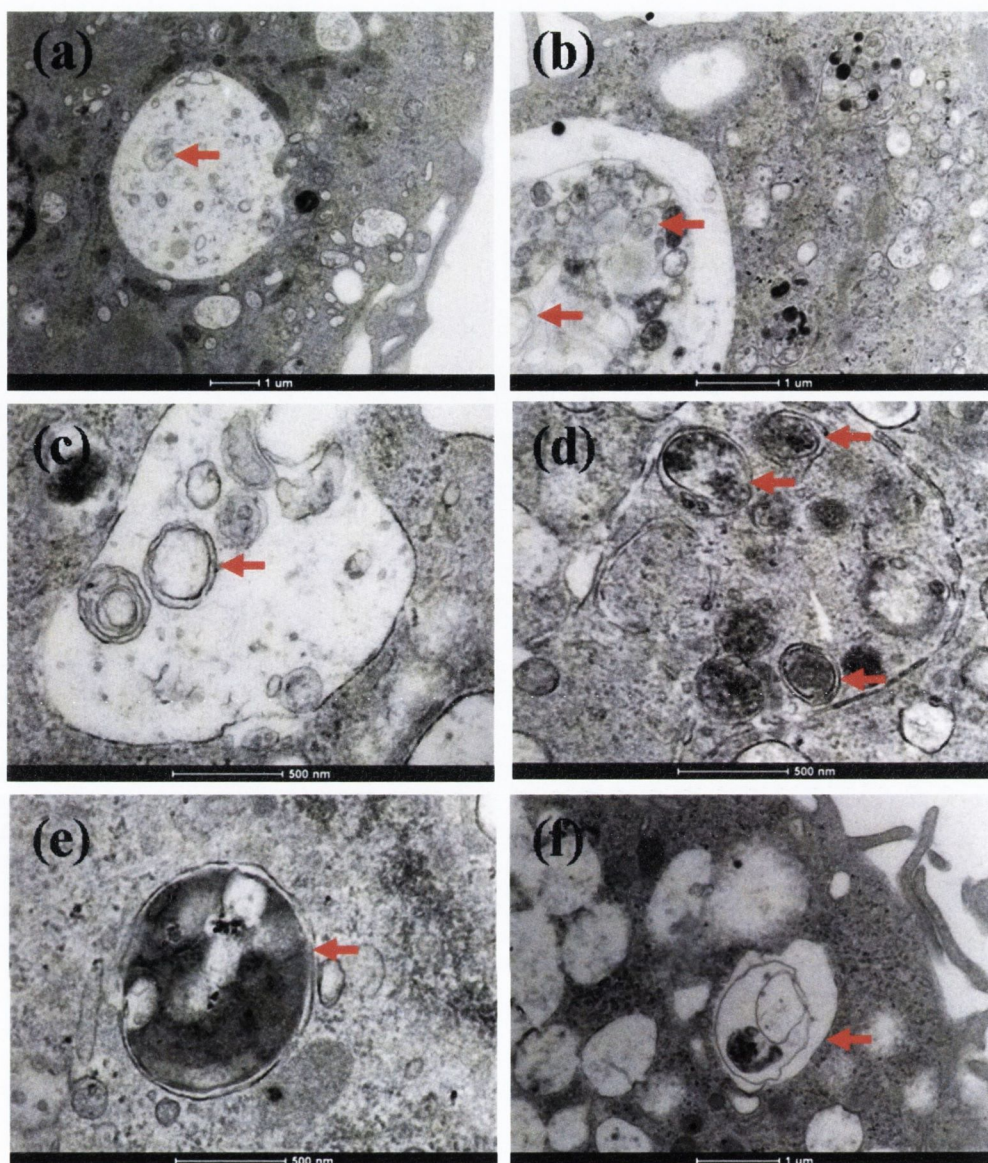


Figure 25 TEM images THP-1 cells following exposure to AgNW for 3 hours. Many examples of the typical autophagic double membrane illustrated (red arrows).

2.4.5. Western blotting confirms increase in LC3 protein

The EM images in the previous section illustrate multiple examples of autophagosomes present in the phagocytic cells following 3 hour exposure to SWCNT or AgNW. This is indicative of an autophagic response by the cells to the NP. A common marker of autophagosomes in western blotting is the light chain associated protein LC3. In mammalian cells LC3 is cleaved at the C-terminal by Atg4 homologues to become LC3-I. Via an ubiquitin-like enzymatic reaction this LC3-I protein conjugates to phosphatidylethanolamine to become LC3-II which then becomes associated with the autophagosome inner and outer membranes (Kaminsky, Abdi et al. 2011). Western blotting was used here to measure the amount of LC3-II protein present in cells.

Chloroquine, which is used here as a positive control, is a commonly used drug to arrest the normal functioning of autophagy and therefore leads to a build up of the LC3 protein. Compared to the untreated cells there is a significant increase in the amount of LC3-II following chloroquine treatment (Figure 26, Figure 27). Also, in the untreated cells a very faint band corresponding to LC3-I can be seen but this band is not present in the chloroquine treated cells or the cells exposed to SWCNT (Figure 26) or AgNW (Figure 27). This is indicative of the conversion of LC3-I to LC3-II during the autophagic process.

There is an increase in LC3-II after 4 hour SWCNT or AgNW exposure which increases even further after 24 hours (Figure 26, Figure 27). The increase in LC3-II in the phagocytic cells confirms that there is an autophagic response by these cells following NP exposure.

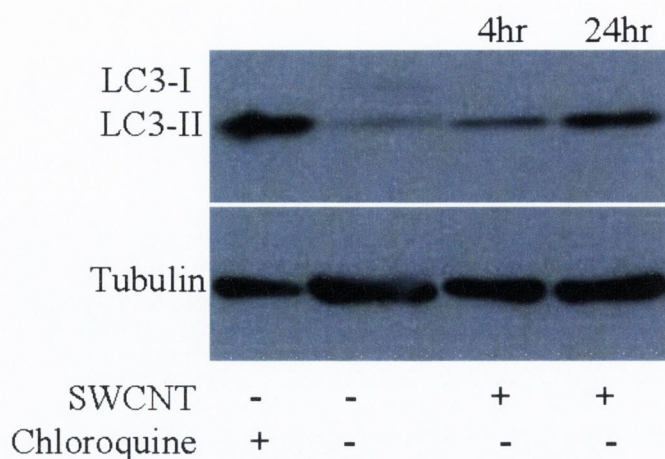


Figure 26 Western blot indicating an increase in LC3-II following treatment of THP-1 phagocytic cells with SWCNT. The cell lysates after the following treatments are shown; first lane from the left is the positive control chloroquine treated, the second lane from the left are untreated control cells, the third lane from the left is following a 4 hour treatment with SWCNT. The far right lane is after 24 hours treatment with SWCNT.

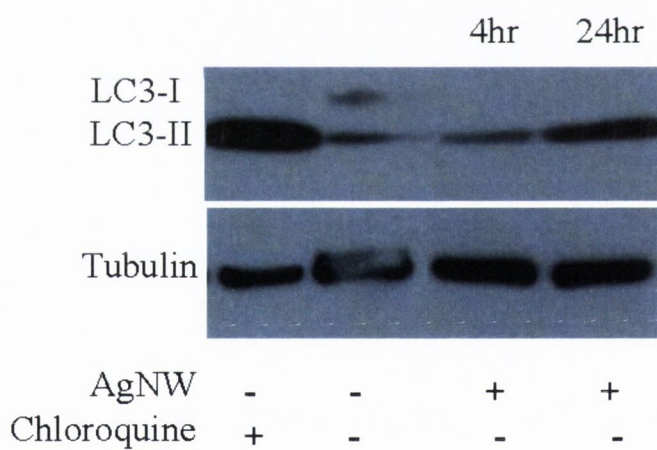


Figure 27 Western blot indicating an increase in LC3-II following treatment of THP-1 phagocytic cells with AgNW. The cell lysates after the following treatments are shown; first lane from the left is the positive control chloroquine treated, the second lane from the left are untreated control cells, the third lane from the left is following a 4 hour treatment with AgNW. The far right lane is after 24 hours treatment with AgNW.

2.4.6. Live cell confocal microscopy confirms autophagic formation and AgNW uptake

Following SWCNT or AgNW exposure, EM imaging revealed an increase in the presence of autophagic vesicles in the THP-1 cells, which is indicative of an autophagic response by the cells. Western blotting confirmed an increase the amount of LC3-II protein in the cells following NP exposure.

To investigate this autophagic response, BMDM LC3-GFP cells were employed enabling fluorescent imaging of the autophagosome puncta. The LC3 protein is located on the inner and outer membrane of autophagic vesicles (Kabeya, Mizushima et al. 2000). In the control cells it is dispersed evenly throughout the entire cell Figure 28 (b). Following chloroquine treatment, which arrests the autophagic process and causes a dramatic build up of non-degraded autophagic vesicles in the cell, LC3 located on these vesicle membranes can be seen in Figure 28 (d) as large green puncta.

Following AgNW or SWCNT exposure, an increase in LC3 accumulation, well above the basal level, can be seen in the form of green puncta Figure 28 (f and h). The presence of these green autophagosomes following NP treatment confirms the significant autophagic response of the cells to the NP.

The surface plasmon resonance of the AgNW (described in section 1.1.2) allowed for imaging of these wires using 488 nm. It was therefore possible to monitor their uptake and transport within the cell using live cell confocal microscopy. Figure 29 is made up of a number of live cell confocal movie frames taken over 135 seconds of GFP-LC3 cells following exposure to AgNW after 6 hours. There are two striking features observed here, firstly the marked increase in green autophagosomes following AgNW treatment and secondly that the AgNW are not only internalized by the cells but they are moved around inside the cell. The cell also shows no signs of acute toxicity and appears to be attempting to process the ingested wires.

Multiple images were captured of AgNW within autophagosomes, confirming a direct link and association of their formation with the presence of the AgNW. For example Figure 30, Figure 31 and Figure 32 all represent images captured during different experiments where the autophagosomes are seen to be surrounding and decorating the AgNW in an attempt to process or degrade the wires. Figure 30 (c) illustrates the diffuse distribution of LC3 in an untreated control cell. Figure 30 (d) illustrates a phagocytic cell following 3 hour exposure to AgNW where a number of internalized wires are seen. One of these wires is almost completely ingested and decorated by autophagosomes.

Another typical example of the AgNW within autophagosomes can be seen in Figure 31 (a) where a wire is held within one very large autophagosome and a couple of smaller ones along its length. No co-localisation of the lysosomes (red) and the autophagosomes (green) is observed in this image (Figure 31). Co-localisation would indicate lysosome and autophagosome fusion which is one of the final steps in the autophagic process, however it has not occurred yet. Figure 32 illustrates the incomplete internalization of an AgNW but shows how the autophagosomes have encapsulated part of the internalised wire.

Figure 33 presents a series of sequential live cell confocal movie frames taken over a ~193 second period illustrating the fusion process of the lysosomes and the autophagosomes which must occur to ensure complete degradation of the autophagosomal contents. A number of these movies and images were taken to establish whether or not the presence of the AgNW or SWCNT blocked this process. Clearly the fusion process proceeds as the lysosomes (red) and autophagosomes (green) can be seen to co-localise over time (Figure 33). If this process were to be arrested it would be detrimental to the cells, causing an accumulation of autophagic vesicles resulting in incomplete degradation of autophagosomal contents such as proteins or NP.

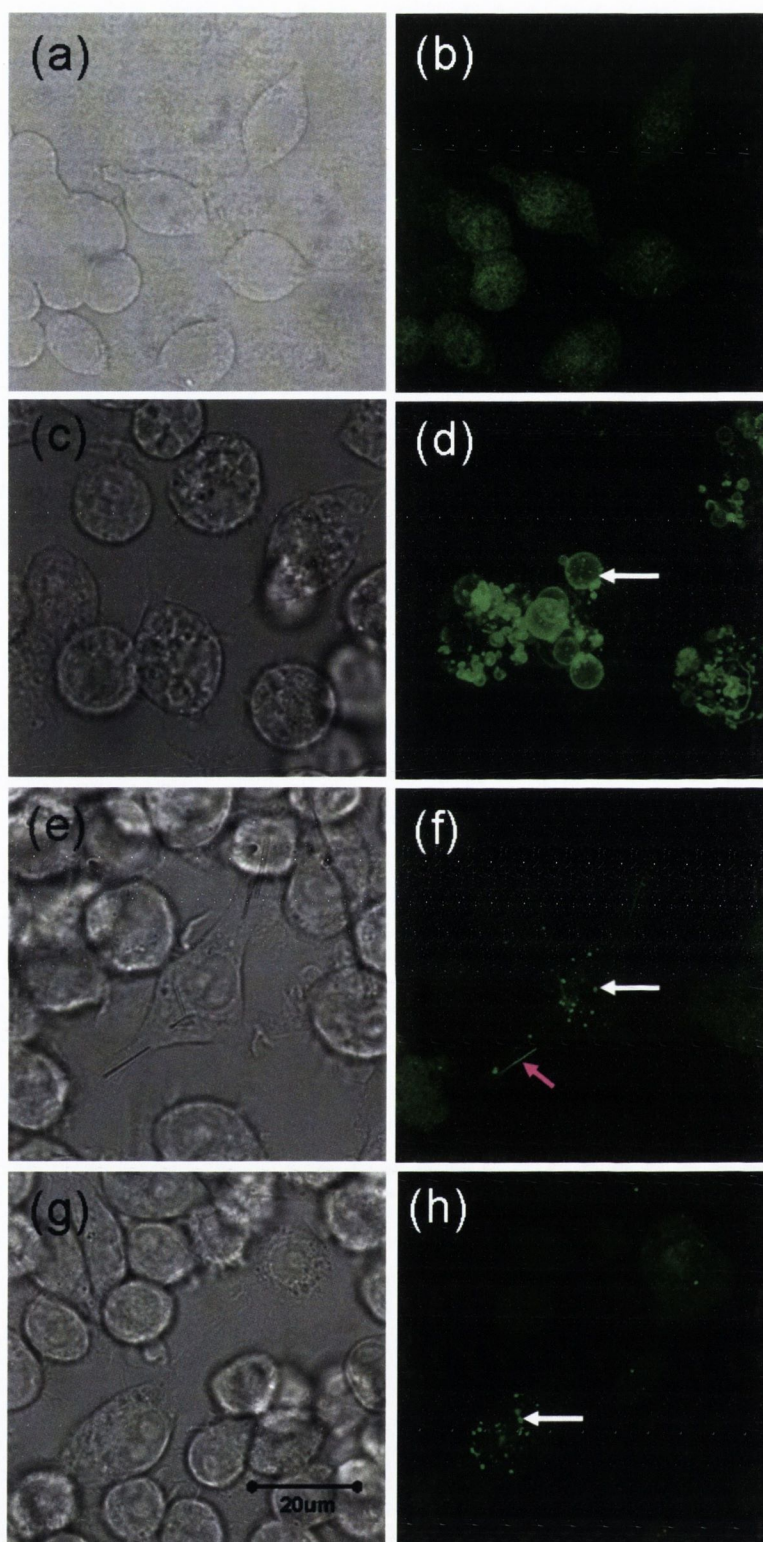


Figure 28 Live confocal images of BMDM LC3-GFP cells. (a&b) control cells no treatment, (c&d) chloroquin treated cells, (e&f) AgNW treated and (g&h) SWCNT treated cells. The control cells show the LC3 protein dispersed evenly throughout the cell with no autophagosomes, whereas in all of the treated cells there is an increase in the number of autophagosomes and they can be seen as bright green puncta (white arrows, d, f, and h). The surface plasmon resonance of AgNW enabled their imaging within the cells (pink arrow).

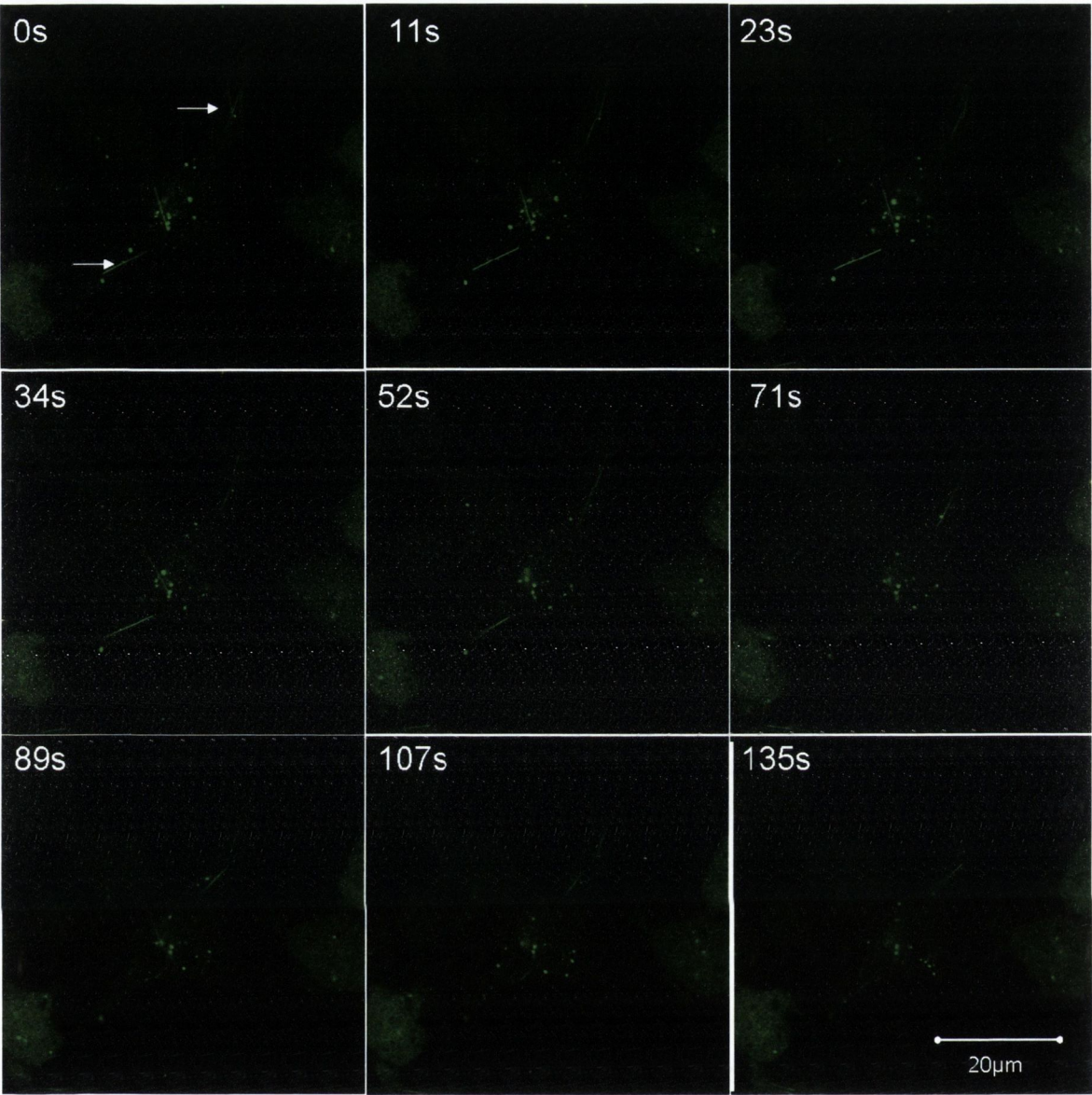


Figure 29 Live confocal microscopy movie stills of BMDM-LC3 (green GFP-LC3) cells following AgNW exposure. A number of live images were taken over 135 seconds. The time at which each was taken indicated. The white arrows in the first image at 0s indicate the area where AgNWs can be seen in the cell. Over time movement of the wires within the cell can be seen along with movement of the autophagosomes.

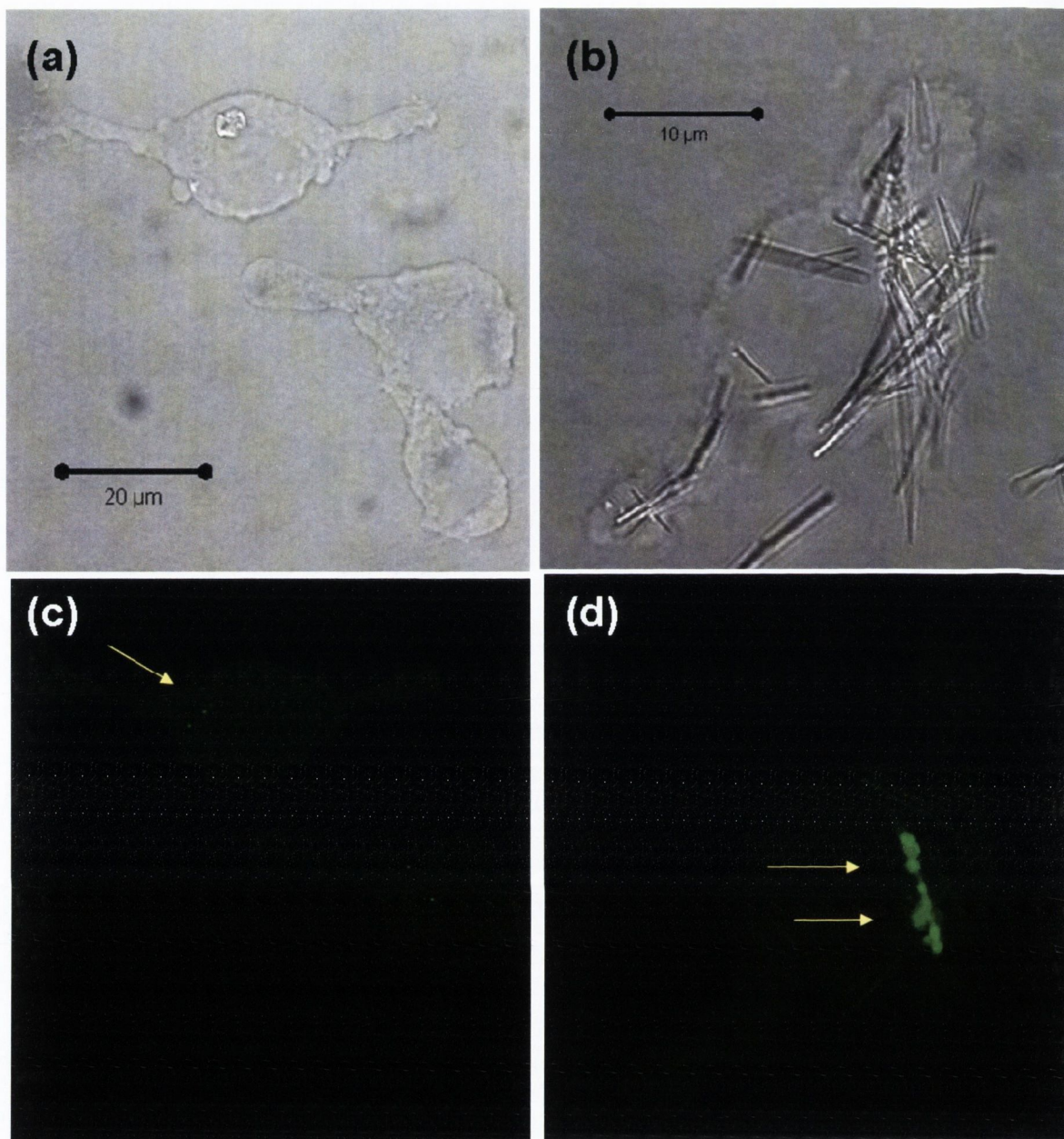


Figure 30 Brightfield and confocal images (green GFP-LC3) of BMDM-LC3 cells; (a and c) are control cells where the LC3 is distributed diffusely throughout the cells, (b and d) is a cell following 3 hours exposure to AgNW. Unlike the control cells the LC3 is now located in discrete puncta highlighting the autophagosomes decorating the length of one of the internalized wires (yellow arrows).

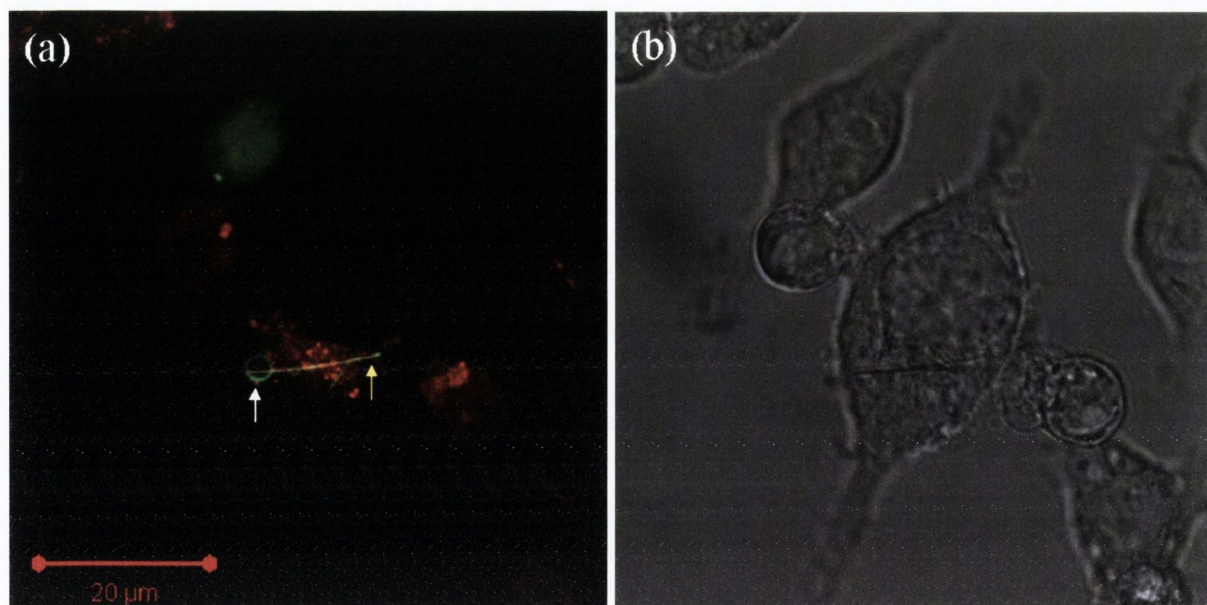


Figure 31 Live confocal and brightfield images of BMDM-LC3 cell following 6 hours AgNW exposure. An internalized AgNW (yellow arrow) is decorated by green autophagosomes one of which is indicated by the white arrow, the red vesicles are lysosomes.

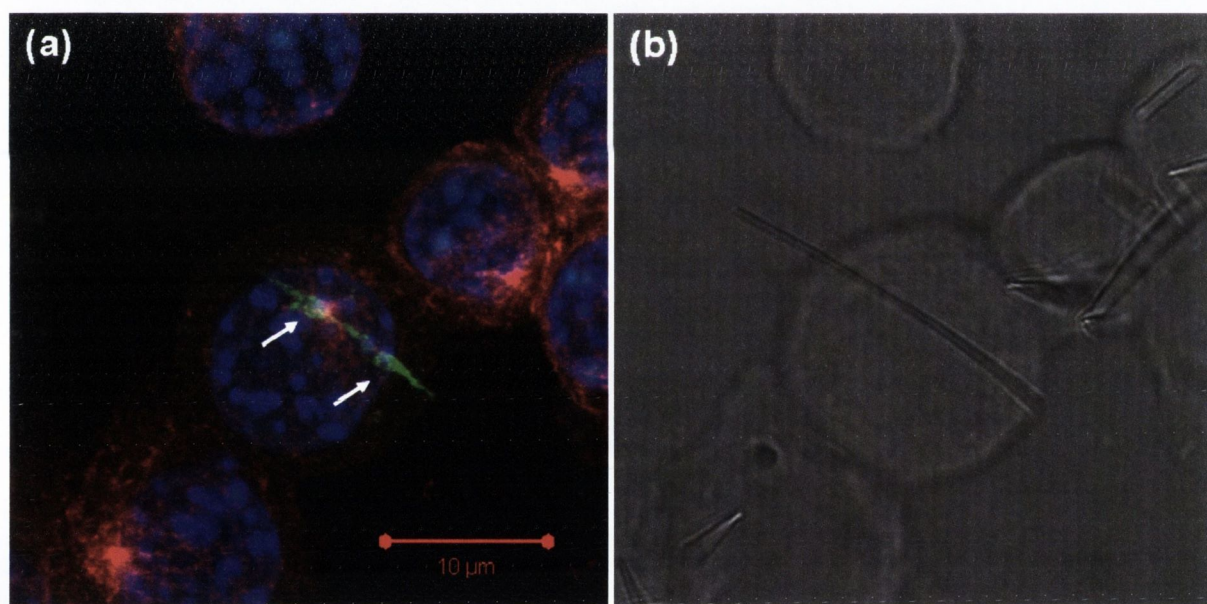


Figure 32 Live confocal images of BMDM-LC3 cells following 24 hour exposure to AgNW. The cells have been stained with Hoechst (blue nucleus), red actin and green autophagosomes. Two large autophagosomes are engulfing the internalized AgNW (white arrows).

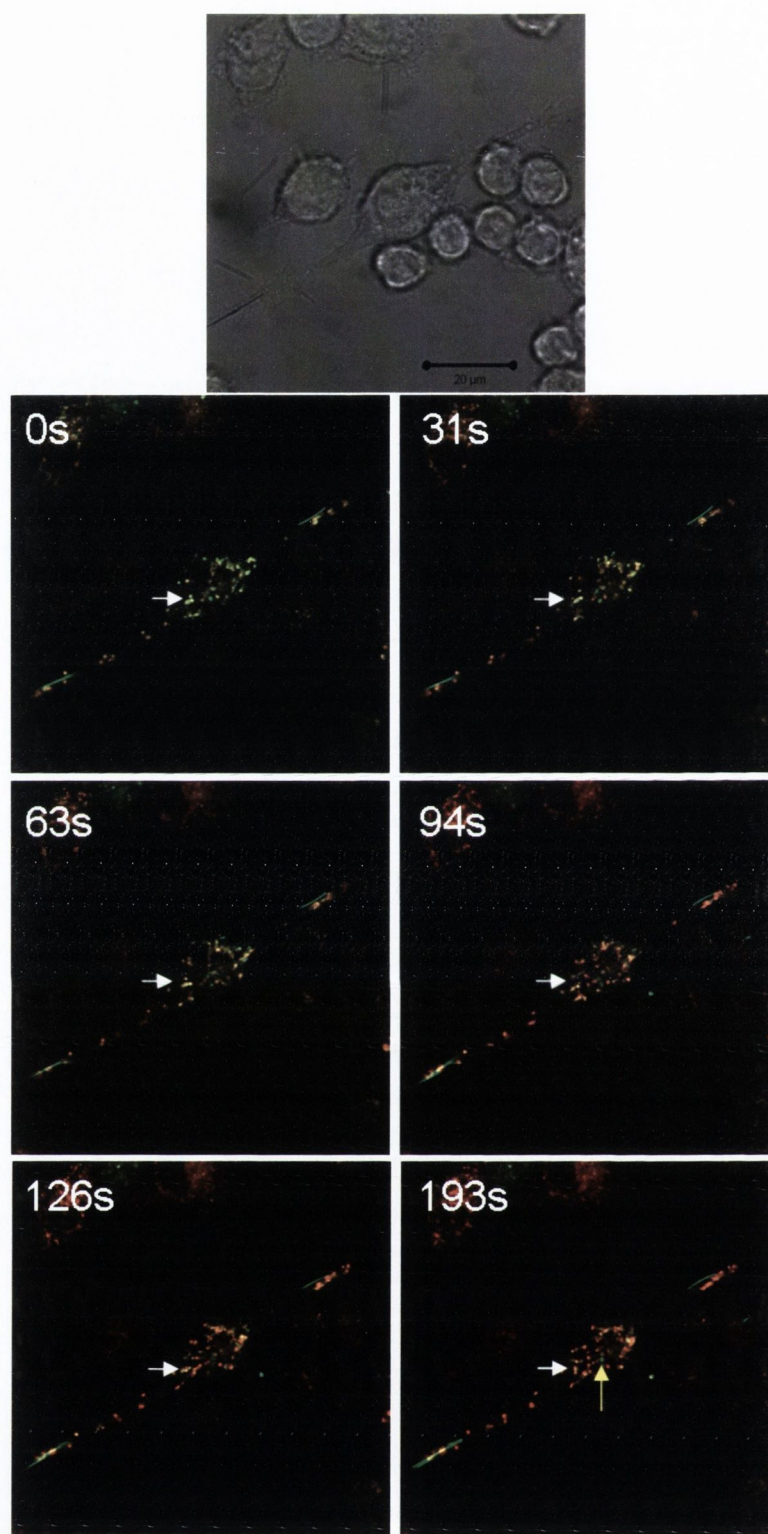


Figure 33 Brightfield and live confocal movie stills of BMDM (GFP-LC3) cells with LysoTracker (red). The dynamic co-localisation of the red lysosomes with the green autophagosomes is illustrated. This shows that exposure to the AgNw does not arrest autophagy.

2.4.7. Quantification of autophagic response following nanoparticle exposure

So far this study has revealed and confirmed an autophagic response by phagocytic cells to SWCNT or AgNW by EM, Western Blotting and confocal microscopy. To quantify the level of autophagy present in these cells, epi-fluorescent microscopy was used to image and count the number of green GFP-LC3 autophagosomes (puncta) present in the cells following their exposure to SWCNT or AgNW. The cells nuclei and actin were also stained (Figure 34 and Figure 35).

The grid described in section 2.3.15 was filled in for each of the treatments. It was found that 100% of cells treated with chloroquine contain puncta whereas only about 5% of the untreated control cells contain puncta which is due to the basal level of autophagy present in the cells (Figure 36).

40% of cells exposed to either SWCNT or AgNW contained puncta (Figure 36). Only 20% of cells grown on the SWCNT thin films or AgNW thin films contained autophagic puncta. This increase in autophagy observed in the cells grown on the thin films could be caused by a release of the NP from the polymer matrix during cutting of the thin films (~ 6mm diameter) to fit into experimental plates. A significant increase in autophagic response is observed in both exposure scenarios but on a different magnitude.

Figure 37 demonstrates the average number of puncta per cell following each treatment. Chloroquine treated cells had the highest number whereas control and SWCNT had the least. Both the thin films and AgNW treated cell had almost the same number of puncta per cell. However this graph merely compliments Figure 36 which best reflects the extent of increased autophagy following nanoparticle and thin film treatment.

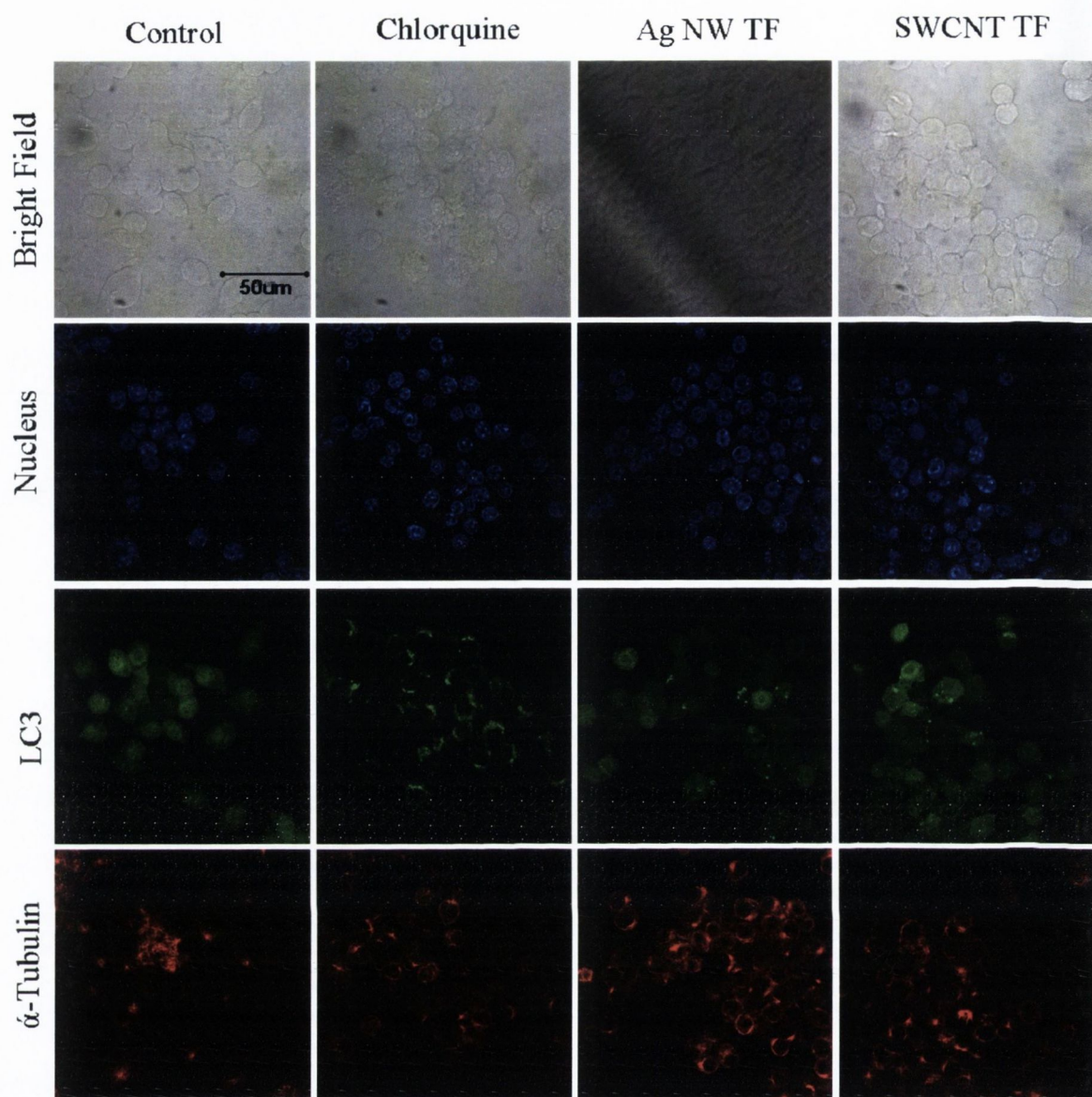


Figure 34 Brightfield and fluorescent images taken of BMDM: untreated, chloroquine treated, and those grown on SWCNT thin films and AgNW thin films. Blue – nuclei, green – LC3 and red – α -tubulin. These images were used to quantify the amount of autophagosomes present following treatments.

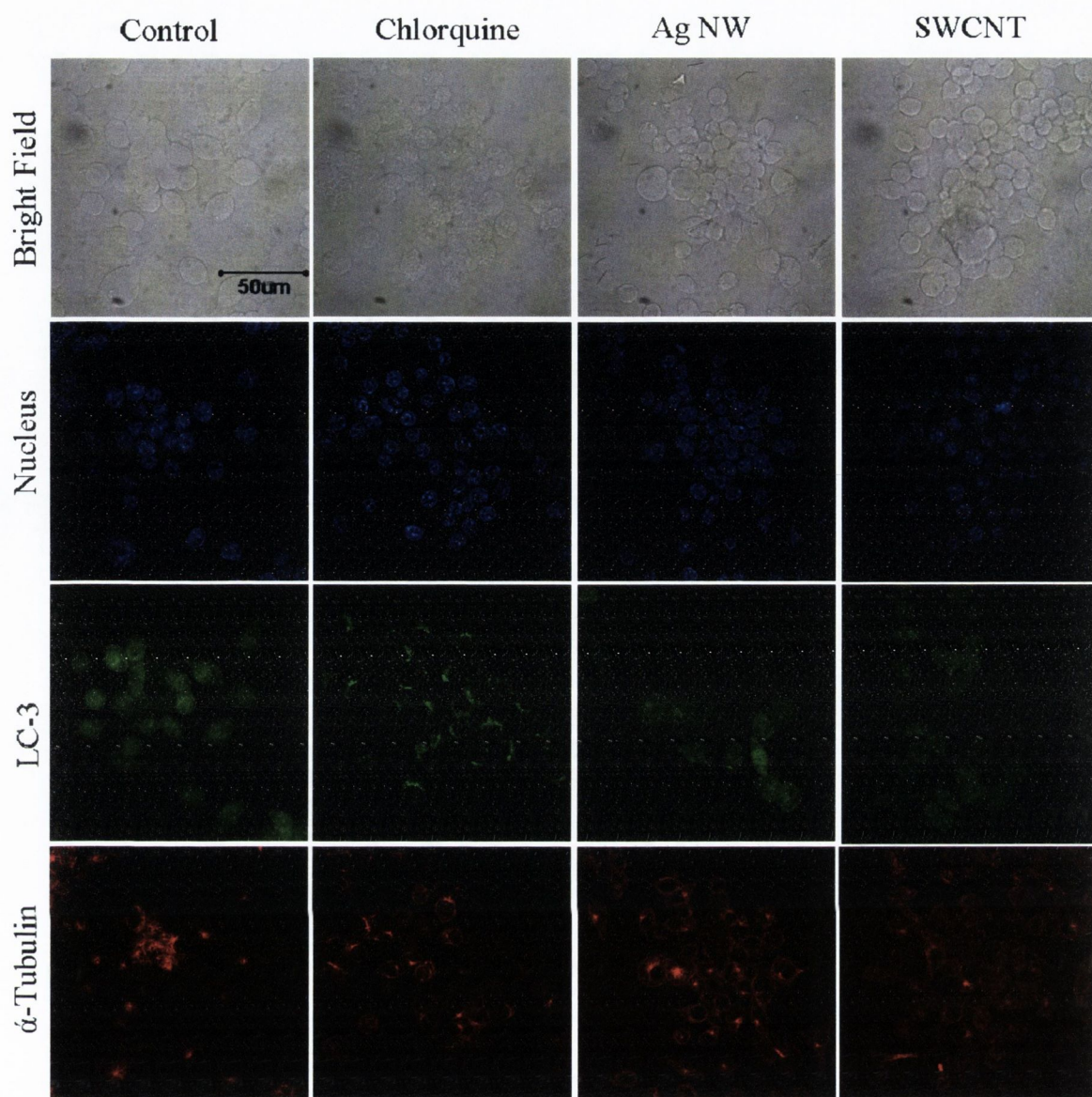


Figure 35 Brightfield and fluorescent images taken of BMDM: untreated, chloroquine treated, SWCNT exposure and AgNw exposure. Blue – nuclei, green – LC3 and red – α -tubulin. These images were used to quantify the amount of autophagosomes present following treatments.

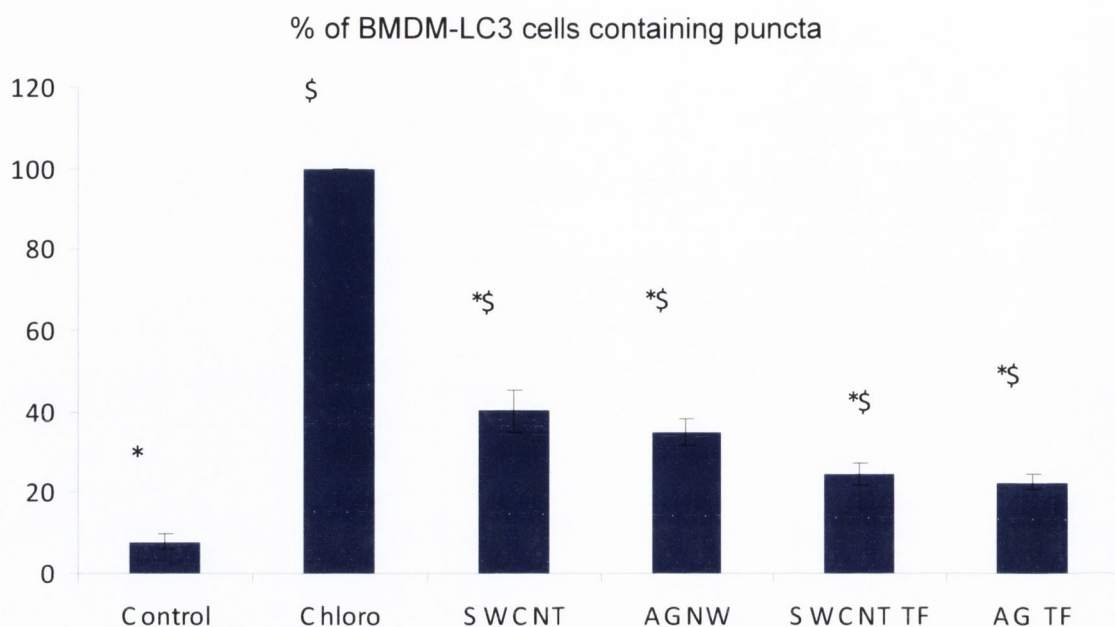


Figure 36 Quantification of the % of BMDM-LC3 cells that contain autophagic vesicles (puncta) following various nanomaterial treatments. 100% of cells treated with chloroquine contain puncta whereas only about 5% of the control cells do which represents the basal level of autophagy present. ~ 40% of cells exposed the SWCNT and AgNW contain puncta compared to ~ 20% of cells grown on SWCNT or AgNW thin films. Data represent the mean of n=3 experiments. Data were analysed by ANOVA with Tukey post hoc correction. \$ Significant to untreated control, * Significant to Chloroquine treated

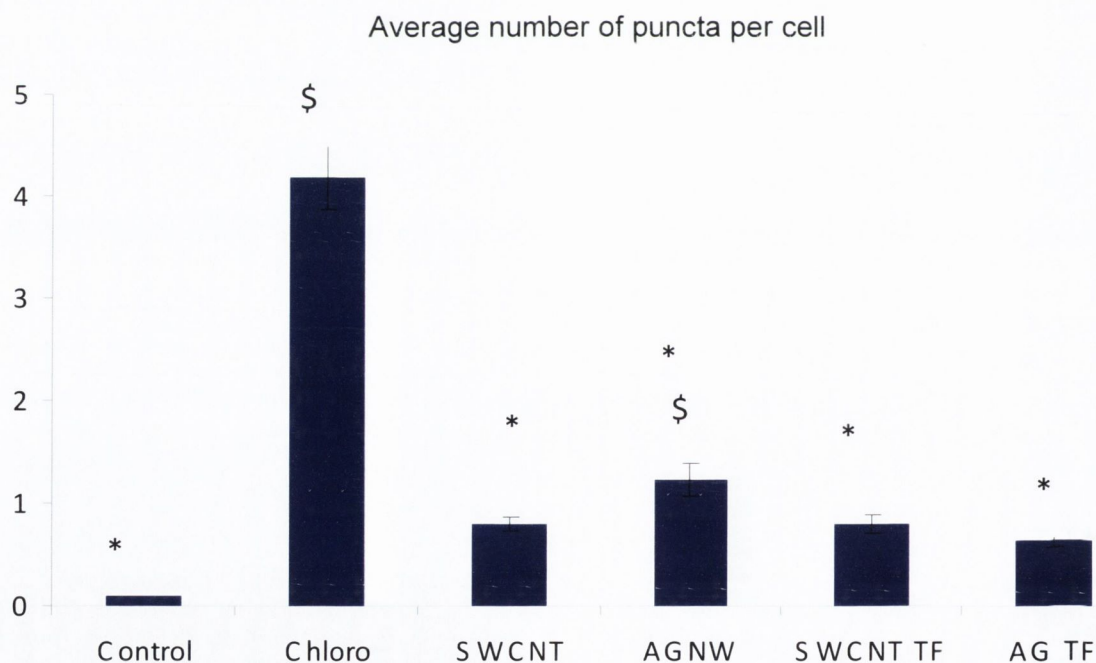


Figure 37 Average number of autophagosomes per cell following treatment with Chloroquine, SWCNT or AgNW and their enabled thin films. Data represent the mean of n=3 experiments. Data were analysed by ANOVA with Tukey post hoc correction. \$ Significant to untreated control, * Significant to Chloroquine treated

2.5. Discussion

Two different scenarios of cell exposure to NP were investigated, in one case the NP were exposed to cells in a loose, unbound state while the second scenario was based on the NP embedded into a polymer matrix to make transparent, flexible thin films. To demonstrate and quantify an increase in autophagy following both NP exposure scenarios a combination of electron microscopy, western blotting, confocal and epi-fluorescent microscopy was used. A priori assumption would be that since the NP are firmly embedded into the thin film polymer matrix no loose NP can exert their effect on the cells. However a significant increase in autophagic response was measured in the phagocytic cells following both exposure scenarios, but of a different magnitude.

The loose AgNW and CNT induced a 40% increase in autophagy in cells whereas there was only a 20% increase in autophagy in cells grown on thin films. However, results from the HCS or MTT cell viability assays did not indicate acute toxicity following the exposure of phagocytes to the AgNW, CNT, or thin films. Perhaps the increase in autophagy observed here can explain some of the controversy over NP toxicity. It provides further insight into the cellular mechanisms initiated following SWCNT or AgNW exposure. It is likely that the results reported here are not strictly limited to the NP under investigation in this study but representative of NP in general. It is a field that is underexplored and warrants further investigation. This finding highlights the importance of stringent control and regulation from the manufacturing stage of material and devices incorporating NPs to the end of their life cycle.

It is still unknown what exactly triggers the formation of autophagosomes following the engulfment of the NP and initiates this stress response from the cells. A number of factors could be driving this process namely NP size, shape, surface area, charge, ion-release and residual contaminants from the manufacturing stages. Interestingly it was possible to image and confirm uptake of the AgNW, which were fluorescing green (due to surface plasmon effects) within the cells following uptake by the phagocytes. These wires were seen to be transported within the cell rapidly and multiple images were captured of

the wires surrounded and decorated by autophagosomes in an attempt to digest and process them within these autophagic vesicles. One of the final steps in the autophagic process is the fusion of the autophagosome with a lysosome to form an autophagolysosome which then degrades its contents. Disruption of this process could lead to an accumulation of autophagosomes and incomplete degradation of the vesicle contents. Using a red lysosomal stain, it was possible to confirm co-localisation of the red lysosomes with the green autophagosomes. It was demonstrated here that firstly SWCNT or AgNW induced an autophagic response in the phagocytic cells and secondly they did not disrupt the autophagic process at any stage. A possible degradation route, common to two physically and chemically different NP has been uncovered.

An intriguing possible application of this autophagic response lies in the area of disease conditions where the autophagic process is disrupted. The medical implications inferable from our demonstrating in this study that SWCNT and AgNW induce autophagy could have possible therapeutic benefits. Autophagic dysfunction is linked or contributes to a number of diseases such as pathogenesis of cancer, liver and immune disease, pathogen infection, and neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's disease (Bursch and Ellinger 2005). For example in neurodegenerative diseases insufficient autophagy activation leads to an accumulation of long lived misfolded proteins (Cheung and Ip 2009). Whereas in tuberculosis the pathogenic mycobacteria survive within phagosomes of the phagocytic cells by arresting the autophagic process thus preventing degradation of the phagosomal contents (Alonso, Pethe et al. 2007; de Chastellier, Forquet et al. 2009). One cannot expect direct treatment with NP suspensions as medicinal drugs. Perhaps NP that are biologically inert or bio-degradable synthesised to mimic the physical and chemical properties of the NP that are proven to induce an autophagic response. The exact NP properties initiating this autophagic response must first be established, then suitable NP made of biodegradable material be used for treatment.

The degradation of NP following their uptake within cells and tissue is another key question that will be addressed in the next chapter.

2.6. Conclusion

This study demonstrated that unbound NP increased the level of autophagy in 40% of cells, whereas only 20% of cells grown on thin films resulted in an increase in autophagy. Live cell confocal microscopy enabled imaging of autophagosomes within the phagocytes following nanoparticle uptake but also confirmed uptake and internalisation of the AgNW. Evaluation of the autophagic response induced by different NP exposure scenarios has been achieved providing further insight into the consequences of NP exposure during different stages of their life cycle. These studies therefore suggest that nanoparticles induce an autophagic response in human phagocytes which may have significant implications for human health and the environment.

Using a range of biochemical and microscopic techniques it has been possible to demonstrate that free NP induce a higher level of cytotoxicity in phagocytic cells than NP incorporated in thin films. Both SWCNT and AgNW induced a marked increase in autophagy providing further insight into the cellular mechanisms ensued following NP exposure. Surprisingly an increase in autophagy was also observed in phagocytes that were grown on the NP thin films, which could be due to NP release during the processing (cutting) of the thin films. This finding is of particular importance highlighting the need for control and regulation of NPs throughout their entire life cycle.

Live confocal microscopy not only enabled imaging of autophagosomes within the phagocytes following NP uptake but was also invaluable in confirming uptake and internalisation of the AgNW. The NPs used here were fluorescent in the green channel which meant it was possible to image them within living cells. This is another significant finding as it is imperative that imaging of NP throughout their entire life cycle is possible on a cellular level and relate this to the overall health following exposure.

Chapter 3

3. Investigation of carbon nanotube biodegradation *in vitro* and *in vivo* using Raman spectroscopy

Work described in this chapter (section 3.4.2) has been published in part in Nature Nanotechnology (2010) 5: 354-359 “*Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation*”. The full paper is included in Appendix 3 of this thesis.

The results presented in sections 3.4.4 and 3.4.5 have been submitted for publication.

3.1. Introduction

The rise in technological applications and consumer products containing engineered carbon nanotubes raises obvious concerns for the possible adverse effects on human health and the environment. The previous chapter demonstrated that minimal toxicity was observed in cells following exposure to SWCNT however they did induce autophagy in phagocytic cells. Raman spectroscopy is optimized in this chapter to image SWCNT within cells and tissue and to measure their integrity.

SWCNTs have been found to induce pulmonary inflammation (Shvedova, Fabisiak et al. 2008; Shvedova, Kisin et al. 2008), oxidative stress (Thurnherr, Su et al. 2009) and early onset fibrosis. Another health concern is that CNT have shown to be biopersistent due to their inherent physical and chemical durability and often being compared to asbestos (Osmond-McLeod, Poland et al. 2011). The exposure of workers to asbestos in the building industry has been described as the greatest occupational health disaster in modern history. Asbestos fibres that are inhaled into the lungs have been found to reach and be retained in the pleura and subsequently cause mesothelioma (Becklake, Bagatin et al. 2007). However, following the inhalation of large amounts of particles by smokers and coal miners, the pleura is not a common site of damage. The natural defense of the lungs moves these particles towards the throat and mouth in an attempt to expel them. This method of expulsion is not possible with asbestos fibres. The long, straight fibrous nature of carbon nanotubes has lead to much speculation and research into whether or not they will pose the same kind of risks as asbestos. Bonner *et al* found that mice that had been

treated with CNT by inhalation, the CNT traveled to the pleura and there was evidence of scarring and fibrosis at week 2, 4, 6 and 14 weeks post exposure (Ryman-Rasmussen, Cesta et al. 2009). None of these symptoms were found in mice that had been treated with carbon black, which is also composed of graphene, but in a compact particulate fashion. These studies show that it is the fibrous shape that is important rather than the chemical nature of the nanoparticles and should be taken into consideration when designing CNT for biapplications. The length of the CNT could play an important role in their ultimate fate and biopersistence within cells. There have been many reports on the chemical cutting of CNT *ex vivo* to make them shorter however they do require strong acids and oxidants (Kosynkin, Higginbotham et al. 2009). It is of fundamental importance to look at the ultimate fate of CNT and whether it is possible that they may be degraded at any stage following use in industry/laboratory, perhaps by bacteria in the air or soil or by cells in humans following exposure. The degradation of carboxylated and pristine SWCNT in horseradish peroxidase (HRP) was carried out by Star *et al* where they found the carboxylated CNT degraded to a much higher degree (Allen, Kotchey et al. 2009). They suggest that a strong adsorption the HRP facilitated such degradation whereas the hydrophobic nature of the pristine tubes repel the HRP thus preventing the enzyme's oxidative effects. Bianco and his group investigated the degradation of oxidized SWCNT and oxidized MWCNT in the presence of either phagolysosomal stimulant fluid (PSF) or HRP (Russier, Menard-Moyon et al. 2010). Using TEM and Raman they found that the SWCNT were degraded to a much higher degree than the MWCNT and that the "more perfect" nanotubes degraded to a lesser extent indicating that the surface functionalisation (carboxyl) plays a critical role in the degradation process.

The ultimate fate of CNT in humans following exposure is determined by how our immune system deals with these foreign bodies. As discussed earlier phagocytes are the first line of defense and use their potent oxidant generating enzymes to kill invading pathogens or in the case of nanoparticles degrade them (Sutherland, Mahoney et al. 1993; Hampton, Kettle et al. 1998; Allen and Stephens 2011). Little or no research has been carried out in the field to measure the integrity of CNT following exposure to cells. This is an essential area to be addressed with CNT applications where possible exposure routes constantly on the rise.

3.2. Aims and Objectives

Raman spectroscopy is employed to image and assess the integrity of SWCNT following their exposure to phagocytic cells and lung tissue. It is demonstrated that this non-invasive, non-destructive, spectroscopic method enables clear discrimination between intact and degraded SWCNT in biological systems. The Raman spectra of SWCNT have a number of distinct bands (S. Reich, C. Thomsen et al. 2004) making it possible to measure their integrity with particular emphasis on any change in the relative intensities of the D to G band (Jorio, Souza et al. 2002). Since neutrophils (or polymorphonuclear leucocytes) and macrophages represent the first line of innate immune defence, they were exposed to SWCNT at different time points. The key metabolic component of these cells, utilised in neutralising pathogens, is the enzyme called myeloperoxidase (MPO) (Nathan 2006). Therefore mouse lung tissue sections of wild type and MPO knock-out mice, following exposure by pharyngeal aspiration of carboxylated CNT were sectioned at 1, 7, and 28 day post exposure and investigated.

The aims of this part of the study are:

- To employ Raman spectroscopy to image and measure the integrity of SWCNT within phagocytic cells.
- To optimize chemometric methods for the analysis of Raman spectral data.
- To employ Raman spectral mapping of tissues and chemometric data analysis to image and measure the structural integrity of SWCNT within the tissue

3.3. Materials and Methods

3.3.1. Preparation and characterisatation of single walled carbon nanotubes

SWCNT (CNI Inc., Houston, TX) produced by the high pressure CO disproportionation process (HiPco) technique employing CO in a continuous-flow gas phase as the carbon feedstock and $\text{Fe}(\text{CO})_5$ as the iron-containing catalyst precursor, and purified by acid treatment to remove metal contaminants were used in the study. Chemical analysis trace metal (iron) in SWCNT was performed at the Chemical Exposure and Monitoring Branch (DART/NIOSH, Cincinnati, OH) using nitric acid dissolution and inductively coupled plasma-atomic emission spectrometry (ICP-AES). Analysis revealed that SWCNT comprised of 0.23 weight % iron. SWCNT were routinely tested for bacterial endotoxin (LPS) contamination using the endpoint chromogenic LAL method. The mean diameter and surface area of SWCNT was 1–4 nm and 1040 m²/g. Surface area was determined by Brunauer, Emmett, and Teller (BET) analysis, and the diameter and length was measured by TEM.

The chemical cutting of SWCNT was performed as follows: Purified SWCNT were dispersed in 3:1 mixture of concentrated H_2SO_4 and 30% aqueous H_2O_2 and sonicated in ultrasonic bath (Branson 1510 Sonifier®, output power of 70 W at 40 KHz) for 24 hrs at 0°C. The dispersion was then heated to 70°C for 10 min for “polishing” the nanotubes. This solution was then diluted 10-fold by deionized water and filtered through PTFE membrane (100 µm pore size). The collected sample was thoroughly washed with deionized water and vacuum dried at 110°C for 30 min.

Obtained short SWCNT were dispersed in 25 mM HEPES buffer (pH 7.4; containing 150 mM NaCl) by sonication.

3.3.2. Cell Culture and tissue preparation

3.3.2.1. *Neutrophils*

Human neutrophils were isolated by a procedure utilizing Histopaque (Sigma, St. Louis, MO, USA). Briefly, human buffy coat (Central blood bank, Greentree, PA, USA) was layered on top of a density gradient (Histopaque 1.077/1.119, Sigma, St. Louis, MO, USA) and subjected to centrifugation as described in the manual (700 g 45 min at room temperature without brake). The neutrophil-rich supernatant (between the layers of Histopaque) was carefully aspirated and washed twice with calcium and magnesium free PBS; thus obtained cells were suspended in RPMI and adjusted to 25×10^6 cells/ 3ml before use. All uptake assays were performed in cell culture medium without serum. After incubation with Nanotubes ($25 \mu\text{g}/10^6$ cells) for 0, 2, 4, and 8 hours, neutrophils were washed twice with PBS and fixed using 2.5 % paraformaldehyde and then cyto-spun onto 1.5-mm thick cover glass (Fisher Scientific). These samples were provided by Dr. Najarjun and Prof. Valerian Kagan, Centre for Free Radical and Antioxidant Health, University of Pittsburgh.

3.3.2.2. *Macrophages (THP-1 cell line and Primary Macrophages)*

Human monocytic leukemia THP-1 cell line (American Type Culture Collection, USA) were maintained in RPMI 1640 (with Glutamax) (Gibco/BRL, Invitrogen, Carlsbad, CA) containing 10% FCS (Gibco/BRL). Prior to treatment cells were plated onto coverslips (13mm borosilicate glass; VWR International, West Chester, PA) in 8-well Labteks, at a density of 0.5×10^5 cells/ml and differentiated with 100nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 72hrs and then treated for 24, 48 and 72 hours with $1 \mu\text{g}/\text{mL}$ of the three different CNT.

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat of anonymous healthy donors (provided, with permission, by the Irish Blood Transfusion Service) by centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway) density gradient, washed and re-suspended in RPMI-1640 culture medium, supplemented with 10% pooled human serum type AB (Sigma), 50U/ml fungizone (Gibco/BRL, Invitrogen, Carlsbad, CA)

and 50 µg/ml cefotaxime (Melford Laboratories, UK). Cells were seeded onto Labtek₈₅, non-adherent cells were removed by washing the wells with HBSS at 24hrs and fresh medium was added. The medium was replaced, subsequent to washing to remove any remaining non-adherent cells, every 2-3 days. MDMs were cultured for 7 to 10 days before treating with 1 µg/mL of the three different CNT.

Three different SWCNT were used here for the THP-1 and the primary macrophages; A = pristine SWCNT, B = SWCNT + BSA and C = covalently modified SWCNT. The SWCNT were provided by Dr. Anton Knyazev, École polytechnique fédérale de Lausanne.

3.3.2.3. *Mouse lung tissue sections*

Specific pathogen-free adult (8-10 week) female C57BL/6 mice (w/t) and B6.129X1-MPO mice (MPO k/o) were supplied by Jackson Lab (Bar Harbor, MN) and weighed 20.0 ± 1.9 g when used. Animals were housed one mouse per cage receiving HEPA filtered air in AAALAC-approved NIOSH animal facilities. All animals were acclimated in the animal facility under controlled temperature and humidity for one week prior to use. Animals were supplied with water and certified chow 5020 (Purina Mills, Richmond, IN) ad libidum, in accordance with guidelines and policy set forth by the Institute of Laboratory Animals Resources, National Research Council. All experimental procedures were conducted in accordance with a protocol approved by the NIOSH Institutional Animal Care and Use Committee. The absence of MPO in MPO k/o mice was proved by Northern blot or Western blot analysis of bone marrow. Neutrophils and monocytes from both peripheral blood and bone marrow of MPO k/o animals failed to exhibit endogenous peroxidase activity.

Mouse pharyngeal aspiration was used for particulate administration. Briefly, after anesthetization with a mixture of ketamine and xylazine (62.5 and 2.5 mg/kg subcutaneous in the abdominal area), a suspension (approximately 50 µl) of SWCNT prepared in PBS (40 µg/mouse) was placed posterior on the throat and the tongue which was held until the suspension was aspirated into the lungs. Control mice were administered sterile Ca²⁺ + Mg²⁺-free PBS vehicle. The mice revived unassisted after approximately 30–40 min. All mice in SWCNT and PBS groups survived this exposure procedure. This technique

provided good distribution of particles widely disseminated in a peri-bronchiolar pattern within the alveolar region as was detected by histopathology. Animals treated with the particulates or PBS recovered easily after anesthesia with no behavioral or negative health outcomes. Mice were sacrificed on days 1, 7 and 28 following the exposures. These samples were provided by Prof. Anna A. Shvedova, National Institute for Occupational Safety and Health, West Virginia.

3.3.3. Raman spectroscopy

3.3.3.1. *Raman spectrometer set-up*

A novel hybrid platform combining Raman spectroscopy and atomic force microscopy was used for the investigation of nanomaterials with living cells. A number of months were spent optimising and calibrating both of these techniques for measuring nanomaterials and cells in both liquid and dry phase. Figure 5 shows the instrument set-up with the inverted microscope for Raman measurements and the AFM head sits on top. An inverted microscope was the preferred option for the investigation of biological samples.

3.3.3.2. *Point spectra of carbon nanotube bundles in neutrophils*

The cover glass was placed and clipped down on the inverted Raman microscope. All point Raman spectra were recorded using a Renishaw inVia Raman spectroscopic microscope, with a cobalt solid state laser operating at a wavelength of 473 nm. All Raman spectra were recorded for 2 seconds, at 10 % laser power using a 100x oil immersion objective. For each spectrum a static grating (2400 l/mm) scan was taken over the range 678.28 cm^{-1} to 2231.82 cm^{-1} (centred at 1500 cm^{-1}). Detection of the Raman scattered light was performed using a peltier cooled CCD detector. Calibration was carried out daily using a piece of silicon to ensure the 520 cm^{-1} line was not out by more than 1 cm^{-1} . An average of at least 20 Raman spectra were taken of each of the samples in Table 2.

Table 2 Summary of CNT and neutrophil samples. At least 20 point Raman spectra were taken for each of these samples. The spectra were then imported into Unscrambler for chemometric analysis.

Sample	Exposure time (hours)
CNT Alone	0
CNT + PMN	0
CNT + PMN	0.5
CNT + PMN	2
CNT + PMN	4
CNT + PMN	8

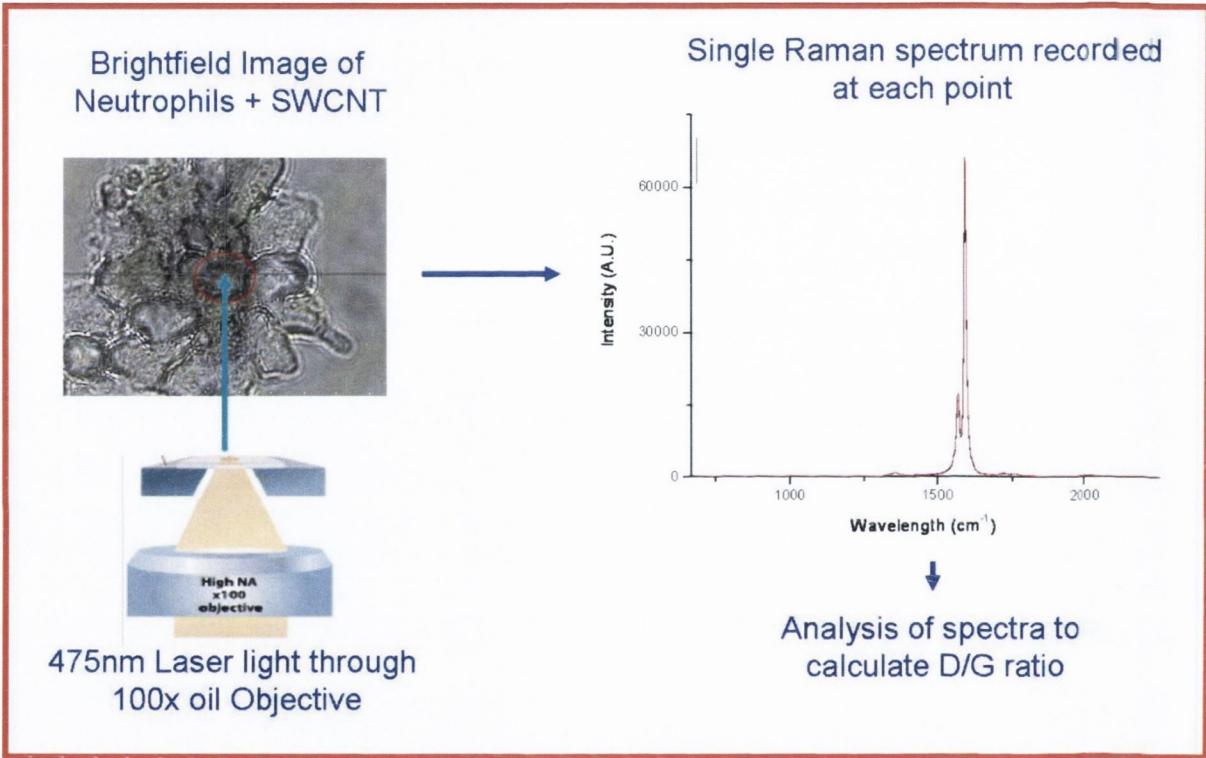


Figure 38 Schematic illustrating laser excitation path from the objective to the sample of SWCNT in human neutrophils, illustrating brightfield image of cells following SWCNT exposure and a typical Raman spectrum acquired. Single Raman spectra were taken and imported into Unscrambler for analysis.

3.3.3.3. Raman maps of macrophages following SWCNT exposure

A Ntegra Spectra Raman spectroscopic microscope (NTMDT and Renishaw) was used, with a cobalt solid state laser operating at a wavelength of 473 nm. 3 Raman maps, 32 x 32 points, were taken for each of the samples shown in Table 3. Each map took ~ 40 minutes to complete. All Raman spectra were recorded for 5 seconds, at 100% laser power

using a 100x oil immersion objective. For each spectrum a static grating (2400 l/mm) scan was taken over the range 678.28 cm^{-1} to 2231.82 cm^{-1} (unlike the neutrophils it was centred at 2700 cm^{-1} to include the 2D band). Detection of the Raman scattered light was performed using a Peltier cooled CCD detector. Calibration was carried out daily using a piece of silicon to ensure the 520 cm^{-1} line was not out by more than 1 cm^{-1} . Analysis of the collected Raman maps was carried out using Matlab (see section 3.3.5 for a full description).

Figure 39 shows a schematic of the work flow, indicating how Raman maps were acquired and processed for all the THP-1 and primary macrophage cells. A $20\text{ }\mu\text{m} \times 20\text{ }\mu\text{m}$ area over the cells was scanned (indicated in the brightfield image) made up of 32×32 points, the spectra are then imported into Matlab where K-means clustering was performed and the I_D/I_G ratio is calculated. I_D/I_G ratio is well documented method for measuring the integrity of SWCNT (Giordani, Bergin et al. 2006; Ni, Fan et al. 2007).

Table 3 Raman maps were taken for each of these samples and analysis was carried out using MatLab software. The name of each sample indicates the cell type (PM or THP-1), the CNT exposure time and the type of SWCNT used.

Sample No.	Sample Name	Sample No.	Sample Name
1	PM 24 Hour Alone	13	THP1 24 Hour Alone
2	PM 24 Hour SWCNT A	14	THP1 24 Hour SWCNT A
3	PM 24 Hour SWCNT B	15	THP1 24 Hour SWCNT B
4	PM 24 Hour SWCNT C	16	THP1 24 Hour SWCNT C
5	PM 48 Hour Alone	17	THP1 48 Hour Alone
6	PM 48 Hour SWCNT A	18	THP1 48 Hour SWCNT A
7	PM 48 Hour SWCNT B	19	THP1 48 Hour SWCNT B
8	PM 48 Hour SWCNT C	20	THP1 48 Hour SWCNT C
9	PM 72 Hour Alone	21	THP1 72 Hour Alone
10	PM 72 Hour SWCNT A	22	THP1 72 Hour SWCNT A
11	PM 72 Hour SWCNT B	23	THP1 72 Hour SWCNT B
12	PM 72 Hour SWCNT C	24	THP1 72 Hour SWCNT C

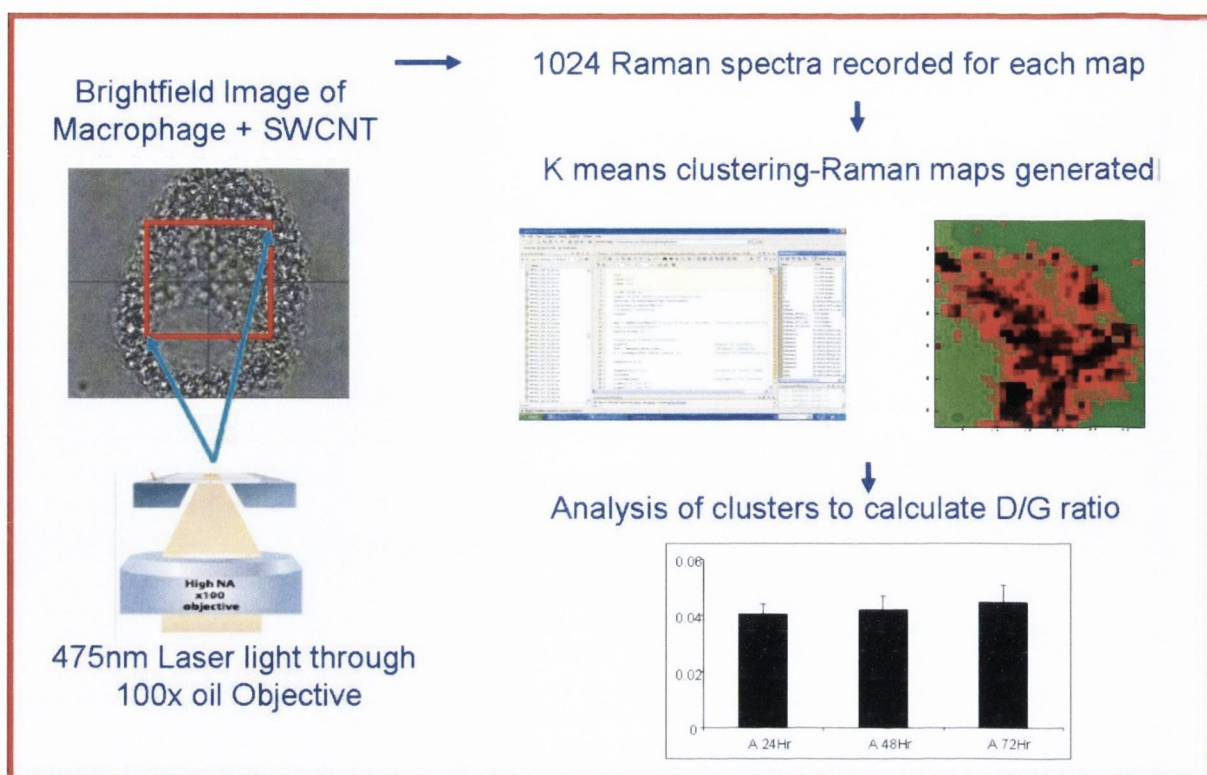


Figure 39 Schematic indicating how Raman maps were acquired and processed for all the THP-1 and primary macrophage cells. A 20 μm x 20 μm area over the cells was scanned (indicated in the brightfield image) made up of 32 x 32 points. The spectra are then imported into matlab where K-means clustering is performed and the I_D/I_G ratio is calculated.

3.3.3.4. Raman maps of mouse lung tissue sections following SWCNT inhalation

Raman maps of lung tissue sections were acquired using an Ntegra Spectra Raman system (NT-MDT, Russia) 473nm cobalt laser operated at 10%, 100x oil objective, 2 s exposure of static spectra centred at 2070 cm^{-1} . Ten maps (32 x 32 points, 20 μm x 20 μm or 10 μm x 10 μm) were recorded for each tissue section. The following were scanned for both the wild type (w/t) and myeloperoxidase knock-out mice (MPO k/o); 3 x 1 day, 3 x 7 day and 3 x 28 day. All of the Raman maps were then imported into Matlab 7 (The Mathworks, USA) for analysis, where all of the spectra were normalised, smoothed, baseline corrected and K-means clustering was carried out. The I_D/I_G ratio was also calculated.

Table 4 List of wild type and myeloperoxidase knock-out mice lung samples use din this study. Ten Raman maps were recorded for each sample.

Lung samples of wild type and MPO knock out mice (May 2010)					
Strain	Group	Exposure	Animal #	Time point	Raman map
B6 129X1-MPO	2	CNT 40ug/mo	26	28 day	Y
B6 129X1-MPO	2	CNT 40ug/mo	39	28 day	Y
B6 129X1-MPO	2	CNT 40ug/mo	40	28 day	Y
C57BL/6	1	PBS	74	28 day	Y
C57BL/6	2	CNT 40ug/mo	68	28 day	Y
C57BL/7	2	CNT 40ug/mo	69	29 day	Y
C57BL/8	2	CNT 40ug/mo	79	30 day	Y
B6 129X1-MPO	1	PBS	102	7 day	Y
B6 129X1-MPO	2	CNT 40ug/mo	106	7 day	Y
B6 129X1-MPO	2	CNT 40ug/mo	107	7 day	Y
C57BL/6	1	PBS	143	7 day	Y
C57BL/6	2	CNT 40ug/mo	147	7 day	Y
C57BL/6	2	CNT 40ug/mo	148	7 day	Y
B6 129X1-MPO	1	PBS	165	1 day	Y
B6 129X1-MPO	2	CNT 40ug/mo	169	1 day	Y
B6 129X1-MPO	2	CNT 40ug/mo	170	1 day	Y
C57BL/6	1	PBS	204	1 day	Y
C57BL/6	2	CNT 40ug/mo	209	1 day	Y
C57BL/6	2	CNT 40ug/mo	210	1 day	y

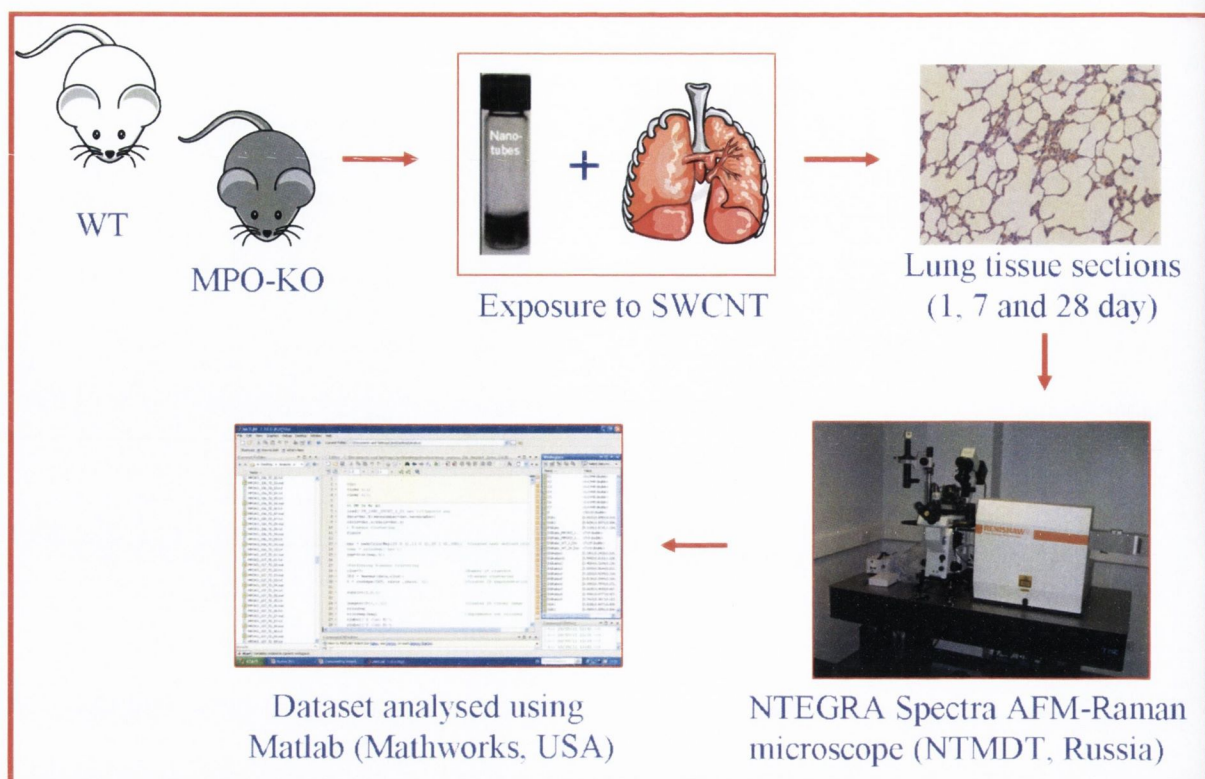


Figure 40 Schematic indicating experimental set up of wild type and MPOKO mice; exposure to SWCNT by pharyngeal aspiration, lung sections taken at 1, 7 and 28 days post exposure, Raman maps acquired and then data analysis carried out using MatLab.

3.3.4. Chemometric analysis using Unscrambler

Data analysis was carried out using the Unscrambler chemometrics software package (v9.8, CAMO, Norway). Principal component analysis (PCA) was used for the classification of samples based on whether or not they contained SWCNTs and the degree to which they had degraded or not. This is done by analysing the data and reducing it to a number of principal components (PC's), each of which describes different aspects of the sample set. In this study, the number of variables (wavenumbers per spectrum) is 1016 and the number of PC's to be retained was set to 4. The scores plot obtained in PCA represents the new coordinate system of the reduced number of variables. PC1 describes the largest possible variation in the dataset i.e. it accounts for most of the information. PC2 is orthogonal to the first PC and PC3 is orthogonal to PC2 and so on (Figure 41).

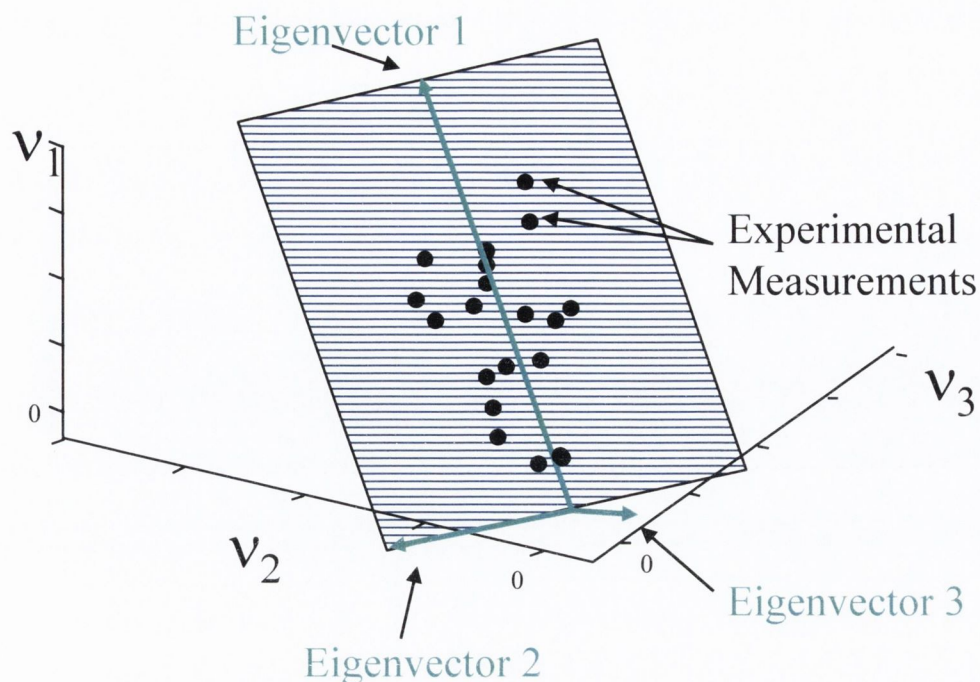


Figure 41 A scores plot obtained in PCA represents the new coordinate system of the reduced number of variables. PC1 describes the largest possible variation in the dataset i.e. it accounts for most of the information. PC2 is orthogonal to the first PC and PC3 is orthogonal to PC2 and so on.

When developing analytical methods based on spectroscopic techniques, the data pre-processing step is a vital part of the overall process. Spectral data pre-processing can enable the removal (or minimisation) of unwanted features such as cosmic rays, noise, background scatter, and fluorescence from spectra. These unwanted features interfere with the complex Raman spectrum. When multivariate analysis is applied to a dataset, no discrimination is made between these unwanted features, and the actual spectrum by the computer program being used. All of the spectral data is processed and therefore incorporated into the model produced. However, by eliminating these features using various pre-processing methods before the application of multivariate analysis enables a reduction in unwanted aspects in the model and it also makes the distribution of the variables more suitable for multivariate analysis.

The following pre-processing techniques were used here;

Smoothing; Smoothing was carried out to reduce the amount of inherent noise present in each of the Raman spectra. The Savitzky-Golay algorithm was used which fits a polynomial to successive curve segments replacing the original values with more regular variations. A 5-point average was carried out on this dataset using second order polynomial.

Derivative pre-processing: By taking the 1st derivative of a spectrum, one can reduce two types of interference at once, baseline and noise. Similar to the Savitzky-Golay smoothing method, the derivative of the spectral curve is taken along a “window” of points. However, it is the derivative of the points that is used here and not the average. The 1st derivative is expected to remove constant offset (baseline), while the 2nd derivative is expected to remove constant offset and sloping baselines. Here, the 1st derivative was used by applying a Savitzky-Golay (Savitzky and Golay 1964) derivitisation with 7-point window averaging and assuming a 2nd degree polynomial order.

Normalisation allows for the reduction of the inherent weighing present within Raman spectra. There are a number of methods of normalisation such as mean, max, and range normalisation. In max-normalisation each spectrum is scaled so that the most intense point in each spectrum is set to a constant value (Adams 2004) (usually 1.0).

3.3.5. Chemometric analysis using MatLab

Firstly, all of the Raman maps acquired for the primary macrophage cells, the THP-1 cells and the lung tissue sections were exported from Raman Nova software as ASCII files. All of the [,], and ; were deleted from these files before they were imported into Matlab. Importing the files into Matlab was a very slow and time consuming process as each Raman map matrix had to be unfolded and converted into the correct format for analysis. Code was written in MatLab with help of Dr. Aidan D. Meade, Dublin Institute of Technology.

3.3.5.1. Primary Macrophage and THP-1 cell analysis:

Code was written in MatLab to carry out the following pre-processing and data analysis on the all of the Raman maps collected.

- Maps of the phagocytic cells (Table 3) were imported into MatLab where the hypercube was unfolded (Figure 42).
- The data was normalized to an area of the spectrum
- The data was smoothed using a Savitzky-golay filter (13, 5, 0)
- All spectra were baseline corrected using a “rubber-band” baseline
- A 1st order polynomial baseline correction was applied.
- K-means clustering was carried out
- The I_D/I_G ratios of all of the clusters were calculated

All of the data generated on the I_D/I_G ratios was then taken into Excel to calculate statistics and generate graphs.

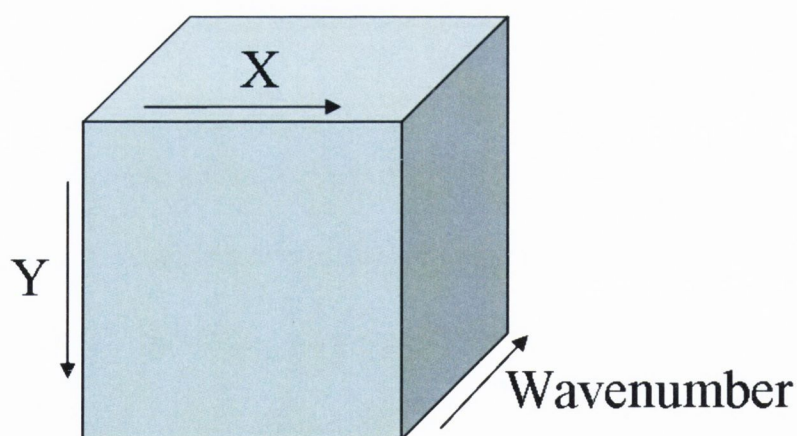


Figure 42 Schematic of a hypercube. X and Y represent the position of each Raman spectrum on the map, and wavenumber is the vector containing the value at each wavenumber in the spectrum.

K-means clustering was used here because it can easily handle large amounts of data like the Raman maps acquired here. Briefly: The number of clusters in which the spectra are

grouped into is determined by the user (the optimal no. found for this study was 7). For each cluster set one spectrum is selected as the cluster centre. All of the spectra in the dataset are then compared to these cluster centres and assigned to the one they are most similar to. Once all of the spectra in the dataset are assigned to a particular cluster, new cluster centres are assigned by averaging the spectra assigned to that centre. This process is repeated until a stable solution is reached (Nijssen, Bakker Schut et al. 2002; Kolijenoviic, Bakker Shut et al. 2004).

3.3.5.2. *Mouse lung tissue section analysis:*

Code was written in MatLab to carry out the following pre-processing and data analysis on the all of the Raman maps collected.

- Maps of the lung tissue (Table 4) were imported into MatLab where the hypercube was unfolded (Figure 42).
- The data was normalized to an area of the spectrum
- The data was smoothed using a Savitzky-golay filter (13, 5, 0)
- All spectra were baseline corrected using a “rubber-band” baseline
- A 1st order polynomial baseline correction was applied.
- K-means clustering was carried out
- The I_D/I_G ratios of all of the clusters were calculated

All of the data generated on the I_D/I_G ratios was then taken into Excel to calculate statistics and generate graphs.

3.4. Results

3.4.1. Raman spectra of SWCNT

Single walled carbon nanotubes have an elegant Raman spectrum made up of discrete bands (Figure 43), the radial breathing mode at 185 cm^{-1} , the defect vibrational mode at 1355 cm^{-1} , the tangential G^+ and G^- oscillations at 1592 cm^{-1} and 1565 cm^{-1} respectively and the 2D overtone of the D band at 2701 cm^{-1} . The intensity of these bands alone is arbitrary however the relative intensities of one to another carries a great deal of information as does the precise Raman shift or positioning of these bands. Figure 44 shows the contrasting Raman spectra of acquired of SWCNT and carbon black, illustrating the G and D bands. Clearly the pristine SWCNT have sharp bands with the G significantly more intense than the D, whereas in the spectrum of the amorphous carbon black, both D and G are of similar intensity. Throughout this study, the primary interest is in the relative intensity of the D to G band, as this is a well documented method of assessing carbon nanotube integrity (Giordani, Bergin et al. 2006; Ni, Fan et al. 2007). It is expected that the spectrum of SWCNT, following some degree of biodegradation, will have an increase in the D band relative to G ratio following oxidation of the carbon nanotube side wall and begin to resemble that of amorphous carbon.

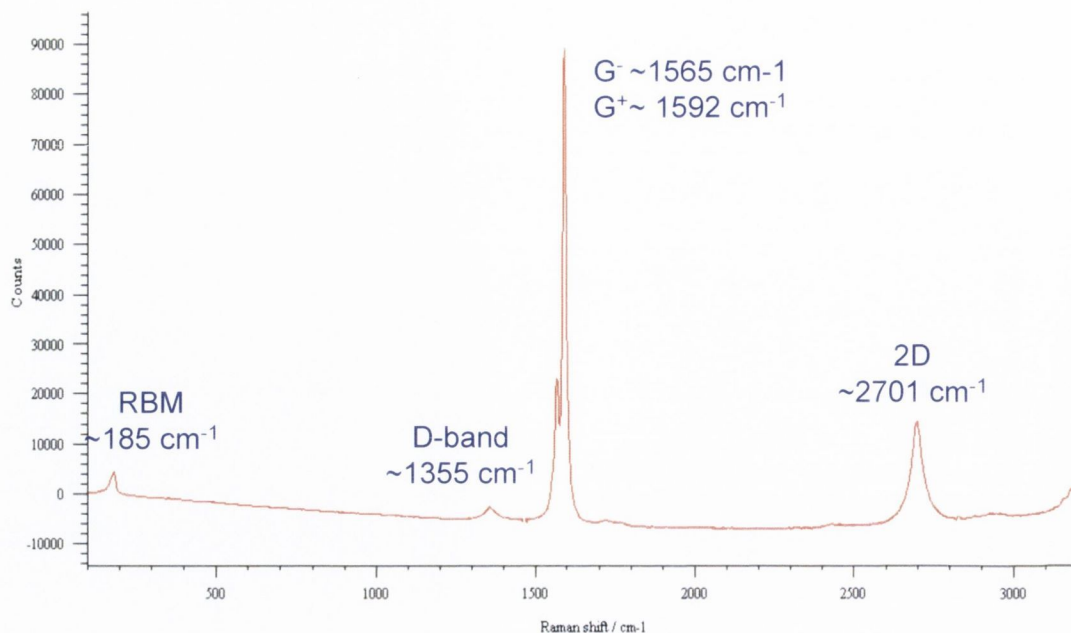


Figure 43 Raman spectrum of SWCNT illustrating the radial breathing mode at 185 cm⁻¹, the defect vibrational mode at 1355 cm⁻¹, the tangential G⁺ and G⁻ oscillations at 1592 cm⁻¹ and 1565 cm⁻¹ respectively and the 2D overtone of the D band at 2701 cm⁻¹.

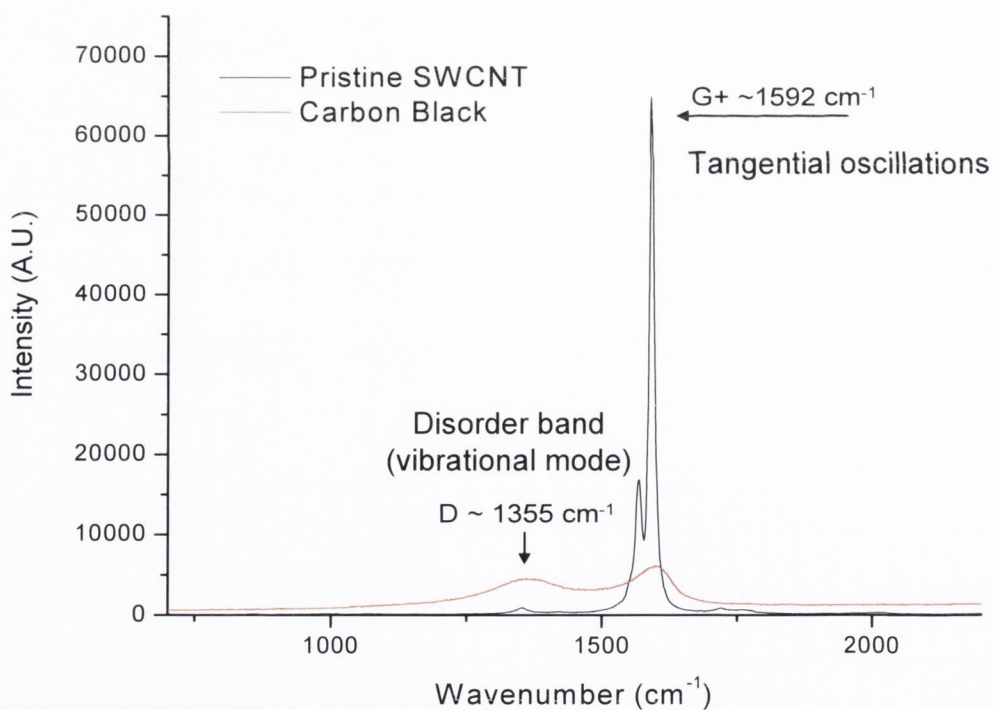


Figure 44 Static Raman spectra, from 678.28 cm⁻¹ to 2231.82 cm⁻¹ (centred at 1500 cm⁻¹), of SWCNT and carbon black with the D and G⁺ bands indicated. This range is acquired throughout as the focus is on the relative intensities of the D to G band following cell and tissue exposure of the SWCNT. This is a well documented method for assessing CNT integrity. A relative increase in the D to G band indicates oxidation or damage to the CNT sidewall

3.4.2. Biodegradation of SWCNT by human neutrophils investigated using Raman spectroscopy

The biodegradation of single walled carbon nanotubes (SWCNT) by human neutrophils using Raman spectroscopy is shown in this section. Figure 45 illustrates the raw Raman spectra acquired for neutrophils alone, in the absence of SWCNT. No characteristic Raman bands can be seen, only a broad background spectrum, as these spectra were only recorded for 2 seconds which was found to be the optimal acquisition time for the SWCNT. This was done so that all Raman settings were consistent throughout the study and since the primary interest is in the signal from the SWCNT. A longer acquisition time would be required to obtain a better spectrum and study the neutrophil Raman bands.

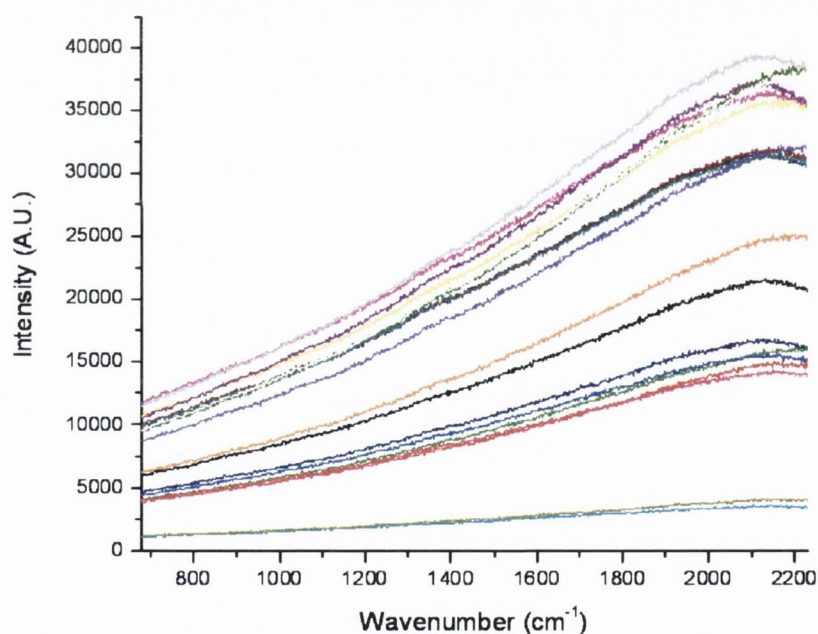


Figure 45 Raw Raman spectra of neutrophils alone. No pre-processing has been carried out on these spectra and they were taken at the optimal settings found for the SWCNT so that all Raman settings were consistent throughout the study.

Figure 46 is an example of the typical Raman spectra and bright-field (BF) images taken of the SWCNT following 0 hours and 8 hours exposure to human neutrophils. The brightfield images show an accumulation of activated neutrophils following SWCNT

exposure. These cells were treated with a high dose of SWCNT which made it possible to identify where the SWCNT bundles were located using white light. Raman spectra were then recorded from areas in the cells that contained these large black bundles of SWCNT. The Raman spectra were recorded from where the cross-wire is located in the BF image. There are striking differences between the Raman spectra of the SWCNT at 0 and 8 hours which indicate degradation. This degradation of the SWCNT is characterised by a loss in the intensity and sharpness of the G^+ and G^- lines ($\sim 1590\text{ cm}^{-1}$ and 1570 cm^{-1} respectively) accompanied by a relative increase in the D band ($\sim 1350\text{ cm}^{-1}$). The SWCNT are no longer made up of intact tubes of conjugated carbons but rather the spectrum resembles that of amorphous carbon. It was also found that in many of the cells at the later time points, the SWCNT appeared to be much more densely packed in specific regions on the cells whereas at the earlier time points they appear to be dispersed more evenly throughout the cell. This would indicate that at the later time points, the SWCNT are packed into vesicles within the cells for processing or degradation. It is proposed that the degradation of the SWCNT in the neutrophils is mediated by the potent oxidating enzyme myeloperoxidase.

In order to quantify the degree to which the nanotubes were being degraded by the neutrophils, the I_D/I_G ratio was calculated. Figure 47 illustrates this ratio following 0, 0.5, 2, 4, and 8 hours post exposure. There is a steady increase in the I_D/I_G ratio with increasing exposure time. This is a well documented method for assessing the integrity of CNTs, where an increase in the I_D/I_G ratio indicates damage to the CNT sidewall. This increase in the D (disorder) band relative to the G band with increasing time, suggests that the graphene sidewall was oxidised by the neutrophils, mediated by myeloperoxidase within these cells. Note there is a slight increase in the I_D/I_G ratio at 0 hours compared to the CNT alone. This is due to a small background contribution from the Raman spectra of the neutrophils even after the pre-processing steps.

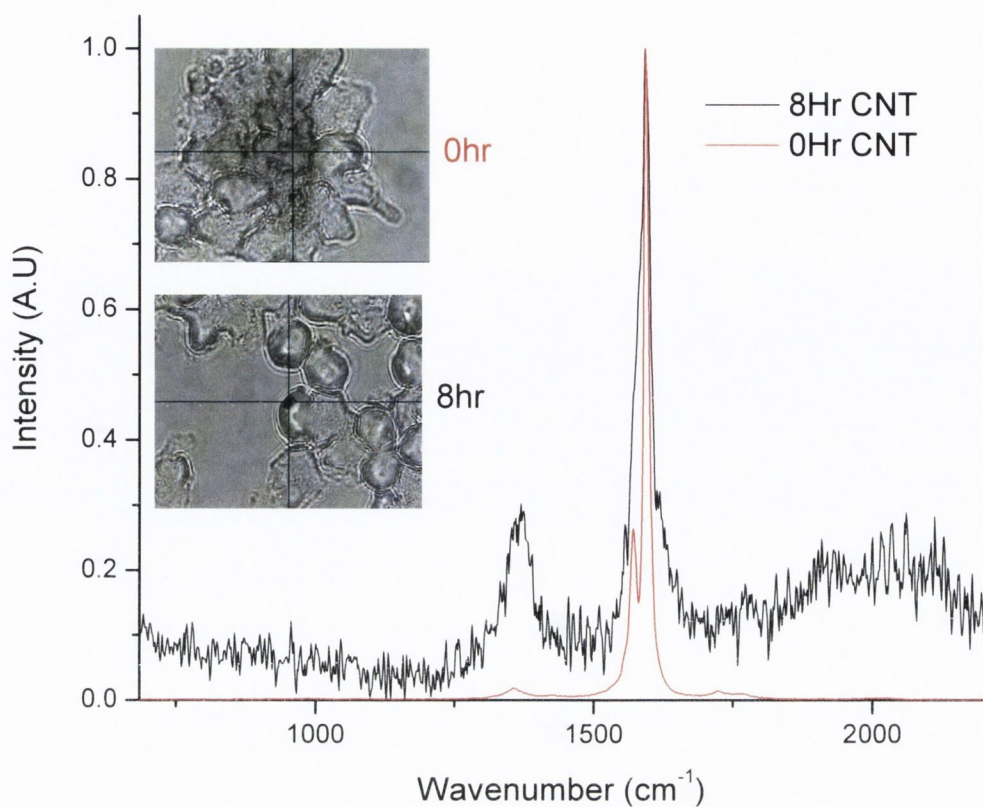


Figure 46 Raman spectra and bright-field images of SWCNT following 0 hour and 8 hour exposure to isolated human neutrophil cells. After 8 hours there is a significant increase in the D band relative to the G band which indicates bio-degradation of the SWCNT.

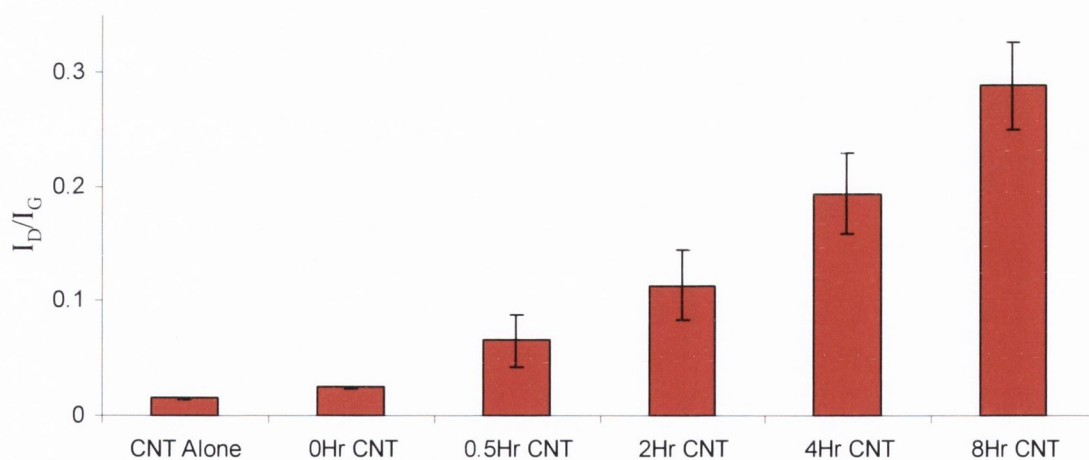


Figure 47 Ratio of D band to G band in the Raman spectra of SWCNT at different time points following exposure to isolated activated human neutrophils. There is an increase in I_D/I_G with time which indicates corresponding degradation of the SWCNT.

3.4.3. Principal component analysis separates pristine from biodegraded SWCNT

Visual inspection of all spectra collected from the samples listed in Table 2 showed that on average the highest degree of degradation was observed after 4 hour and 8 hour exposure of the SWCNT to neutrophils. To analyse the dataset in an objective manner, principle component analysis (PCA) was used. Various pre-processing methods to improve the scatter plots, as described in methods, were applied to the spectra before applying PCA. The best combination proved to be smoothing followed by 1st derivative and then max-normalisation. The score plot acquired following these steps is shown in Figure 48. It indicates good separation of the controls, degraded SWCNTs and pristine SWCNTs and is consistent with the observations made of the raw Raman spectra by eye.

The score plot can be broken down into four quadrants, the two left quadrants contain the “pristine” non-degraded SWCNT spectra whereas the two right hand quadrants contain the spectra of the carbon black, neutrophils alone and the SWCNT that were biodegraded the most (with the neutrophil spectra in upper quadrant and the biodegraded SWCNT and carbon black spectra towards the lower quadrant. This proved to be an objective method for separating out the pristine SWCNT from those that were biodegraded. However, unlike the I_D/I_G results, it does not provide quantitative results on the degree to which the SWCNT were degraded at each time point. It was decided that from here PCA would no longer be used for analysis.

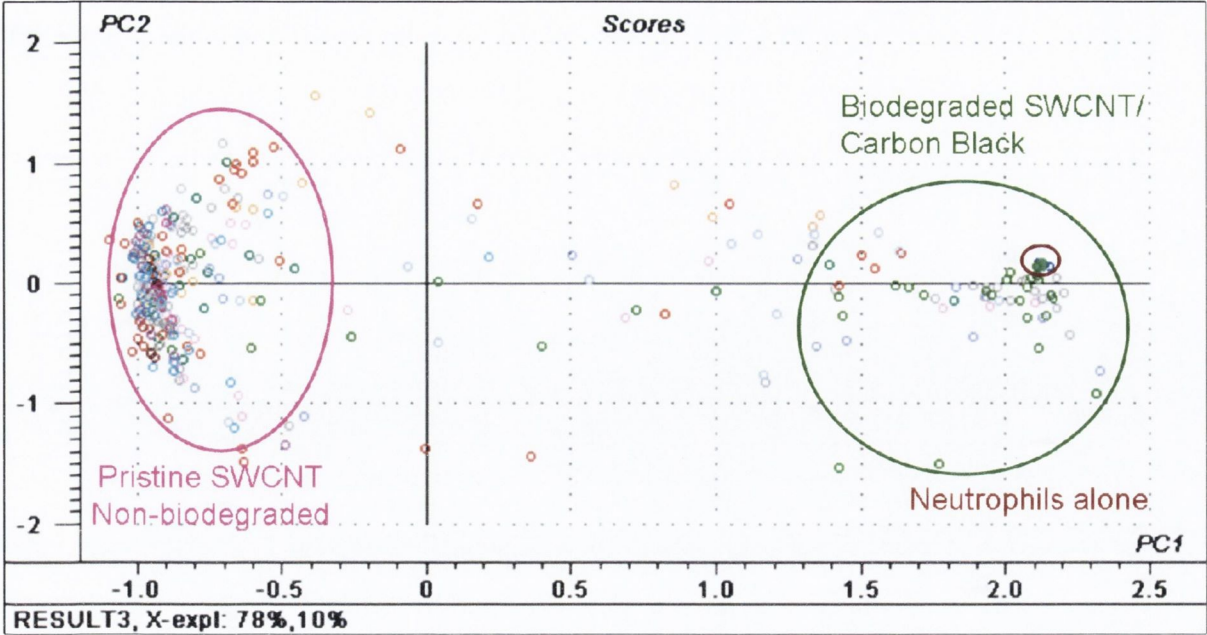


Figure 48 PCA score plot separates the neutrophils alone, carbon black, pristine SWCNT and biodegraded SWCNT. Using the first two PC's, where 88% of variance is used, all of the samples in the data set are successfully clustered into three main groups.

3.4.4. Biodegradation of SWCNT by macrophages investigated using Raman spectroscopy

Here, a measure of the I_D/I_G ratio is calculated for SWCNT in primary macrophages and THP1-cells following K-means clustering. Unlike the analysis of the previous section, which was carried out by taking point spectra of the SWCNT within the neutrophils, here Raman maps of the macrophages were recorded and processed. Following K-means clustering, a map of each 32 x 32 point scan is generated based on the Raman signal at each point. All of the spectra in each map are broken up into 7 clusters with a corresponding colour, with each cluster representing the average Raman spectrum at that point (Figure 49). The strongest and weakest signals from the SWCNT and the tissue are now discernable. However, it is not possible from these images alone to distinguish between pristine and biodegraded SWCNT which is why the I_D/I_G ratio is calculated.

The bright-field image in Figure 49 (A) shows a representative primary macrophage following 24 hour exposure to SWCNT. The red square indicates the area from which the Raman map (1204 spectra) was taken from. Following processing in MatLab, the corresponding Raman map, Figure 49 (B), was generated from the Raman spectral clusters of Figure 49 (C). The black cluster 2 indicates the highest signal from the D and G band of the SWCNT with the red and brown clusters to a lesser intensity. The green clusters 1 and 7 clearly indicate the Raman signal from the primary macrophage cells (Amide I $\sim 1658\text{ cm}^{-1}$, esters/fatty acids $\sim 1744\text{ cm}^{-1}$ and CH_2 bending of proteins/carbohydrates $\sim 1442\text{ cm}^{-1}$).

From all of the collected and processed Raman maps, the I_D/I_G ratio was calculated for the three different SWCNT (A, B and C) following exposure to primary macrophages and THP-1 phagocytic cells for 24, 48 and 72 hours. Figure 50 shows that from 24 hours to 72 hours exposure, the I_D/I_G ratio does not alter significantly in either cell type for any of the three SWCNTs. This is not surprising as the amount of hMPO present in macrophages is markedly less than that in neutrophils (13 ng hMPO per 10^6 macrophages, compared with 1.8 mg hMPO per 10^6 neutrophils). This is indicative of the fact that hMPO is involved in the biodegradation of SWCNT. Noticeably the I_D/I_G ratio for the covalently

modified tubes (SWCNT-C) is higher than SWCNT-A and SWCNT-B, due to the fact that the D band is inherently much higher.

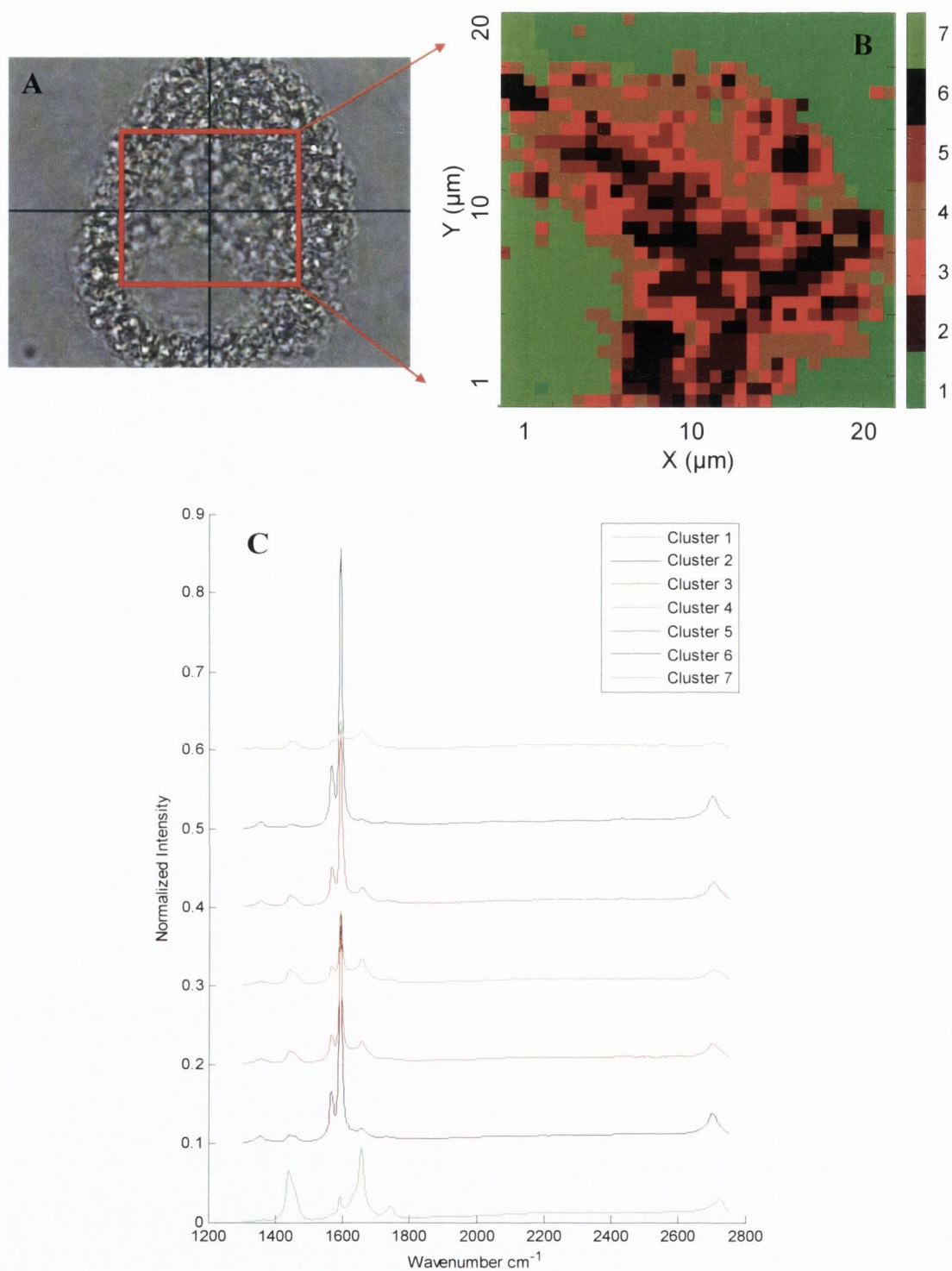


Figure 49 Example of (A) bright-field image of a primary macrophage following exposure to SWCNT, (B) the corresponding Raman map generated from (C) the Raman spectral clusters generated using k-means cluster analysis in MatLab. The red box in (A) indicates the 20 x 20 μm area from which the Raman map covers. The black cluster 2 indicates the highest signal from the SWCNT with the red and brown to a lesser intensity. The green clusters 1 and 7 clearly indicate the Raman signal from the primary macrophage cells (Amide I $\sim 1658\text{ cm}^{-1}$, esters/fatty acids $\sim 1744\text{ cm}^{-1}$ and CH_2 bending of proteins/carbohydrates $\sim 1442\text{ cm}^{-1}$).

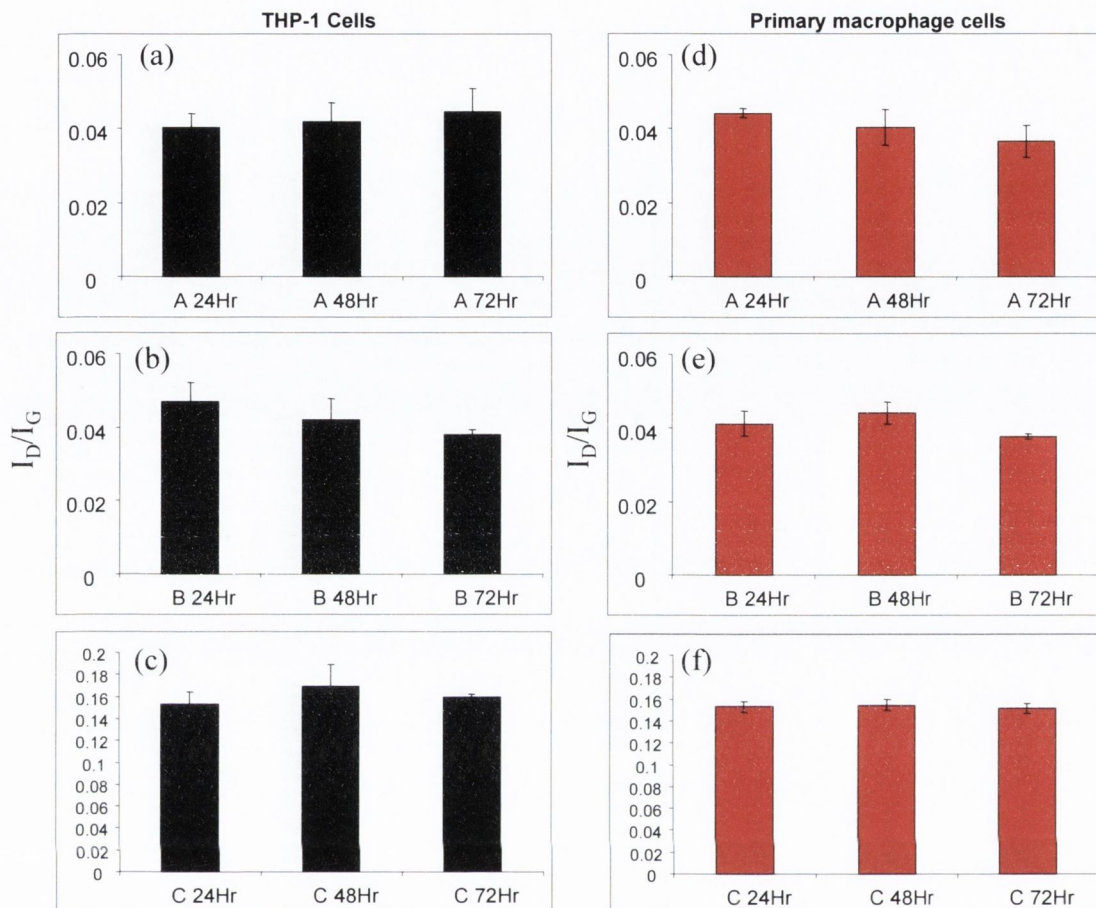


Figure 50 Ratio of D band to G band in the Raman spectra of three different carbon nanotubes (a, d pristine), (b, e pristine + BSA) and (c, f covalently modified) at different time points (24hr, 48hr and 72hr) in both (a-c) THP-1 cells and (d-f) primary macrophages. The D to G band ratio is calculated from Raman clusters shown in Figure 49. There is no significant change in I_D/I_G with time which is not surprising as the macrophage cells contain significantly less MPO than the neutrophils.

3.4.5. SWCNT biodegradation in wild type and MPO k/o mouse lungs investigated using Raman spectroscopy

The experiments presented in the previous sections have shown that the SWCNT are biodegraded in a time dependent manner in neutrophils and to a lesser extent in the macrophages. The involvement of MPO in the degradation process has also been implicated. In this section, if and how they are degraded in the lungs of wild type (w/t) and MPO knock out (k/o) mice is examined. Lung sections were taken at 1, 7 and 28 day post SWCNT exposure to detect firstly if they are degraded in the mouse lungs and secondly to see if they are degraded to a higher extent in the wild type animals.

Figure 51 shows examples of bright-field images of w/t and MPO k/o lung tissue section at 1 day and 28 day post exposure, the red box indicating the area where 32 x 32 Raman spectra were acquired. Following one day post exposure, a large accumulation of SWCNT can be seen in both the w/t and the MPO k/o sections. At 28 day post exposure, there is a dramatic decrease in the amount of SWCNT present in the tissue sections and in particular in the w/t where a greater extent of clearance of the nanotubes appears to have occurred.

Figure 52 is an example of a bright-field image (A) and Raman map (B) generated of a w/t lung section at 1 day which is based on the clusters per pixel (C). From the map it is clear to see the black and red areas correspond to the accumulation of carbon nanotubes and the green and brown areas to the cells in the surrounding area. The signal from the SWCNT is very strong at this time point. Ten of these bright-field images and Raman maps were generated for every sample up to 28 days. An example of the Raman maps with their corresponding Raman spectral clusters at different time points can be seen in Figure 53. The most striking feature of this image is the difference in the intensity and abundance of the SWCNT spectra.

To quantify the level of degradation in the tissue, the I_D/I_G Raman band ratios were calculated for the w/t and MPO k/o at various time points (Figure 54). There is a steady increase over time in the I_D/I_G ratio of the SWCNT in w/t mice, with a significant

difference ($p=0.00696$) between the 1 day and 28 day samples. However, this is not the case for the 7 day MPO k/o samples where the I_D/I_G appears to reach a plateau, whereas the w/t continues to increase up to 28 days which would imply an increased level of SWCNT biodegradation in the w/t mice. There is a significant difference ($p = 0.0277$) between the degradation of the SWCNT by the w/t and MPO k/o at 28 days. This is another indication that hMPO is involved, but not exclusively, in the biodegradation of the SWCNT.

In all of the results shown, it is assumed that the I_D/I_G of carbon nanotube bundles rather than single tubes is measured. Firstly, in order to locate the SWCNT for recording the point spectra or maps, they had to be visible by microscope and secondly the signal of individual tubes would be more difficult to pick up as it would be buried in that of the cells or tissue.

For W/T 1day vs 28day $p=0.00696$ For MPO-/- 1day vs 28day $p=0.07599$
For W/T vs MPO-/- 28day $p=0.02767$ (Figure 54).

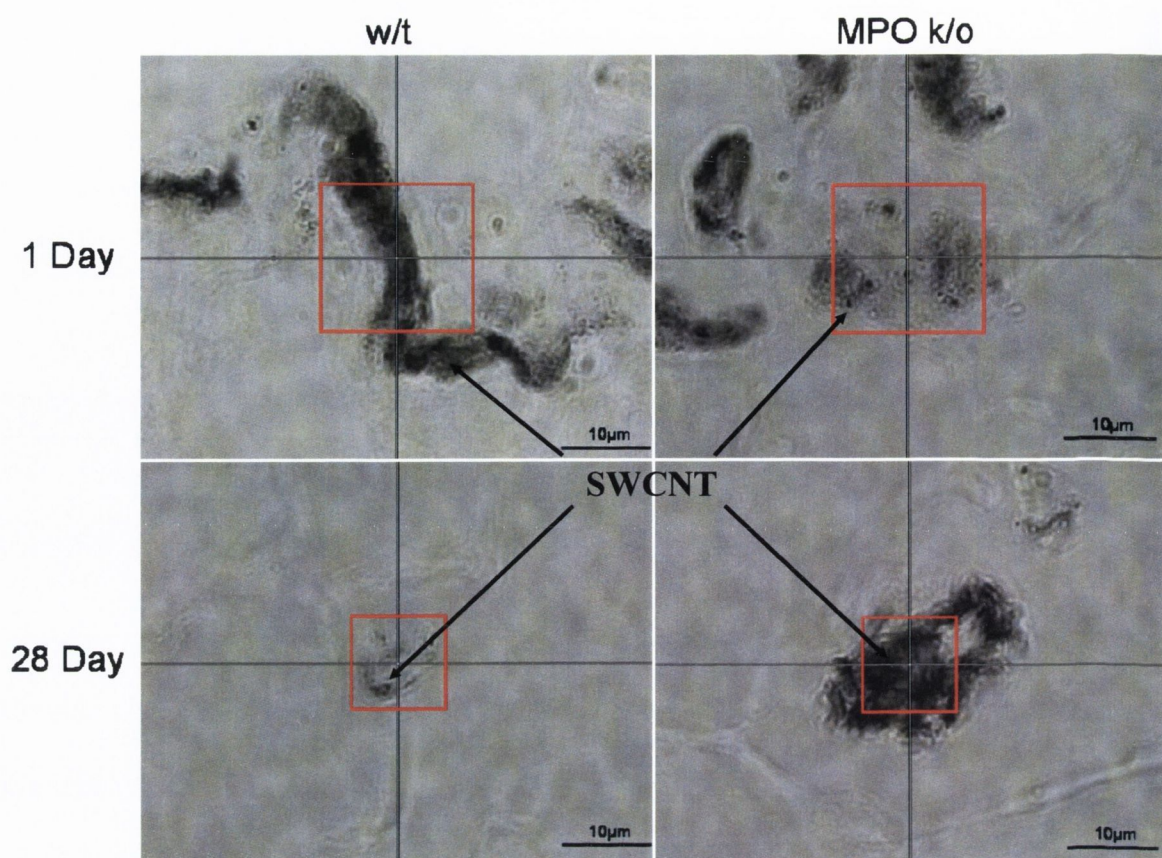


Figure 51 Bright-field images of wild type and MPO knock out mice following exposure to SWCNT at 1 day and 28 day. The red squares indicate the areas from which 1024 Raman spectra were recorded to generate a Raman map. It is clear to see at 1 day there is a large accumulation of SWCNT in both w/t and MPO k/o samples. At 28 day there is a significant clearance of the SWCNT in both samples but to a much higher degree in the w/t.

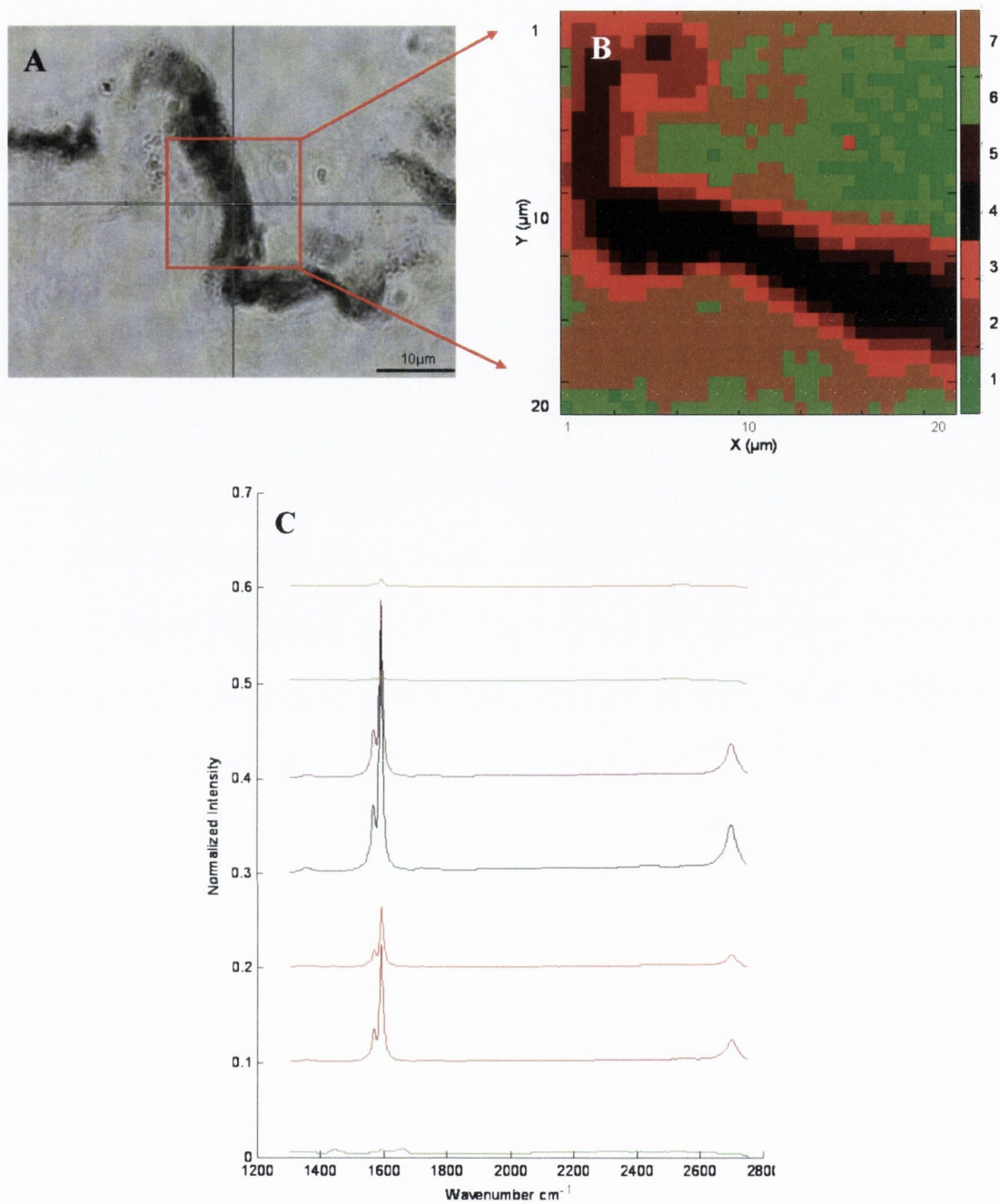


Figure 52 W/t lung tissue section at 1 day following SWCNT exposure. (A) Bright-field microscopic image illustrating area where 20 x 20 μm scan was taken, (B) Raman map based on all Raman spectra from the scan broken into 7 clusters (C) reflecting SWCNT integrity.

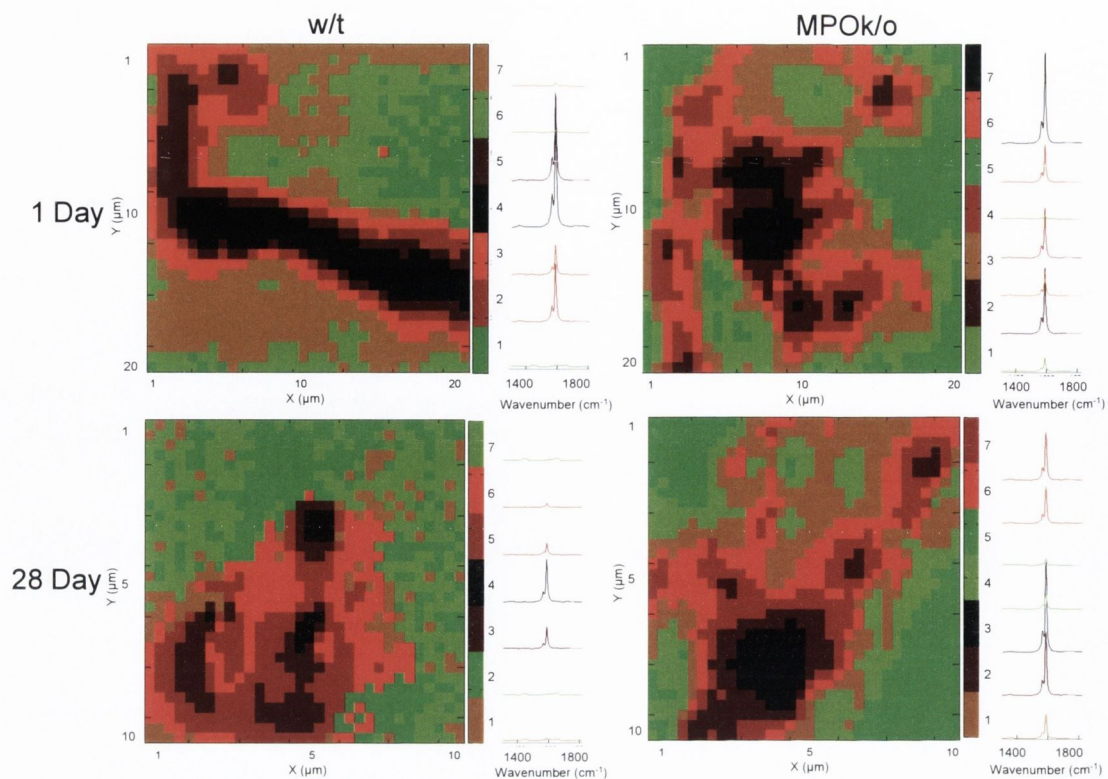


Figure 53 Raman maps generated from the areas indicated in Figure 51. The black pixels indicate the highest signal from the SWCNT and the red and brown to slightly less intensity. Whereas the green signals are either background or tissue contributions and are not included when calculating the D to G ratio.

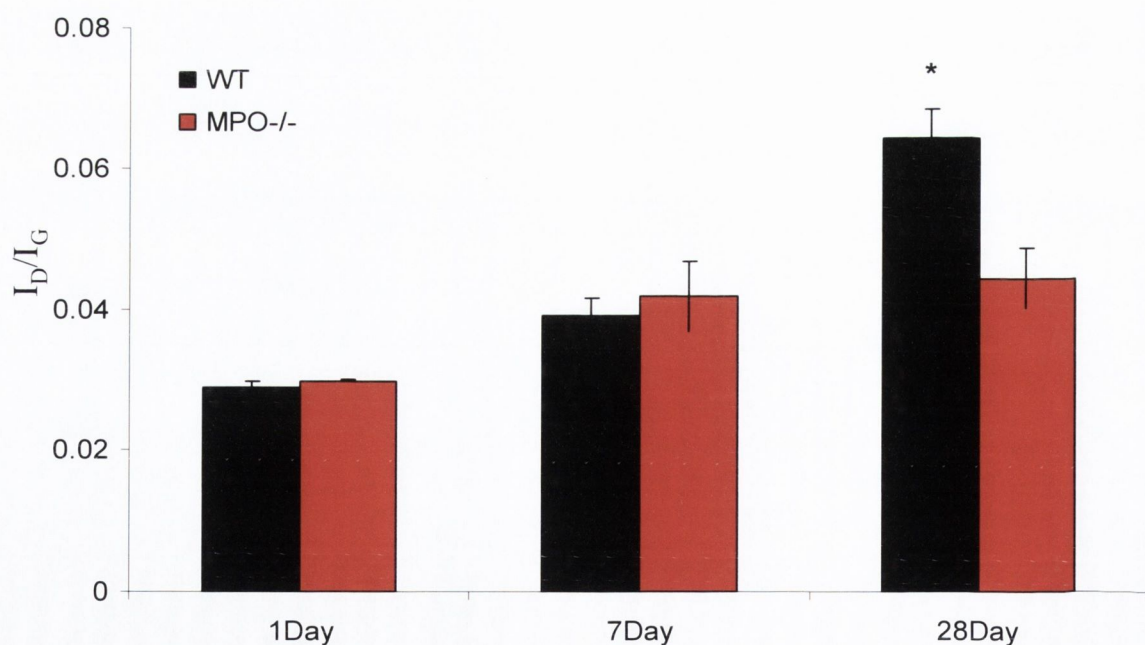


Figure 54 Ratio of D band to G band in the Raman spectra of carbon nanotubes at different time points in the lung tissue of both w/t and MPO^{-/-} mice. There is an increase in the I_D/I_G with time which is higher in the w/t mice. A significant difference ($p = 0.0277$) between the I_D/I_G of the w/t and MPO^{-/-} is observed at 28 days (* $P < 0.05$ vs MPO^{-/-})

3.5. Discussion

Research on biological effects of CNT has mainly focused on the toxicity of the CNT on the cells or tissue they have been exposed to. Much attention has also been on the ultimate fate of the CNT and whether or not they are expelled from the lungs or cells and cleared following exposure. A number of groups have looked at the degradation of CNT *ex vivo*. However, little or no research has been carried out measuring and quantifying the location and integrity of the CNT *in vitro* and *in vivo*. Using Raman spectroscopy, it has been demonstrated here that SWCNT are degraded in a time dependent manner by human neutrophils and to a much lesser extent in macrophages. It was also shown that they were degraded in a time dependent manner in w/t mice compared to MPO k/o mice. Both of these results lead to the conclusion that MPO is involved in the degradation process but not exclusively. Raman spectroscopy proved to be a successful, objective, non-invasive, non-destructive, label-free method of assessing the biodegradation of the SWCNT *in vivo* and *in vitro*. This technique could be applied to any cell type or tissue section and could be measured on wet or dry samples. It could also be utilized on other nanoparticles that have a Raman signal that can easily be separated from the cell or background signal. The method of chemometrics used here to carry out analysis on the vast amount of Raman spectral data collected proved to be an ideal technique to map and locate the SWCNT within the cells and tissue and also to pull out useful information on the integrity of the tubes from their ID/IG ratio. This method of K-means cluster analysis could also be used on other large datasets.

The detection limit of this method was not fully explored in this study. However a very low concentration relative to that used in the synthesis of the SWCNT thin films was used. The macrophages (cell line and primary cells) were treated with a concentration of 1 µg/ml and the Raman signal was really high with only 10% of laser power needed. This highlights the potential of this technique for use in detecting very low concentrations of CNT in cell and tissue following possible exposure. It could be used to scan large biological regions to locate and image CNT in cells or tissue and then move on to measure their integrity.

There is however a number of draw-backs with this protocol. On average each scan took about 40 minutes to complete, therefore with regards to high throughput, this is not practical and can lead to a bottle-neck when it comes to high volumes of sample measurements. There is a need for a faster method of scanning the samples if this technique is to be used practically for real-time results and analysis. Raman spectroscopy as a technique is already making leaps and bounds in the medical industry. Much research has already been carried out on Raman spectroscopy as a potential diagnostic tool (Lyng, Faoláin et al. 2007) and method for measuring toxicity induced by CNT (Knief, Clarke et al. 2009).

The next logical step for future work here is to investigate the biodegradation of MWCNT in cells and tissue. Perhaps they would be degraded to a much lesser extent and much slower due to the concentrically arranged layers of graphene which must be successively degraded. The Raman signal from MWCNT is not as strong and would perhaps require longer scanning time to acquire adequate intensity.

A sample of SWCNT is generally made up of semi-conducting and metallic SWCNT. One way of figuring out the electronic properties of CNT is to use the Kataura plot which uses the RBM mode (from the Raman spectrum) to calculate the diameter of the tubes and plot it against the energy separation. Raman spectroscopy is also a well documented technique for identifying the electronic properties of SWCNT based on the shape of the G band. Where the G band is Lorentzian in shape for semiconducting CNT and it is more broadened in shape and down shifted in metallic CNT (Cheng, Debnath et al. 2011). In this study 473 nm was used as the excitation wavelength and the majority of the SWCNT look to be semi-conducting from their G band however this is an area that should be further investigated. It would be interesting to measure the amount of biodegradation of the semi-conducting compared to the metallic SWCNT and establish if one type undergoes a higher degree of degradation than the other.

3.6. Conclusion

Raman spectroscopy in conjunction with chemometric analysis of the large data sets acquired proved to be an ideal technique for measuring the integrity of SWCNT in cells and in tissue, an area which has previously never been addressed. Calculating the I_D/I_G ratio of the collected Raman spectra proved to be a robust, objective method for assessing the biodegradation of these SWCNT. By calculating this ratio it was possible to measure the degree to which the tubes were degrading over time in different samples. SWCNT were degraded by neutrophils in a time dependent manner, mediated by MPO, with the highest level of degradation seen at 8 hours. Using this technique we found that SWCNT were not degraded to the same extent in macrophages and this correlated with their significantly lower MPO contents.

Over the course of 28 days SWCNT were degraded to a much higher degree in w/t mice than MPO k/o. This reiterated the fact that MPO is somehow involved in the degradation of the SWCNT but not exclusively, as up to 7 days post exposure the MPO k/o and w/t mice showed a similar I_D/I_G ratio that then reached a plateau in the MPO k/o. Experiments carried out by our collaborators found that following pharyngeal aspiration, SWCNT induced an inflammatory responses in both w/t and MPOk/o mice as evidenced by increased production of pro-inflammatory cytokines (TNF- α , IL-6 and MCP-1) 1 day after the exposure. An increase in the numbers of PMNs present in BAL was also observed which were slightly weaker in MPO k/o mice *vs* w/t animals (Shvedova, O. Kapralova et al. 2012). The increase in PMN response in the w/t mice ties in with the increased SWCNT degradation observed.

Overall Raman spectroscopy proved to be an objective, non-invasive, label free method of locating, imaging and measuring the integrity of SWCNT within cells and tissues.

Chapter 4

4. Intracellular distribution and subcellular targeting of CdTe quantum dots

The work presented in this chapter has been published in part in Small (2008) 4 (11); 2006-2015, “*CdTe nanoparticles display tropism to core histones and histone rich cell organelles*”. The full paper is included in Appendix 3 of this thesis.

4.1. Introduction

Imaging techniques for AgNW and SWCNT within biological setting were investigated and optimized in the previous chapters. An important underlying cellular mechanism following SWCNR or AgNW exposure was uncovered and a method for measuring SWCNT integrity was established.

Another key task in nanobiology is the elucidation of the mechanisms by which nanoparticles interact with specific intracellular targets which is explored in this chapter. Unmodified luminescent semiconductor nanoparticles or quantum dots (QDs) are capable of a strikingly rapid accumulation in the nuclei and nucleoli of living human cells, driven by processes of yet unknown nature. It is hypothesised that such a strong tropism of QDs could be mediated by the charge-related properties of the macromolecules presented in the nuclear compartments. The complex microenvironment encountered by QDs in the nuclei and nucleoli of live cells is primarily composed of proteins and other biopolymers such as DNA and RNA, and with this in mind, the human phagocytic cell line THP-1 was utilized in this work as a model system. Nuclear lysates, purified protein and nucleic acids solutions were also used to investigate the interaction of QDs with intranuclear macromolecules.

4.2. Aims and Objectives

A combination of technological approaches including live cell confocal microscopy, fluorescent lifetime imaging (FLIM), spectroscopic methods and zeta potential measurement were used to find the ultimate location of negatively charged QD within phagocytic cells was investigated.

The aims of this part of the study are:

- To image and establish the ultimate location of negatively charged QD within phagocytic cells.
- To investigate the dynamic modifications of QD properties following their interaction with the biological domains within the phagocytic cells using FLIM.

4.3. Materials and Methods

4.3.1. Cell culture

4.3.1.1. *Phagocytic cells*

Human monocytic leukemia THP-1 cells (American Type Culture Collection, USA) were maintained in RPMI 1640 (with Glutamax) (Gibco/BRL, Invitrogen, Carlsbad, CA) containing 10% FCS (Gibco/BRL) and 100 μg of penicillin/mL and 100 mg of streptomycin/mL (Sigma, P4333). Prior to treatment, cells were plated onto coverslips (13mm borosilicate glass; VWR International, West Chester, PA) in 8-well Lab-Teks, at a density of 0.5×10^5 cells/ml and differentiated with 100nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 72 hours. Just before confocal imaging, the cells were incubated for 30 minutes with the QDs (1) TGA capped CdTe and (2) cysteamine-capped CdTe, at a final concentration of 1×10^{-6} M (supplied by Dr, Stephen Byrne, TCD). The cells were then washed with warm (37°C) medium before imaging.

4.3.1.2. *Epithelial cells*

HEp-2, epithelial cells (adherent), Source Gift from EuroImmune (Freiburg, Germany) were cultured and maintained at 37°C, 5% CO₂ in minimum essential medium (Eagle) with Earles Salts (Sigma M5650), 10% foetal bovine serum; 2mM/L L-glutamine; 100 μg of penicillin/mL and 100 mg of streptomycin/mL (Sigma, P4333). Prior to treatment, cells were plated onto coverslips (13mm borosilicate glass; VWR International, West Chester, PA) in 8-well Lab-Teks at a concentration of 1×10^5 cells/mL and incubated for 48 hrs. Just before confocal imaging, the cells were incubated for 30 minutes with the TGA capped CdTe QDs at a final concentration of 1×10^{-6} M (supplied by Dr, Stephen Byrne, TCD). The cells were then washed with warm (37°C) medium before imaging.

4.3.2. QD preparation

Thioglycolic acid (TGA) capped CdTe and cysteamine-capped QDs were prepared according to published procedures (Byrne, Corr et al. 2006; Rogach, Franzl et al. 2007). QDs were stored at 4 °C in the dark and used as received but, before incubation with the cells, they were diluted in the appropriate warm (37 °C) medium to reach the desired final concentration of 1×10^{-6} M. Using the polynomial fit described by Yu et al, (Yu, Qu et al. 2003), the calculated diameter of the TGA capped and cysteamine capped QDs was 3.2 nm and 3.32 nm respectively.

4.3.3. DNA/RNA/Nuclear lysate preaparaion

Core histones (Medical Supply Co. Ireland) were diluted in water to 5 mg/mL stock solution following suppliers instructions. DNA and RNA were provided by Dr. Eugene Dempsey (TCD) and the nuclear lysate (~ 12 mg/mL) was provided by Dr. Stephen McDonald (TCD).

4.3.4. Confocal microscopy

Confocal images were collected using a 100x oil immersion objective on a Nikon TE 300 microscope using an Ultra View Live Cell Imager confocal microscopy workstation (Perkin Elmer Life Sciences). The laser excitation used was 488nm with a broad emission filter at 525nm.

4.3.5. Fluorescent lifetime imaging

Fluorescence lifetime images were collected with the FLIM system (Microtime200 time-resolved confocal microscope system, PicoQuant) equipped with Olympus IX71 inverted microscope (40x dry objective). The samples were excited by 480 nm picosecond pulses generated by a PicoQuant, LDH-480 laser head controlled by a PDL-800B driver.

The setup was operated at a 20-MHz repetition rate with an overall time resolution of ~ 350 psec.

4.3.6. Modified dot-blotting

Evaluation of the QDs interactions with bovine serum albumin (BSA), DNA, RNA, core histones and nuclear lysates was carried out by a modified dot-blot technique on nitrocellulose membranes. 2 μ l of each BSA, RNA, DNA and core histones at a concentration of 5 mg/mL were bound to the nitrocellulose along with 2 μ l of nuclear lysate at ~ 12mg/mL. The membrane strips with bound biopolymers were flooded with QDs and H₂O mixture (4×10^{-6} M) and allowed to incubate for ~ 45 min at 37°C, 5 % CO₂ (to mimic the conditions close to the intercellular environment). The nitrocellulose and QDs were then washed vigorously 3 times with de-ionised water and then UV images were collected using the trans-illuminator.

4.3.7. Absorption and photoluminescent measurements

The absorption and the photoluminescence (PL) spectra were recorded using a Cary 50 Conc. UV/Vis spectrophotometer and a Cary eclipse fluorescence spectrometer respectively with an excitation of 450 nm. The samples were placed in quartz cuvettes for measurement. Spectra of QDs alone was taken with 3 mL of water and 20 μ l of QD, with the absorbance at ~0.2. All histone and BSA solutions were made up to 5 mg/mL and added to the QD-water solution 5 μ l at a time.

4.3.8. Zeta potential measurements

Zeta potential and size measurements of the QDs were carried out using a Zetasizer, Model Nano (Malvern Instruments). Size and charge measurements were taken of all the solutions from section 4.3.7.

4.4. Results

4.4.1. Confocal imaging confirm nuclear localization of intracellular QD

As demonstrated in our previous study, small green-emitting negatively charged CdTe QD are capable of rapid nuclear accumulation in cells of a phagocytic nature (Nabiev, Mitchell et al. 2007). Figure 55 (A) shows the accumulation of 3.2 nm sized TGA capped CdTe QD (negatively charged) in the nucleus and nucleoli of the THP1 cells following 30 min incubation. Figure 55(B) shows the predominant membrane-associated fluorescence pattern and discrete cytoplasmic aggregates of the 3.2 nm sized cysteamine capped CdTe QD (positively charged) also following a 30 minutes incubation. However, no nuclear or nucleoli accumulation was observed. Experiments with the cysteamine-capped QD effectively rule out the size of particles as the reason for the different ultimate locations of these QD within the THP1 cells. It was thought that perhaps the surface charge of the QD might dictate firstly, the mechanism by which they were taken into the cell and secondly their ultimate destination within the cell. As seen in Figure 55 (A), the QD clearly highlight the nuclear area and two characteristic areas of increased fluorescence intensity corresponding to the nucleoli. It has been suggested that such nucleolar tropism of negatively charged QD is mediated by molecular interactions with positively charged histones. Another example of this can be seen in Figure 56 where green CdTe 2.5 nm QD localize in the nucleus and nucleoli of the THP-1 cells, whereas bigger red QD remain in the cytoplasmic region. However, the tropism to the nuclear region was considered, as size effects have been addressed earlier (Nabiev, Mitchell et al. 2007).

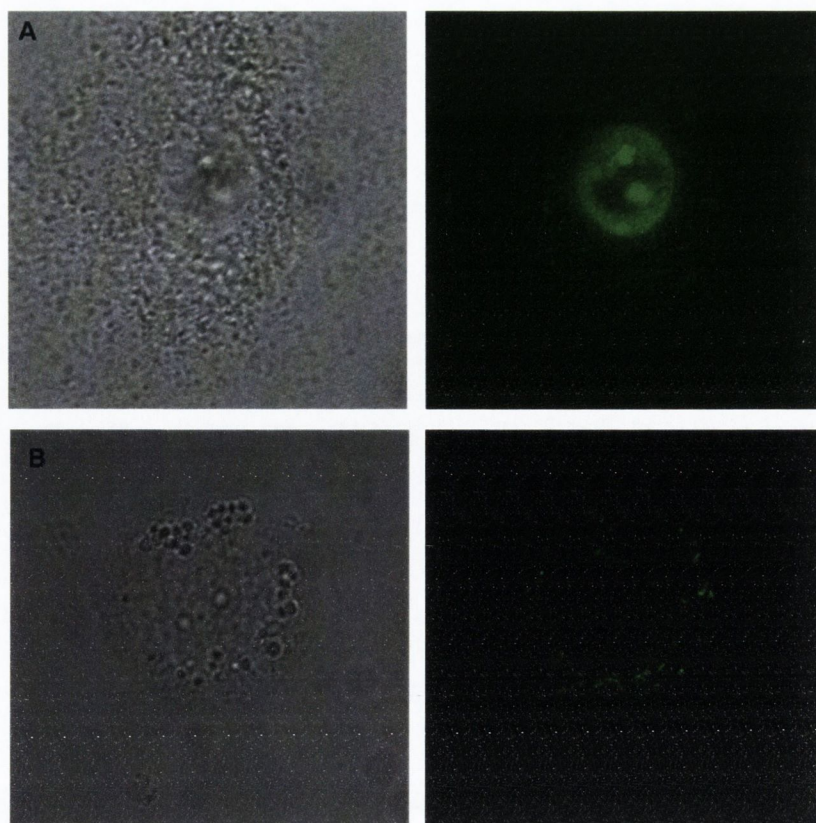


Figure 55. Transmitted light and single plane (middle part of the nucleus level) confocal image of the THP1 cell after 30 min exposure to (A) TGA capped (negatively charged) CdTe QDs. The confocal image was taken using 488 nm excitation and a long band-pass emission filter (525 nm), the green colour shown here is a pseudo colour applied after collection and does not reflect the true emission wavelength (546 nm) of the QDs. (B) Cysteamine capped (positively charged) CdTe QDs – this confocal image was taken using 488 nm excitation and a long bandpass filter (505 nm) again the green colour shown here is also a pseudo colour applied after collection and does not reflect the true emission wavelength (558 nm) of the QDs. Different uptake patterns between the negatively and positively charged QDs can be seen.

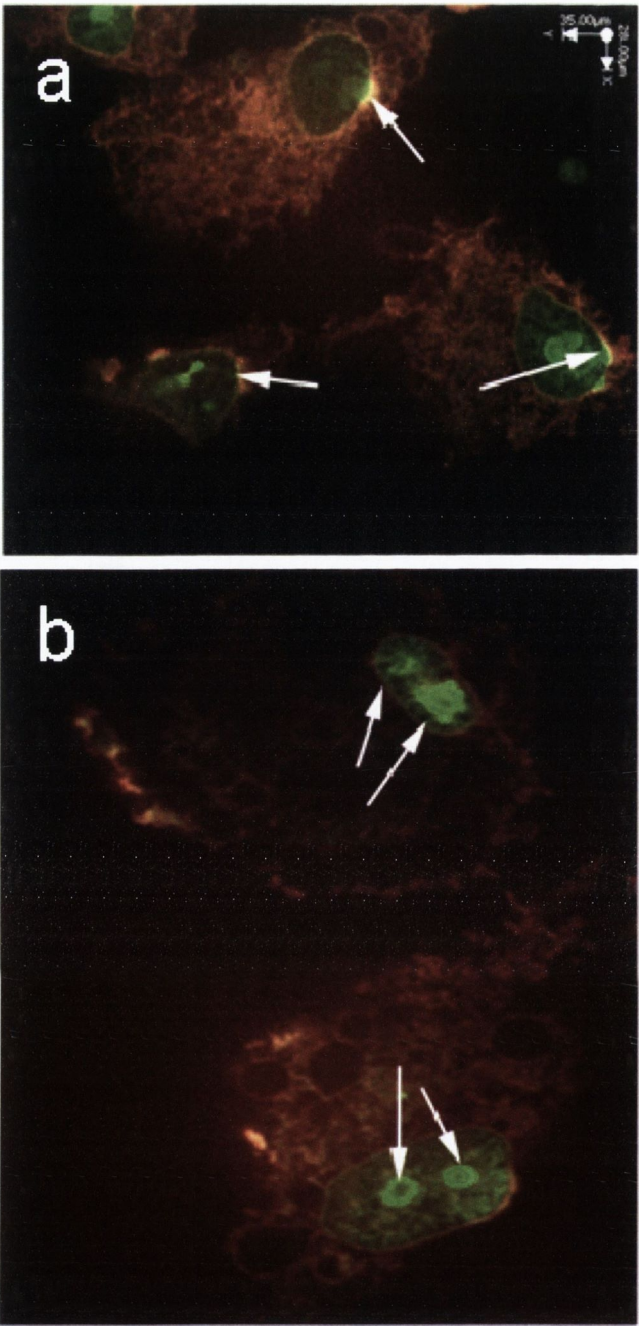


Figure 56 Intracellular compartmentalization of green and red CdTe QDs in THP-1 macrophage cells. Similar to Figure55 (a) and (b) illustrate (white arrows) how the green 2.5 nm QDs localize in the nucleus and nucleoli of the cells, whereas the red QDs remain in the cytoplasmic region.

4.4.2. FLIM revealed reduced lifetime of nucleus-targeting QDs

If these interactions between QDs and histones take place, they are likely to have a distinctive effect on the physio-chemical properties of the QDs, affecting their intrinsic photo-luminescence characteristics. This hypothesis was first addressed by implementing whole cell fluorescent lifetime imaging (FLIM). Each pixel in the FLIM image gives the fluorescent lifetime at a particular position in space (x, y), while monitoring the entire PL spectrum. Lifetime-based contrast is valuable because the lifetimes of probes often depend on their local environment. Figure 57 shows the FLIM images taken at a single focal plane of THP1 cells at the level of the nucleus with (B) and without (A) exposure to QDs. The THP 1 cell without the QDs shows an even distribution of auto-fluorescence lifetimes across the entire cell with the lifetime ranging from 1.5 ns to 6 ns. However, the FLIM image of the THP 1 cell with QDs clearly shows a strong contrast between the QDs lifetimes depending on their location within the cell. The QDs detected in close proximity to the cell membrane have a longer lifetime compared to QDs which were internalised and confined to endosomal compartments, or to QDs which were located in the nuclear (nucleoli) area.

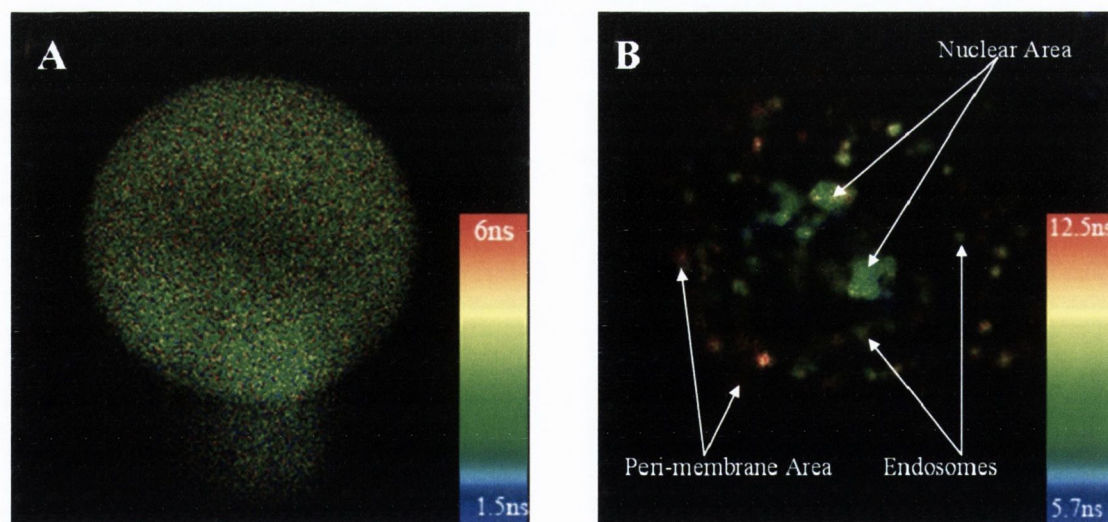


Figure 57 FLIM images taken in a single focal plane (nucleus level) of (A) THP1 cell without QDs and exhibiting an even distribution of PL lifetime and (B) THP1 cells incubated with CdTe QDs showing a variation in PL lifetime within different parts of the cell. The image was collected at 250×250 pixel resolution with 4096 time channels; 2 ms acquisition time per pixel and a total recording time of 6.63 min. Image size: $39.50 \mu\text{m} \times 39.50 \mu\text{m}$.

For comparison, FLIM imaging of Hep-2 epithelial cells following incubation with CdTe TGA capped QDs was also carried out. In keeping with the previous results from the THP-1 cells, the cells alone showed a low level of auto-fluorescence photoluminescence (Figure 58 (A)). The cells treated with QDs displayed lifetimes that were more distinct and localized depending on the location of the QDs within the cells (Figure 58 (B)). The different lifetimes observed here allow for cell barriers, cytoplasmic regions, nuclear regions and nucleoli to be easily distinguished in a label free manner. In both cell lines this variation in lifetime is due to the interaction of the QDs with different macromolecules with the cells.

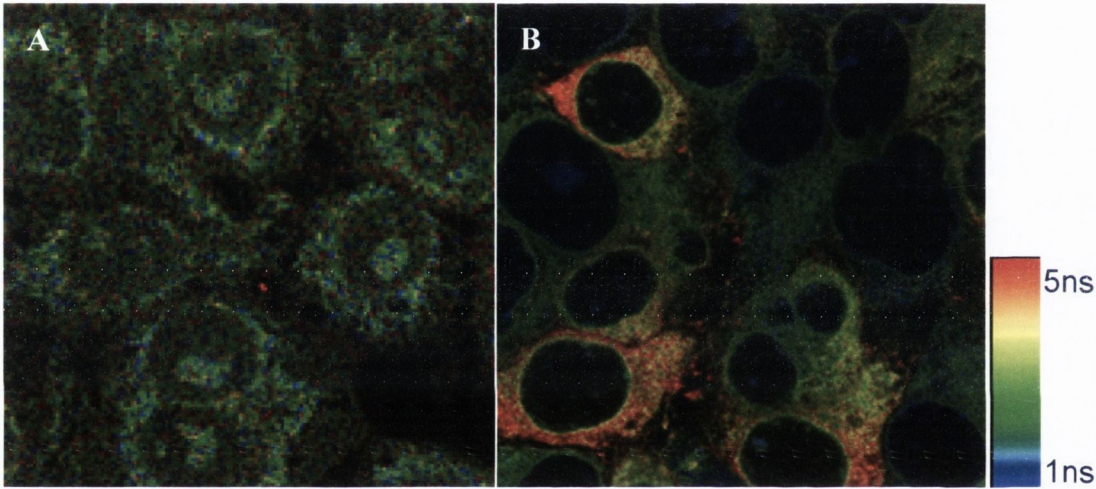


Figure 58 Fluorescent lifetime (FLIM) images of epithelial cells (A) alone, illustrating a low level of autofluorescence from the cells and (B) following exposure to CdSe QDs where the PL of the QDs is reduced in the nuclear compartment compared to the cytoplasmic region.

However, as a whole, the PL decay associated with the QDs is significantly longer than auto-fluorescence (Figure 57), but is shorter than the PL lifetime of the original aqueous solution of QDs (Figure 65, 16-27 ns). To compare these differing decays we calculated the average PL times (τ_{av}) that describe the mean time taken for a photon to be emitted:

$$\tau_{av} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i}$$

The partial factors α_i and partial lifetimes τ_i were estimated from a fit of i-exponential function of the form $IPL(t) \propto \sum \alpha_i \exp(-t/\tau_i)$ to the experimental PL decay curves (Figure 59;Table 5). Here IPL is PL intensity, t denotes time and α_i represents a weighting of the various decay time components τ_i . Strikingly, the average lifetime associated with the PL of the CdTe QDs is almost three times longer than the lifetime of the auto-fluorescence and measurable luminescence from the QDs and can be detected up to 100 ns after the pump pulse (Figure 59). Among other factors, this long lifetime demonstrates the benefits of using QDs as intracellular imaging agents to facilitate the discrimination between labeled and label-free organelles (or intrinsic cellular components).

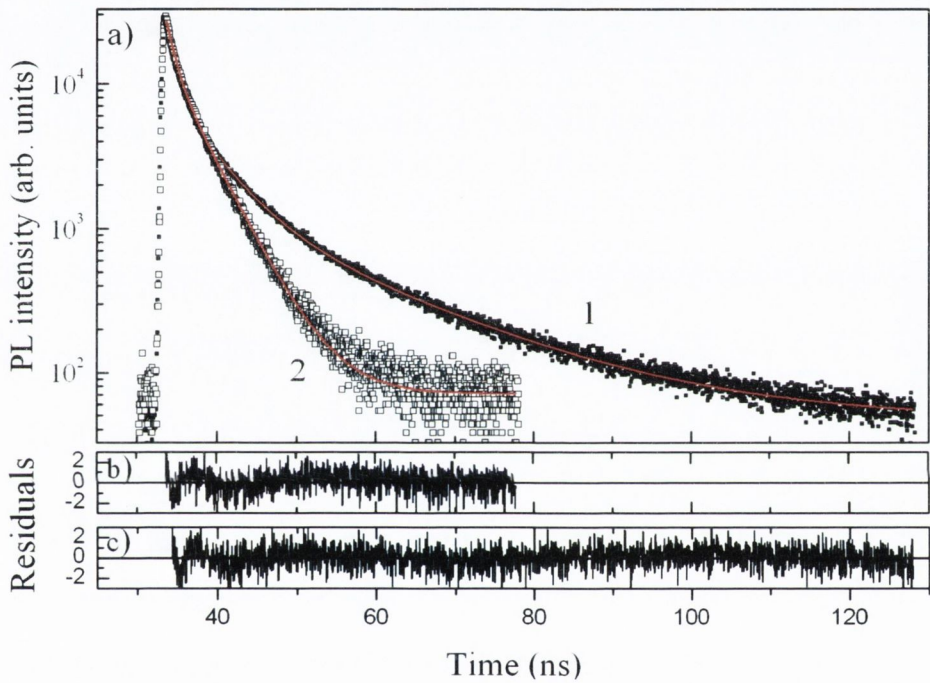


Figure 59. Time-dependent PL intensity decays for THP 1 cells with (1) and without (2) QDs (a). Results of multi-exponential analysis of decay curves are shown by thick lines, (b) and (c) corresponding residuals.

Table 5 Multi-exponential fit parameters to the observed PL decay of THP 1 cells with and without QDs

Imaging	τ_1 (ns)	α_1	τ_2 (ns)	α_2 (ns)	τ_3 (ns)	α_3	τ_{av} (ns)
Without QDs	0.976	0.49	3.996	0.51	-	-	3.41
With QDs	1.115	0.51	4.653	0.41	17.725	0.08	9.23

A further feature of QDs PL decay dynamics, which is clearly seen in Table 5, is the multiexponential decay kinetics. The sum of at least three exponential functions is required

to achieve a satisfactory fit to the QDs PL decay data yielding a reasonable plot of weighted residuals and χ^2 value of 1.1 (Figure 59;

Table 5 Multi-exponential fit parameters to the observed PL decay of THP 1 cells with and without QDs

). The observed multiexponential decay kinetic reflects fluctuations in nonradiative relaxation pathways (Fisher, Eisler et al. 2003) and is indicative of a broad lifetime distribution caused by the diverse intracellular micro-environments. It has been shown that PL decays can be best understood by a model of continuous distributions of decay times when fluorophores (QDs in this case) are embedded in non-uniform environments (Eftink 1991). In this case, fitting procedures cannot distinguish sufficiently between, for example, a single Gaussian distribution of lifetimes and the sum of two exponentials, or a bimodal Gaussian distribution and the sum of at least three exponentials (James, Liu et al. 1985; Demas and DeGraff 1993). Thus, a description based on discrete lifetime components should only be regarded as truly representing discrete molecular states if supported by supplementary data (Carraway and Demas 1991

). Therefore, to gain a better insight into the spatial distribution of the lifetimes, the PL dynamics were evaluated from PL lifetime histograms (Figure 60, Figure 62). The histograms were obtained from multi-exponential fitting procedure carried out for each pixel of the scan.

The lifetime distribution recorded for THP 1 cells without QDs (Figure 60 (a)) revealed pronounced heterogeneity of the auto-fluorescence lifetimes with an average value of 3.9 ns (Peak full-width-at-half-maximum (FWHM) = 1.9 ns). In contrast, the decay time distribution analysis shows that the CdTe QDs luminescence decay kinetics involve processes with two distinctive groups of lifetimes (Figure 60 b and c) with the maxima values depending on the cellular compartments where the QDs are localized. In the nuclear area (Figure 57, nuclear area) the histogram shows lifetime distributions that consist of two maxima centered at 2.5 and 8.5 ns with FWHM values equal to 2.5 and 4.0 ns, respectively. Clearly, the short-lived component strongly overlaps with the auto-fluorescence signal. Analysis of the QDs lifetime map in the region of the cell membrane reveals a pronounced shift of the long-lived component indicating an increase in PL lifetime (Figure 60 c). This component is centered now at 11 ns with FWHM value of 5.5 ns.

A FLIM image of a smaller area of the Hep-2 cells was acquired (Figure 61) from which PL histograms were generated (Figure 62). A similar pattern of lifetime distributions is seen in the Hep-2 cells (Figure 62) where the average PL lifetime in the nuclear region (A) is ~ 2 ns and ~ 5 ns in the cytoplasmic region (B). There is a broader distribution of PL lifetimes in the cytoplasmic region whereas in the nuclear region it is a narrow discrete distribution. This would suggest that the QDs are interacting with specific macromolecules in the nucleus as apposed to a broader range in the cytoplasm.

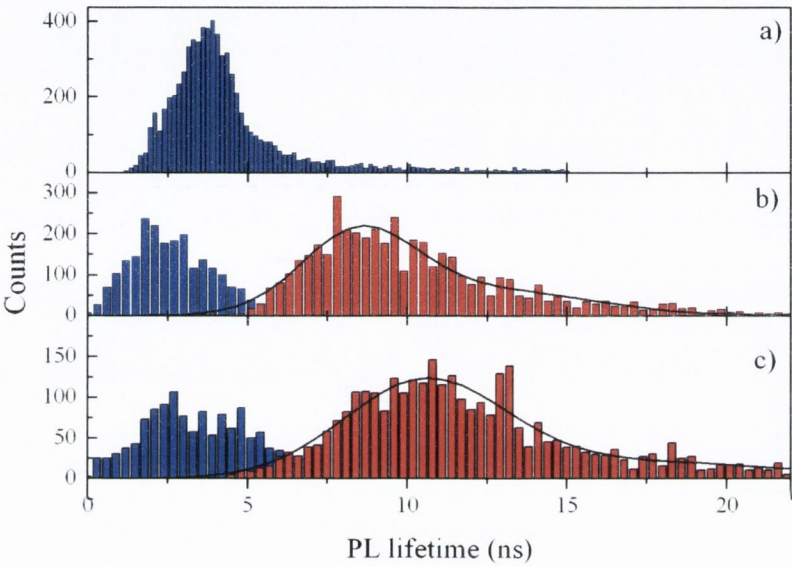


Figure 60. Lifetime histograms obtained from FLIM images of THP 1 cells without QDs (a) and with QDs (b and c). The lifetime distribution recorded for THP 1 cells without QDs (a) shows pronounced heterogeneity of the auto-fluorescence lifetimes with an average value of 3.9 ns. The envelope curve in (b) and (c) is presented as guide for eye. Here two distinctive groups of lifetimes are shown with their maxima values depending on the cellular compartments where the QDs are localized. The histograms in b, are those that correspond to the lifetimes in the nuclear area and the histograms in c, are those corresponding to the perimembrane area (see Figure 57). In both cases it is a significant reduction in lifetime compared to that of the QDs in water, 16-27 ns (Figure 65), however those in the nuclear region show the greatest reduction in lifetime (b).

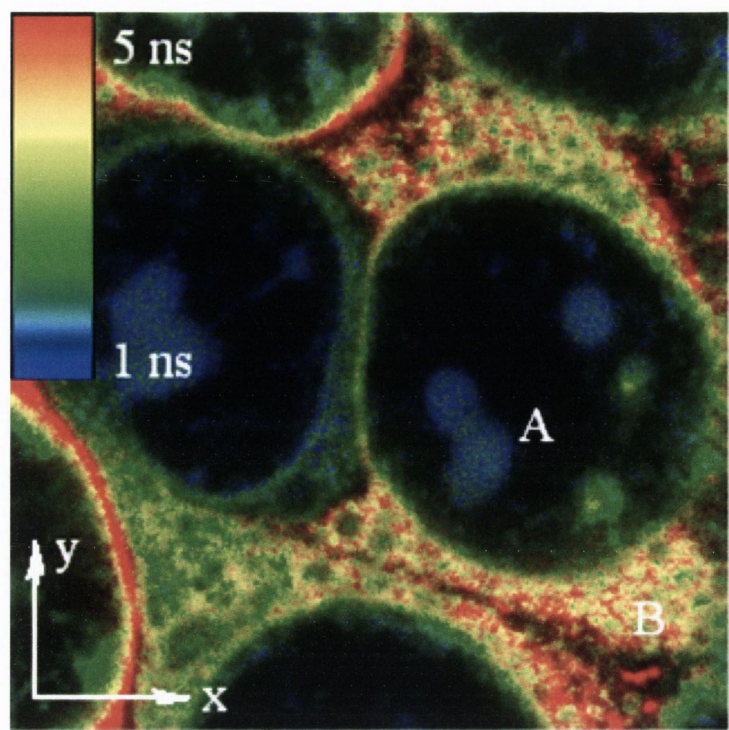


Figure 61 Fluorescent lifetime (FLIM) image of epithelial cells following exposure to CdSe QDs (A) nuclear region and (B) cytoplasmic region. A FLIM image is generated based on the photoluminescent lifetime (PL) of the fluorophore signal and not the intensity. Localization of the QDs within the different cell compartments dramatically affects the QDs' photoluminescence lifetimes, which is due to the interaction of the QDs with the intercellular macromolecules. Clearly the PL of the QDs is reduced in the nuclear compartment compared to the cytoplasmic region.

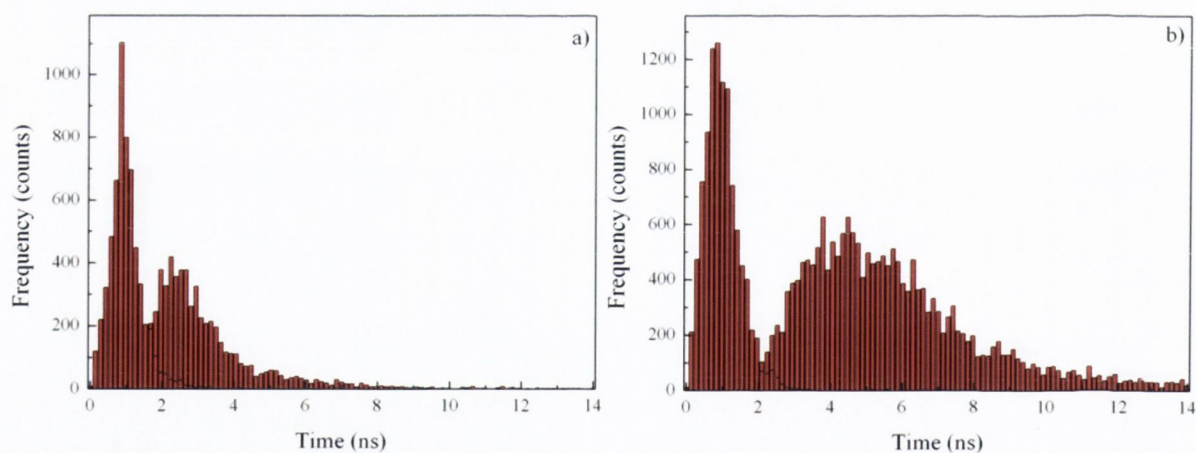


Figure 62 PL lifetime histograms obtained from regions A (a) and B (b) of Figure 61. The average lifetime from (a) the nuclear region is discrete and is around 2.5 ns whereas the average lifetime from (b) the cytoplasmic region is about 5 ns and has a broad distribution of PL lifetimes.

One of the universal characteristics of QDs is that they exhibit a bi-exponential decay radiative lifetime (Wang, Qu et al. 2003). The shorter lifetime is usually around several nanoseconds and can be attributed to the intrinsic recombination of initially populated core states, (Bawendi, Carroll et al. 1991) whereas the longer lifetime is usually around tens of nanoseconds with its origin still in question. However, recent studies by Wang et al (2003) suggest the involvement of the surface states in the recombination process in colloidal QDs (Wang, Qu et al. 2003). In FLIM the image contrast is independent of the local PL intensity instead it is based on the fluorescence lifetime in each region of the cell.

The possible reason for the QDs lifetime reduction observed in the nucleus and nucleoli could be due to aggregation of the QDs, mediated by the binding of the QDs to biopolymers within the nuclear and nucleolar compartments. The main mechanism of PL lifetime reduction in this case would most probably be related to a transfer of electrons from the photoexcited semiconductor nanoparticle to the cation bound at the surface of intranuclear organelles, which involves a chemical reduction of the cationic groups. The fact that the most pronounced modification has been observed for the surface associated component of the life-time distribution (with short-lived component intact) provides strong support for creditability to this mechanism. This hypothesis is further investigated here with the focus on the biopolymers present in abundance in the nuclear compartment.

4.4.3. Densitometric evaluation of direct QD-histone binding

Confocal microscopy (Figure 55 (A)) demonstrated that the CdTe, TGA capped negative QDs utilised in this study enter the nucleus and nucleoli of the THP1 cells. From the FLIM data obtained, the lifetimes of these QDs depends greatly on their cellular compartmentalisation. The question of whether the interaction of QDs with individual biopolymers that occur in abundance in the nucleus could be reproduced in a direct *in vitro* binding system is therefore addressed. It is demonstrated here that QDs preferentially bind to core histones over other nuclear macromolecules.

DNA, RNA, core histones, BSA and nuclear lysate interaction with QDs was performed by a modified dot-blot technique, as described in Methods. BSA has been shown to increase the luminescence and have a stabilising effect on QDs (Wang, Kuo et al. 2006) so it was used here as a control. Figure 63 B shows clearly that the QDs bind only to core histones and the nuclear lysate. No binding of the QDs to BSA, RNA or DNA was observed.

This binding of the negatively charged QDs to the core histones could be attributed to the fact that the core histones are approximately 30-40 % positively charged due to the presence of the amino acids lysine and arginine on their N-termini, (Hansen, Tse et al. 1998) whereas the DNA and RNA are negatively charged, (Draper 2004) resulting in a net negative force between them and the QDs. The nuclear lysate also shows strong binding of the QDs, with the intensity even exceeding the purified core histone proteins. However, since the total estimated protein load in the nuclear lysate (~12 mg/mL) was significantly higher than the concentration of the purified core histone protein solution used (5 mg/mL), a direct quantitative comparison may not be applicable in this case. In addition, the presence of some other macromolecule(s) with similar properties in the whole nuclear lysate, or any changes in histone-QDs affinity due to the protein purification steps, or a combination of both of these factors can potentially contribute to this phenomenon.

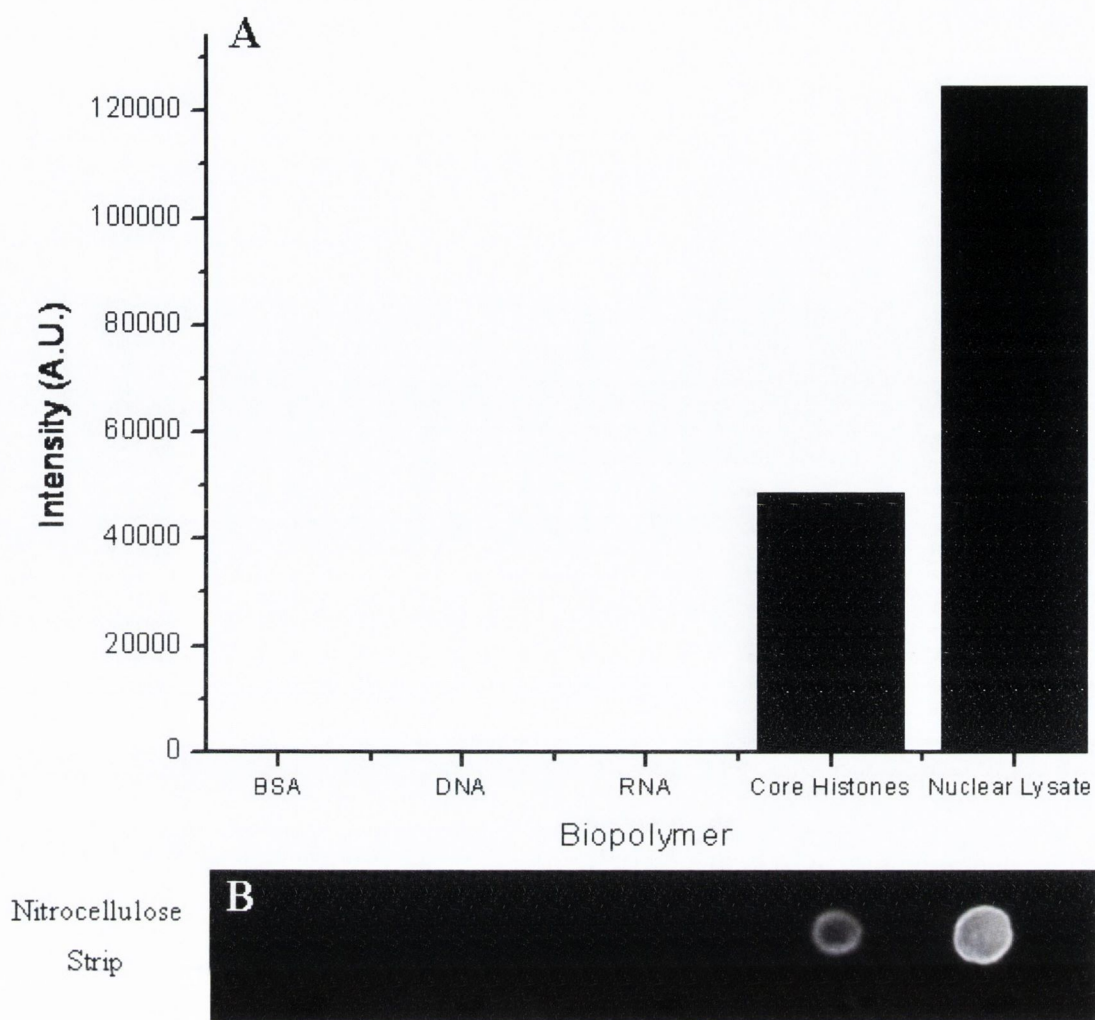


Figure 63 (A) Densitometry results obtained for the QDs bound to the biopolymers on the nitrocellulose demonstrate no detectable binding of the QDs to the BSA, RNA or DNA but strong interaction with the core histones and nuclear lysate. Note that all the tested solutions were equilibrated by the relevant purified macromolecule concentration (5 mg/mL) whereas the nuclear lysate contained ~12 mg/mL total detectable protein. (B) Shows the UV image taken of the Nitrocellulose with QDs clearly detectable only where the core histones and nuclear lysate were bound.

4.4.4. UV-Vis spectra illustrate red-shift following QD-histone interaction

The absorption and emission spectra of the QDs in water and with varying concentrations of core histones, BSA and foetal calf serum (FCS) were recorded. Figure 64 (A – B) shows the absorption and PL spectra obtained for QDs mixed with increasing amounts of FCS and BSA, the original mixture was 2mL of water + 10 μ L of QD (4×10^{-4} M) and the proteins were added in steps of 5 μ L (stock concentration of 5 mg/mL). In both cases the absorbance peak remains in the same position ~ 522 nm, even with increasing amounts of FCS or BSA.

The intensity of the QDs PL spectra upon mixing with both the FCS and BSA show dramatic increases in intensity with increasing amounts of each protein. In both cases the spectra appear to reach a plateau and the intensity remains the same even after further additions of proteins (up to 0.125 mg/mL). This enhancement in luminescence emission of the QDs is due to the interaction of the BSA and FCS molecules with the surface of the QDs and their capping of remaining surface defect states this reduces the amount of non-radiative decay channels and thus increases the PL efficiency. Mattoussi et al (Mattoussi, Mauro et al. 2000) report on the enhancement of the PL of CdSe-ZnS QDs upon bio-conjugation with MBP-zb. They suggest that the proteins interact with the alkyl-COOH capped QDs in solution and that these interactions alter the electrostatic/polar environment of the inorganic core, thus affecting the efficiency of the core electron-hole recombination and leading to the observed increase in PL intensity. Mamedova et al (Mamedova, Kotov et al. 2001) prepared BSA-CdTe conjugates using a glutaric dialdehyde (G) linker. They also found an increase in PL and attribute it to the resonance energy transfer from the tryptophan moieties of the albumin to the CdTe nanoparticles. Kumar et al (Kumar, Mital et al. 2001) observed an increase in the PL intensity of adenine capped CdS QDs by about threefold.

The absorbance and PL spectra recorded for the QDs mixed with increasing amounts of core histones are shown in Figure 64(C). The peak absorbance of the QDs remains at ~ 522 nm, as shown previously. However, there is a dramatic shift off the baseline even after the very first addition of the core histones. This would indicate that

there is a change occurring within the QD ensemble due to the interaction of the QDs with the core histones. The PL spectra of the QDs mixed with the core histones is also significantly different from those obtained from the QDs mixed with BSA and FCS. A red shift from 546 nm to 557 nm and reduction in PL was also observed. Initially there is a small increase in the PL intensity and then a dramatic reduction with an eventual plateau. Significantly, this reinforces the very different interactions the TGA-capped QDs have when mixed with the core histones compared to the other proteins. A study by Yaroslavov *et al*, (Yaroslavov, Sinani et al. 2005) reports the interaction of negatively charged CdTe QDs with positively charged poly (*N*-ethyl-4-vinylpyridinium bromide-co-4-vinylpyridine), PEVP. They found that the addition of the PEVP in increasing amounts to a solution containing the QDs resulted in a decrease in the fluorescence that eventually stabilised. A similar observation is made here where the addition of positive core histones to the negative QDs in water resulting in a red PL shift and corresponding decrease in the PL intensity. They suggest that the observed quenching is due to the electrostatic interactions of the positive PEVP with the negative QDs resulting in electron transfer (Yaroslavov, Sinani et al. 2005). This could possibly explain the quenching and the red shift observed here. On the other hand, the luminescence quenching accompanied by a red shift of the PL band may be attributed to short range dipole-dipole interaction in QD assemblies. This phenomenon has been reported for films of close-packed QDs of different sizes (Kagan, Murray et al. 1996; Micic, Jones et al. 1998; Crooker, Hollingsworth et al. 2002). In the case presented here, formation of these assemblies is mediated by the positive core histones *via* an of electrostatic attraction.

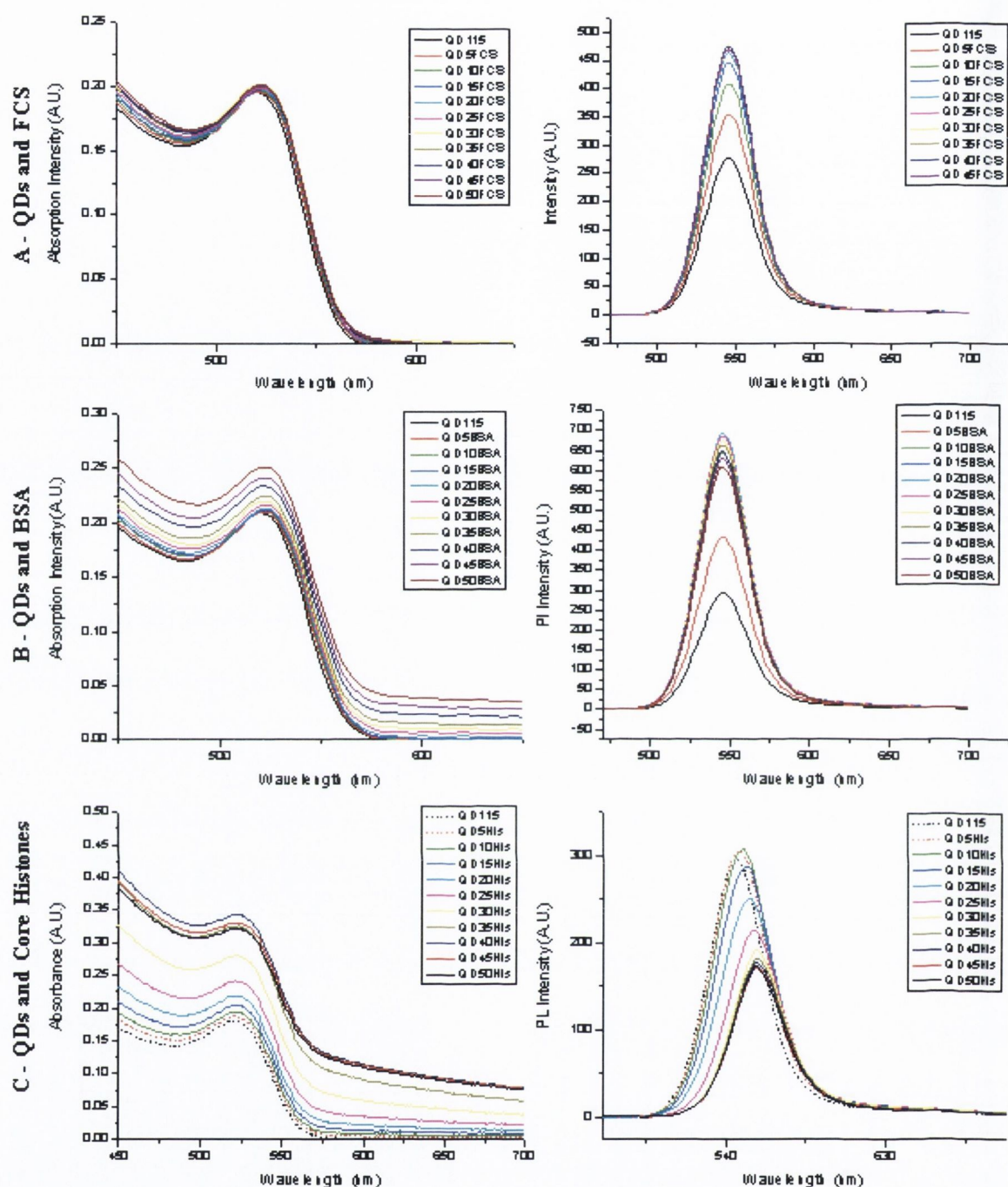


Figure 64 (A-C). Absorption and PL spectra of CdTe TGA capped QDs when exposed to various biopolymers. There is no significant shift off the absorption baseline and an enhancement of PL for both QDs in FCS and BSA. In contrast there is a dramatic shift off the absorption baseline after the first addition of core histones to the QDs in water and a dramatic decrease in PL intensity accompanied by a red shift.

4.4.5. QD charge shifts from negative to positive following histone interaction

Two main observations were noted from the spectral data, firstly the absorption values of the QDs when mixed with histones were shifted significantly from the baseline, indicating a change to the surface properties of the QDs or more likely enhanced light scattering by QD-histone nanocomposites. Secondly, a red shift and a considerable reduction in intensity were observed for the PL spectra of the QDs and histones, indicating aggregation and quenching of the sample. Thus, by measuring the size and charge of the QDs before and after the addition of increasing amounts of the proteins, we intended to corroborate our spectroscopic findings with physical net size and charge measurements. The BSA and core histones used have net negative and positive charges respectively (Hansen, Tse et al. 1998; Lee, Chen et al. 2007). When mixed with water alone the QDs measured 3.4 nm in hydrodynamic diameter and had a charge of -28.9 mV, which is the expected average size and charge of these negative TGA capped QDs. After the addition of 10 μ L of BSA (5 mg/mL) to give a final concentration of 0.025 mg/mL, there was a shift in their size to 12 nm and a corresponding change in their net charge to - 20 mV. A significant increase in size (181 nm) was observed when the BSA concentration was brought to 0.125 mg/mL with a shift in the charge now only - 8 mV. The first addition of the core histones (10 μ L – final concentration 0.025 mg/mL) to the QD and water mixture resulted in a different outcome to that of the BSA. There was a significant increase in size from 4 nm to 150 nm and the charge was - 18 mV. However, the most striking measurements occurred when the core histone concentration was brought to 0.125 mg/mL, the size jumped to 225 nm and the charge shifted from negative values to positive to + 15mV, demonstrating the effect which the positively charged core histones are having on the negative QDs.

BSA is a relatively large (5.5 nm x 5.5 nm x 9 nm) (Patil, Sandberg et al. 2007) molecule compared to that of the QDs and the iso-electric point for BSA is pH 4.78, above this point it is considered to be negative (Patil, Sandberg et al. 2007). Our experiments were carried out in water (pH 7) so we can consider the BSA to be negatively charged (Brewer, Glomm et al. 2005). However, BSA is not uniformly charged, there are three

domains: domain I is -11, domain II is -7 and domain III is +1 (Scharbin, Janicka et al. 2007). Therefore we cannot rule out the possibility that some of the negative QDs in solution are interacting with some of the positive moieties on the BSA surface, leading to a higher positive Zeta potential.

Table 6 Charge and size results recorded for CdTe QDs when mixed with water and various concentrations of BSA and core histones using the Zetasiser Nano. This table represents the average size and charge of the QDs measured in solution using a DLS system.

Sample (mg/mL)	Size (nm)	Charge (mV)
0.00 protein	3.4	-28.9
0.025 BSA	5.9	-20.7
0.125 BSA	181	-7.13
0.025 C.His	149	-17.9
0.125 C.His	226	+15

4.4.6. FLIM confirms reduction in QD lifetime following histone interaction

As the FLIM method proved to be a very sensitive technique for characterising QDs interactions with nuclear compartments in whole live cells, this approach was also used for studies of direct interaction of QDs and biopolymers *ex-vivo*. Figure 65 shows the fluorescent lifetime image obtained for the QDs in water. A FLIM map is produced within a 16-27 ns lifetime window showing an even distribution of nanoparticles. Once again, two maxima in the lifetime histogram are present: one centered at 6.6 ns and a second long-lived component detected at 22.8 ns. Figure 66 (a, c, e) shows the fluorescent lifetime images of the QDs with increasing amounts of BSA (0.025, 0.075, 0.125 mg/mL). There is no change in the lifetime decay distribution which implies that the negatively charged BSA has no effect on the stability of the QDs in solution. However, both short-lived and long-lived components are now shifted to the longer lifetimes (9 ns and 24 ns respectively) in comparison with the aqueous solution of QDs. These experimental findings indicate partial removal of the nonradiative recombination channels due to the improvement of QDs surface passivation in agreement with the observed dramatic increase in the steady state PL intensity (Figure 64 B). Figure 66(g, i, k)) shows the FLIM images observed when incremental increasing concentrations of core histones were added (0.025, 0.075, 0.125 mg/mL). Even a small amount of the core histones caused a reduction of the mean lifetimes of both components (5 ns and 20.5 ns respectively) as is shown in Figure 66g, indicating aggregation mediated by the presence of the positive histones. An enhanced aggregation of QDs (Figure 66 i,j) caused by the increased concentration of core histones is accompanied by further decreases in lifetimes, once again in agreement with the strong steady-state PL quenching (Figure 64). It should be noted that the presence of big clusters of QDs stabilises the photoluminescent decays: both short-lived (4 ns) and long-lived (17 ns) components of lifetime distributions remain the same in Figure 9i and Figure 9j. This fact taken with saturation of steady-state PL quenching (Figure 64) is indicative of screening of the electrostatic interaction and stabilisation of the electron transfer rate at the provided concentrations of core histones.

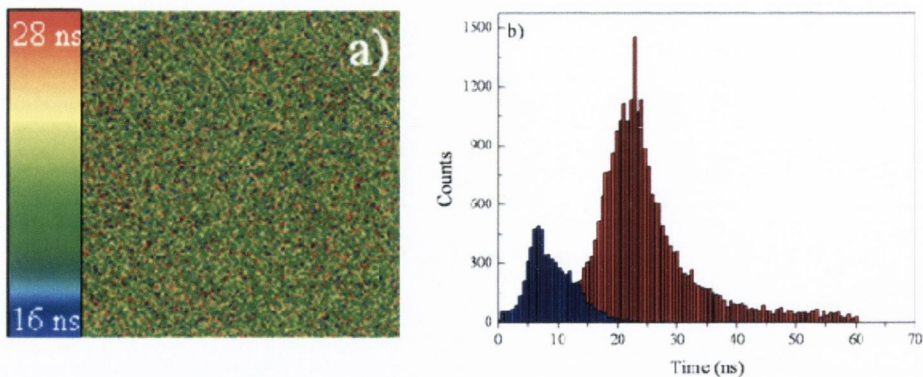


Figure 65. Fluorescent lifetime image (a) and PL lifetime (b) histograms obtained for the QDs in water. The lifetime distribution ranges from 16-27 ns and the image shows an even distribution with no aggregates which would imply that the QDs are distributed evenly with their surface and core intact.

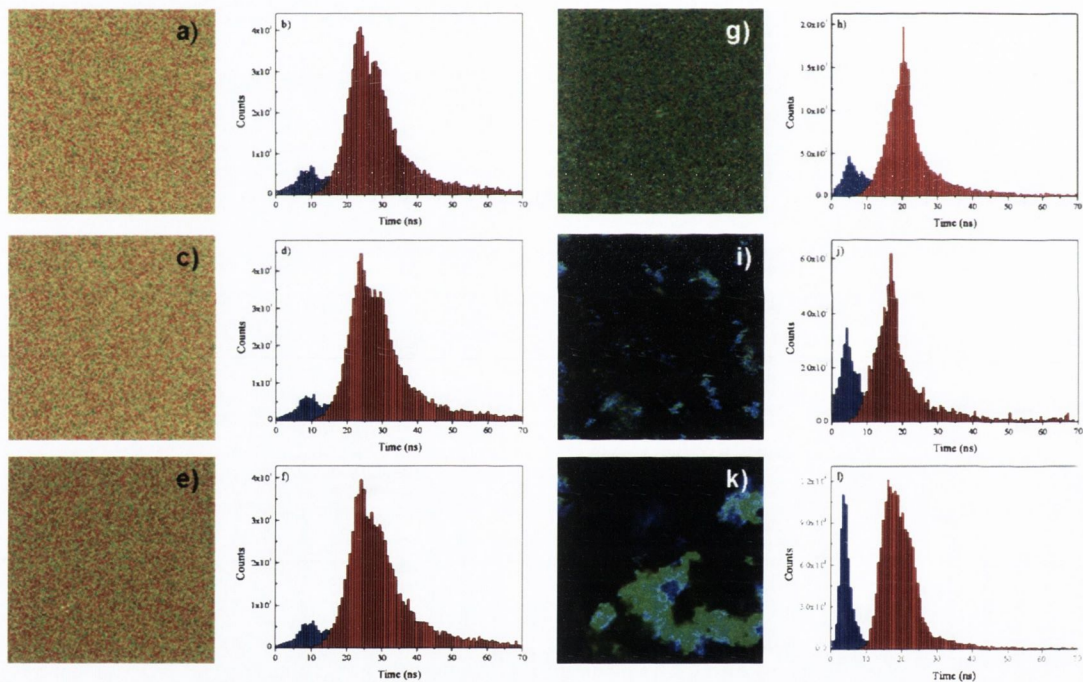


Figure 66. FLIM images of (a, c, e) QDs with increasing amounts of BSA (0.025, 0.075, 0.125 mg/mL) and their corresponding PL lifetime histograms (b, d, f) and (g, i, k) QDs with increasing amounts of core histones (0.025, 0.075, 0.125 mg/mL) along with their corresponding PL lifetime histograms (h, j, l). This colour map is the same as presented in Figure 8.

4.5. Discussion

In this study, the first detailed investigation of the dynamic modification of QD characteristics on their contact with selected biopolymers in live cells and cell-free systems, was performed. The targeting of negatively charged QDs to their ultimate nuclear and nucleolar destinations dramatically affects the QDs photoluminescence lifetimes. It was possible to demonstrate that unmodified CdTe QDs preferentially bind to the positively charged core histone proteins as opposed to DNA or RNA, by using a combination of advanced technological approaches including live cell confocal microscopy, fluorescent lifetime imaging (FLIM), spectroscopic methods and zeta potential measurements. When CdTe QDs bind to the charged core histone, there is a concurrent dramatic shift off the absorption band, and a red-shift and decrease in the photoluminescence (PL) intensity of the QDs. FLIM imaging of the QDs has demonstrated an increased formation of QD-protein aggregates in the presence of core histones, with a resulting significant reduction in the PL lifetime. FLIM technology for the first time enabled to reveal that the localization of negatively charged QDs to their ultimate nuclear and nucleolar destinations dramatically affects the QDs photoluminescence lifetimes and offered thereby a sensitive readout for physical interactions between QDs and their intracellular macromolecular targets. These findings strongly suggest that charge-mediated QDs/histone interactions could provide the basis for QDs nuclear localisation downstream of intracellular transport mechanisms.

QDs showed a particular tropism to the core histones which are abundant in the nucleus, apposed to the DNA or RNA. This results in dramatic changes in their physical and chemical properties. The shift off the absorption baseline, red-shift and decrease in the PL intensity (Figure 64) was dramatic and strongly indicative of the possible associated changes to the QDs surface integrity. At the same time, there is no evidence of deterioration of the QDs core structure, since the relevant absorption wavelengths were not affected (Figure 64). These observations are supported by increased histone/QDs aggregate formation in the presence of QD and by the shift of charge from negative to positive measured after the addition of the core histones to the QDs.

The FLIM images of the QDs (Figure 66) clearly demonstrated an increase in aggregation with the addition of core histones with a significant reduction in the PL lifetime. Despite this strong experimental evidence, we cannot exclude some other possible mechanisms for the reduced QDs lifetime in the nuclear compartments. One possible speculation is based on the fact that the nucleus *per se* presents a strong acidic environment (contributed by nucleic acids) and the PL of the QDs varies with pH and is diminished in acidic conditions (Gao, Kirstein et al. 1998; Boldt, Byrnes et al. 2006). However, it has been recently shown that TGA surface capping makes CdTe QDs extremely stable in physiological conditions at least on the timescale of intracellular imaging experiments (Byrne, Corr et al. 2006; Sussha, Javier et al. 2006). Another assumption builds around the earlier observation that QDs that are very tightly packed and therefore can undergo a reduction in lifetime as a result of the energy transfer between QDs of different sizes from ensemble of nano-particles (Kagan, Murray et al. 1996). In this case electronic energy transfer from the small to the large dots is observed as the decrease in the luminescence lifetime of the small dots is accompanied by increases in the luminescence lifetime of the large dots. It is difficult to explain the 2.5 ns shift of the long-lived component in lifetime histograms presented in Figure 4 b, c, based on this mechanism alone when working with the assumption that the energy transfer parameters are the same for QDs which have aggregated on membrane of the cell and for those interacting in the nucleus. However, one cannot rule out that one or both of these mechanisms also contribute to some extent to the observed changes in QDs during their intracellular compartmentalisation.

The method of QD uptake remains to be answered however it is certain that the transport of QDs within cells is energy dependent and not passive. Nabiev *et al* showed that, in order to reach the nucleus, CdTe QDs exploited the cells active microtubule transport route, whereas cells treated with anti-microtubule agent nocodazole lead to an accumulation of QDs in the cells cytoplasm rather than the nuclear region. However this affinity of CdTe QDs to the nuclei of cells is in many ways a double edged sword.

Histones play a regulatory role in the normal cell cycle. This “tropism” of QDs to the histones during different stages of the cell cycle could be exploited to determine the specific cell cycle of different cell types. However, disruption of normal cell cycle by QDs in healthy cells could be catastrophic. Masinger *et al* 2007, showed that even low level

exposure to anionic CdTe QDs induced nuclear and mitochondrial disruption leading ultimately to genotoxic and epigenetic changes (Maysinger, Lovric et al. 2007). Alternatively, QDs can be used for direct manipulation at the level of subcellular components. Microinjection of QDs or mRNA-QD into the nuclei of Cos7 cells, described by Ishihama *et al* 2009, used oligo-QDs to track the movement of mRNA in the nucleus thus, allowing for single molecule tracking of the diffusion of mRNA in chromatin versus interchromatin regions of the nucleus (Ishihama and Funatsu 2009).

Caution is paramount in the development and interpretation of cytotoxicity methods for assessing these nanomaterials which are unlike any drugs or materials used routinely in labs, and for which most no cytotoxicity kits take account of. It is therefore vital to use a broad range of techniques to measure the toxicity of QDs and other nanomaterials and realise that not one technique will work for all.

4.6. Conclusion

Negatively charged CdTe QDs show a rapid uptake into the nucleus and nucleoli of phagocytic cells. Strong experimental evidence from the present study suggests this nuclear affiliation is charge mediated, driven by the interaction of the negatively charged QDs and the positively charged histones. Spectroscopic results reveal the QD core remains intact whereas the QD surface integrity is affected by the strong interaction with histone proteins. The QDs preferentially bound to histones over DNA, RNA or BSA and induced a reduction in PL lifetime following this interaction.

Finally, in this work, FLIM technology revealed for the first time that the localization of negatively charged QDs to their ultimate nuclear and nucleolar destinations dramatically affects the QDs photoluminescence lifetimes and thereby provides a sensitive readout for physical interactions between QDs and their intracellular macromolecular targets. Charge-mediated QDs/histone interactions could potentially serve as the basis for QDs nuclear distribution downstream of concurrent intracellular transport mechanisms.

Chapter 5

5. General Discussion and Conclusion

It is estimated that about 20-50 million tonnes of e-waste is disposed of globally per year (Ongondo, Williams et al. 2011). It is thought that this figure may be a gross underestimate as evidence suggests that massive amounts of e-waste are being disposed of illegally in developing parts of the world such as China and India. The toxic components from this e-waste leach in the water, soil and air which surround the dumping sites and have knock-on effects which are detrimental to human health and the environment over time. The amount of e-waste produced each year is set to increase as electronic devices become more prevalent and are replaced more frequently.

This thesis concerns itself with the nanomaterials which are commonly incorporated into electronic devices, creating a new problem of 'nano-e-waste' that can have repercussions for human health and the environment. Scenarios whereby humans can potentially be exposed to NP arise throughout the entire life cycle of the NP from their manufacture through to their use and eventual disposal. Traditional cytotoxicity tests have high throughput capacity but have often been shown to lead to inconclusive or false positives when assessing the interaction of cells with nanomaterials (Hirsch, Roesslein et al. 2011). For example Casey *et al* found that the experimental exposure of CNTs to lung cells resulted in low to high toxicity depending on the dye used in the cell viability assay. Monteiro *et al* reported that a number of classical cell viability/toxicity assays alone were not sufficient in assessing the toxicity of SWCNT, C₆₀, carbon black or QD (Monteiro-Riviere, Inman et al. 2009). Traditional cell viability assays do not discriminate between the routes of cell death and do not provide any link between the physiochemical properties of the NP and their effect on the cells or tissue (Nerl, Cheng et al. 2011); this limits the usefulness of these assays beyond the simple quantification cell death. Thus, there is an urgent need for alternative and more definitive tests and techniques tailored specifically for interrogating the interaction of specific nanoparticles with cells or tissue.

Using high throughput screening and MTT assays this study showed that neither SWCNT or AgNW were acutely toxic to phagocytic cells. However, we believe that this

type of screening for the toxic effects of nanomaterials is no longer sufficient; and through the use of a combination of biochemical and microscopic techniques we explored the interaction between phagocytic cells and NP. The studies outlined in this thesis demonstrate that SWCNT, AgNW and their enabled thin films induce an elevated autophagic response in phagocytic cells. Interestingly this significant finding could resolve some of the controversy from existing toxicity studies. The same autophagic response, to a lesser degree, was observed when phagocytic cells are grown on thin films made from SWCNT or AgNW; prior to this study the NP were assumed to be securely embedded within the polymer matrix and therefore should have had no effect on the cells. This highlights the importance of assessing NP toxicity throughout their entire life cycle, where possible exposure to NP should always be assumed.

A shortage of publications regarding the processes governing the degradation of NP reflects the difficulty of obtaining of these types of measurements. The autophagic response observed here could represent one possible NP degradation route, where the NP is degraded within the autophagolysosome. This is a promising finding for future application of SWCNT and AgNW, illustrating that uptake of NP into the cells induces a mechanism whereby the cell attempts to degrade and process the NP which does not result in acute toxicity. Caution must be taken on the interpretation of this result as autophagy is also known to be a mechanism of cell death and the degradation of the NP within the autophagolysosome could ultimately lead to the death of the cell. Further, more comprehensive, studies on exposure of NP to cells over longer periods than reported here would need to be carried out in order to clarify this.

One of the major challenges facing researchers in this field is how best to image NP within living cells. In this study confocal enabled imaging of AgNW was used to determine their ultimate location within the cell. An intriguing finding was the ease with which confocal microscopy made it possible to image AgNW within live cells. A number of questions now arise as a result of this study. What is the uptake mechanism of the AgNW and SWCNT and what initiates the cells to put them into autophagosomes and try and digest and process them? Fluorescent imaging was suitable for the AgNW, however not all NP autofluoresce so alternative techniques and approaches must be explored.

The uptake and release of NP within biosystems requires a suitable technique that will fulfill two major functions, firstly the ability to image and locate NP within cells and tissue and secondly the capacity for image acquisition in real-time in this water based environment. Raman spectroscopy unlike FTIR has minimal interference from water, is non-destructive, non-invasive and requires little or no sample preparation. It also has the advantage of being a label-free imaging technique requiring no fluorescent staining or tags. The biological applications of Raman spectroscopy have been progressively improving and seminal advances have been made in the past 15 years. In this study Raman spectroscopy was used for imaging and measuring the integrity of SWCNT following their exposure to both cells and tissue. An optimised standard operations procedure was achieved enabling the user to carry out these measurements in any cell or tissue type with minimal preparation.

Unlike the AgNW, the SWCNT do not fluoresce but have a very strong elegant Raman spectrum which makes them ideal candidates for this technique. A unique and novel protocol was created in this work which made it possible to map their location within cells and tissue which is crucial in addressing the question of uptake and ultimate location of the NP following exposure. There has also been a substantial amount of research on the toxic effect of CNT on cells and tissue which doesn't address the question of what happens to the CNT post exposure.

In this study we measured the biodegradation of SWCNT within cells and tissue for the first time. It was possible through the use of chemometric analysis techniques to calculate the relative intensity ratio of two main Raman spectral peaks (D and G bands, I_D/I_G). This revealed a time dependent degradation process driven primarily by MPO in PNM cells. Here the Raman map acquisition and analysis was very time consuming (~ 40 minutes per map) and the biggest limitation of this study. A more rapid method of acquisition and analysis would be desirable for future studies to allow this technique be applied in a more real time setting with an immediate result. Also mapping of larger areas, particularly for tissue would be desirable.

QD are an entirely different NP with one of the most attractive properties being the change in emission wavelength depending on their size. Using FLIM here it was possible to image QDs, based on their PL lifetime, within living cells. The first detailed investigation of the dynamic modification of QD characteristics on their contact with selected biopolymers in live cells and cell-free systems, was performed. It was found that the unmodified CdTe QDs preferentially bound to the positively charged core histone proteins. FLIM technology revealed that the localization of negatively charged QDs to their ultimate nuclear and nucleolar destinations dramatically affects the QDs photoluminescence lifetimes and offered thereby a sensitive readout for physical interactions between QDs and their intracellular macromolecular targets. These findings strongly suggest that charge-mediated QDs/histone interactions could provide the basis for QDs nuclear localisation downstream of intracellular transport mechanisms.

Raman and FLIM not only proved to be imaging techniques but also provided feedback on the chemical and physical state of the SWCNT and QD pre and post exposure. This can be extrapolated and combined with results from other experiments and techniques to give a better picture of where the NP are within the cells, what is happening to them and also what is happening to the cells. It was not explored here but both of these techniques could be used to provide information on the biochemical changes induced by NP on cells. Such studies could provide more comprehensive understanding of the biochemical implications following NP exposure to cells. The relationship between physical and chemical properties of NP and the effects they have on cells could be elucidated. It is that specific patterns may emerge in the spectra or FLIM images for certain NP or cell types.

With such a broad range of NP available with different physical and chemical properties we must realize that no single technique is suitable for investigating the totality of interactions between NP and cells. Using novel adaptive approaches to well established microscopic and spectroscopic techniques, a broad range of new protocols tailored to revealing underlying cellular mechanisms, imaging their distribution and measuring the integrity of individual NP was achieved in this thesis. Evaluation of the autophagic response induced by different NP exposure scenarios has been achieved thus providing further insight into the consequences of NP exposure during different stages of their

lifecycle. Raman spectroscopy was found to be an objective, robust, non-invasive, non-destructive technique for identifying, imaging and assessing the integrity of SWCNT within cells and tissues. FLIM technology for the first time revealed that the localization of negatively charged QD to their ultimate nuclear and nucleolar location affects the PL lifetime of the QD and thereby provides a sensitive readout for the physical interactions between QD and these biological domains. These studies suggest applying the best suited technique for each NP leads to the most useful quantity of information acquired.

We have made observations through the course of this study that once again highlight how little we yet understand about nanoparticles and how human cells and tissues interact with them. This, from certain points of view, should be a matter of concern. Increasing interaction with devices and substances which utilize NPs has led to ever escalating exposure to these relatively new, potentially dangerous, in some cases unnatural, man made materials. The human drive to explore and discover has led us to create these nanoscale substances in order to benefit from their unique properties with little regard for their potentially negative effects. Like the discovery of asbestos we are enamored with their potential before we become cognizant of their dangers. Unlike asbestos, however, nanoparticles have been widely distributed in a relatively short time to the extent that a significant majority of the global population come into contact with them in one form or another on a daily basis. The empirical evidence would suggest that the benefits of NP use far outweigh the possible negative aspects. Studies like this demonstrate the subtle dangers and potential pitfalls of further and more intensive use of NP and highlight the need for further research and awareness that NP compounds are not biologically inert and that more concerted steps need to be taken before we should further implement their use.

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Appendix 1

Cell Lysis Buffer:

HEPES (pH 7.4)	50 mM
NaCl	150 mM
MgCl ₂	1.5 mM,
EGTA	1 mM
Sodium pyrophosphate	10 mM
Sodium fluoride	50 mM
β-glycerophosphate	50 mM
Na ₃ VO ₄	1 mM
Triton X-100	1% (v/v)
Phenylmethylsulphonyl fluoride	2 mM
Leupeptin	10 µg/ml
Aprotinin	10 µg/ml.

N.B. Protease and phosphatase inhibitors are added fresh before lysis

Table A1.1. Components of resolving gel solution for SDS-PAGE

Component	7% gel	8% gel	10 % gel	12 % gel
Distilled water	10.03 ml	9.37 ml	8.03 ml	6.7 ml
1.5 M Tris-HCl; pH 8.8	5 ml	5 ml	5 ml	5 ml
10 % w/v SDS	200 µl	200 µl	200 µl	200 µl
10 % w/v APS	200 µl	200 µl	200 µl	200 µl
TEMED	16 µl	11 µl	10 µl	10 µl
30 % Polyacrylamide	4.67 ml	5.33 ml	6.67 ml	8.0 ml
Total	20 ml	20 ml	20 ml	20 ml

Table A1.2. Components of the stacking gel solution for SDS-PAGE

Component	5% gel
Distilled water	6.8 ml
1.5 M Tris-HCl; pH 8.8	1.25 ml
10 % w/v SDS	100 µl
10 % w/v APS	100 µl
TEMED	10 ml
30 % Polyacrylamide	1.7 ml
Total	10 ml

5X SDS-PAGE sample buffer (Laemmli sample buffer)

Tris-HCl (pH 6.8)	0.312 M
SDS	10 % w/v
2-mercaptoethanol	25 % v/v
Bromophenol blue	0.05 % w/v

Final concentration: Tris-HCl 62.5 mM (pH 6.7), Glycerol 10% (v/v), sodium dodecyl sulphate 2% (w/v), bromophenol blue 0.002% (w/v) containing β - mercaptoethanol 143 mM. This solution was aliquoted and stored at -20°C. This solution was added to the lysate to give a final concentration of 1X.

10X SDS-PAGE Running Buffer

Trizma base	30 g
Glycine	142 g
SDS	10 g

Dissolved in deionised water to a final volume of 1 L. Dilute 1:10 in deionised water before use.

Western blot semi-dry transfer buffer:

Trizma base	5.8 g
Glycine	29 g
SDS	1 g

Dissolved in deionised water to 800 ml and then 200 ml methanol was added to a final volume of 1L.

Western blotting blocking solution

Marvel powder	500 mg
PBST	10 ml

Appendix 2

MatLab code for use in analysis of Raman spectral data:

1. *Primary Macrophage and THP-1 cell analysis*

A. The following code was used to import all the PM and THP-1 Raman maps. Only one example file is shown here “PM_24Hr_SWCNT_A_01.txt”.

```

clc;

close all;

clear all;

tic

%% PM 24Hr A1

[X,Y,wavenumber,hypercube] = renishawimp_am('PM_24Hr_SWCNT_A_01.txt');
    %Imports map

[xAxis yAxis zAxis]=size(hypercube);

a=xAxis.*yAxis;

B = reshape(hypercube,a,zAxis);           %Unfolding the hypercube

data=normaliz(B);                         %Vector normalisation

```

```
[data,wavenumber]=Raman_PreProcess(data,wavenumber);    %Subtract baseline
and smooth
```

```
dat=struct('B',data,'wavenumber',wavenumber,'x',xAxis,'y',yAxis);
```

```
save PM_24Hr_SWCNT_A_01.mat dat
```

```
... this code was repeated for each PM sample
```

```
toc
```

B. The following code was used to carry out K-means cluster analysis, generate Raman maps based on these clusters and calculate I_D/I_G of all the PM and THP-1 Raman maps. Only one file is represented here “PM_24Hr_SWCNT_A_01.txt”. See Table 3 for a full list of all the PM and THP-1 samples analysed.

```
clc;
```

```
close all;
```

```
clear all;
```

```
%% PM 24 Hr A1
```

```
load('PM_24Hr_SWCNT_A_01.mat');%Imports map
```

```
data=dat.B;wavenumber=dat.wavenumber;
```

```
xAxis=dat.x;yAxis=dat.y;
```

```
% K-means clustering
```

```
figure
```

```
map = makeColorMap([0 0 1],[1 0 1],[0 1 0],100);    %Creates user defined
colormap
```



```

%map = colormap('hsv');

num=size(map,1);

%Performing k-means clustering

clus=7; %Number of clusters

IDX = kmeans(data,clus); %K-means clustering

D = reshape(IDX, xAxis ,yAxis, 1); %Creates 2D
representation

subplot(1,2,1)

imagesc(D(:,:,1)) %Creates 2D visual image

colorbar

colormap(map) %Implements our colormap

xlabel('X ( $\mu$  M)')

ylabel('Y ( $\mu$  M)')

%Extracting spectra of clusters

cluster1 = find(0<IDX & IDX < 2);
cluster2 = find(1<IDX & IDX < 3);
cluster3 = find(2<IDX & IDX < 4);
cluster4 = find(3<IDX & IDX < 5);
cluster5 = find(4<IDX & IDX < 6);
cluster6 = find(5<IDX & IDX < 7);
cluster7 = find(6<IDX & IDX<8);

%Calculating mean spectra of clusters

C1 = mean(data(cluster1,:));
C2 = mean(data(cluster2,:));

```

```

C3 = mean(data(cluster3,:));
C4 = mean(data(cluster4,:));
C5 = mean(data(cluster5,:));
C6 = mean(data(cluster6,:));
C7 = mean(data(cluster7,:));

subplot(1,2,2)

[x y]=size(map);
step=round(x/clus);

plot(wavenumber,C1,'Color',map(step,:,:));hold on
plot(wavenumber,C2+0.1,'Color',map(step*2,:,:));
plot(wavenumber,C3+0.2,'Color',map(step*3,:,:));
plot(wavenumber,C4+0.3,'Color',map(step*4,:,:));
plot(wavenumber,C5+0.4,'Color',map(step*5,:,:));
plot(wavenumber,C6+0.5,'Color',map(step*6,:,:));
plot(wavenumber,C7+0.6,'Color',map(end,:,:));

hold off

legend('Cluster 1','Cluster 2','Cluster 3','Cluster 4','Cluster
5','Cluster 6','Cluster 7');

xlabel('Wavenumber cm^(Allen, Kotchey et al.)');

ylabel('Normalized Intensity');hold off

box off

GGR1=C1(find(wavenumber==1658));GGR2=C1(find(wavenumber==1744));GGRatio(1
)=GGR1/GGR2;

GGR1=C2(find(wavenumber==1658));GGR2=C2(find(wavenumber==1744));GGRatio(2
)=GGR1/GGR2;

GGR1=C3(find(wavenumber==1658));GGR2=C3(find(wavenumber==1744));GGRatio(3
)=GGR1/GGR2;

```



```

GGR1=C4(find(wavenumber==1658));GGR2=C4(find(wavenumber==1744));GGRatio(4
)=GGR1/GGR2;

GGR1=C5(find(wavenumber==1658));GGR2=C5(find(wavenumber==1744));GGRatio(5
)=GGR1/GGR2;

GGR1=C6(find(wavenumber==1658));GGR2=C6(find(wavenumber==1744));GGRatio(6
)=GGR1/GGR2;

GGR1=C7(find(wavenumber==1658));GGR2=C7(find(wavenumber==1744));GGRatio(7
)=GGR1/GGR2;

GGRatios1=GGRatio;

DGR1=C1(find(wavenumber==1442));DGR2=C1(find(wavenumber==1658));DGRatio(1
)=DGR1/DGR2;

DGR1=C2(find(wavenumber==1442));DGR2=C2(find(wavenumber==1658));DGRatio(2
)=DGR1/DGR2;

DGR1=C3(find(wavenumber==1442));DGR2=C3(find(wavenumber==1658));DGRatio(3
)=DGR1/DGR2;

DGR1=C4(find(wavenumber==1442));DGR2=C4(find(wavenumber==1658));DGRatio(4
)=DGR1/DGR2;

DGR1=C5(find(wavenumber==1442));DGR2=C5(find(wavenumber==1658));DGRatio(5
)=DGR1/DGR2;

DGR1=C6(find(wavenumber==1442));DGR2=C6(find(wavenumber==1658));DGRatio(6
)=DGR1/DGR2;

DGR1=C7(find(wavenumber==1442));DGR2=C7(find(wavenumber==1658));DGRatio(7
)=DGR1/DGR2;

DGRatios1=DGRatio;

GGRatio_C_72=[GGRatios1; GGRatios2; GGRatios3];

DGRatio_C_72=[DGRatios1; DGRatios2; DGRatios3];

```

toc

All of the data generated on the I_D/I_G ratios was then taken into Excel to calculate statistics and generate graphs.

2. *Mouse lung tissue section analysis*

A. The following code was used to import all the mouse lung tissue section Raman maps. Only one file is represented here “WT_1Day_P03_1672_01.txt”. See Table 4 for a full list of all the mouse samples imported.

```
clc;

close all;

clear all;

tic

%% WT 1Day 01

[X,Y,wavenumber,hypercube] =
renishawimp_am('WT_1Day_P03_1672_01.txt'); %Imports map

[xAxis yAxis zAxis]=size(hypercube);

a=xAxis.*yAxis;

B = reshape(hypercube,a,zAxis); %Unfolding the hypercube

data=normaliz(B); %Vector normalisation

[data,wavenumber]=Raman_PreProcess(data,wavenumber); %Subtract
baseline and smooth

dat=struct('B',data,'wavenumber',wavenumber,'x',xAxis,'y',yAxis);
```



```
save WT_1Day_P03_1672_01.mat dat
```

```
toc
```

B. The following code was used to carry out K-means cluster analysis, generate Raman maps based on these clusters and calculate I_D/I_G of all the lung tissue section Raman maps. Only one file is represented here “C_PBS_204_1D_01.txt”. See Table 4 for a full list of all the tissue samples analysed.

```
clc;

close all;

clear all;

tic

%% WT PBS 01

load('C_PBS_204_1D_01.mat');%Imports map

data=dat.B;wavenumber=dat.wavenumber;

xAxis=dat.x;yAxis=dat.y;

% K-means clustering

figure

%Performing k-means clustering

clus=7; %Number of clusters

IDX = kmeans(data,clus); %K-means clustering

D = reshape(IDX, xAxis ,yAxis, 1); %Creates 2D
representation

%Extracting spectra of clusters
```

```

cluster1 = find(0<IDX & IDX < 2);
cluster2 = find(1<IDX & IDX < 3);
cluster3 = find(2<IDX & IDX < 4);
cluster4 = find(3<IDX & IDX < 5);
cluster5 = find(4<IDX & IDX < 6);
cluster6 = find(5<IDX & IDX < 7);
cluster7 = find(6<IDX & IDX<8);

```

```

%Calculating mean spectra of clusters

```

```

C1 = mean(data(cluster1,:));
C2 = mean(data(cluster2,:));
C3 = mean(data(cluster3,:));
C4 = mean(data(cluster4,:));
C5 = mean(data(cluster5,:));
C6 = mean(data(cluster6,:));
C7 = mean(data(cluster7,:));

```

```

%Finding peaks of CNT's

```

```

GGR1(1)=C1(find(wavenumber==1592));GGR2(1)=C1(find(wavenumber==1565));GGR
atio(1)=GGR1(1)/GGR2(1);

GGR1(2)=C2(find(wavenumber==1592));GGR2(2)=C2(find(wavenumber==1565));GGR
atio(2)=GGR1(2)/GGR2(2);

GGR1(3)=C3(find(wavenumber==1592));GGR2(3)=C3(find(wavenumber==1565));GGR
atio(3)=GGR1(3)/GGR2(3);

GGR1(4)=C4(find(wavenumber==1592));GGR2(4)=C4(find(wavenumber==1565));GGR
atio(4)=GGR1(4)/GGR2(4);

GGR1(5)=C5(find(wavenumber==1592));GGR2(5)=C5(find(wavenumber==1565));GGR
atio(5)=GGR1(5)/GGR2(5);

GGR1(6)=C6(find(wavenumber==1592));GGR2(6)=C6(find(wavenumber==1565));GGR
atio(6)=GGR1(6)/GGR2(6);

```



```

GGR1(7)=C7(find(wavenumber==1592));GGR2(7)=C7(find(wavenumber==1565));GGR
atio(7)=GGR1(7)/GGR2(7);

DGR1(1)=C1(find(wavenumber==1357));DGR2(1)=C1(find(wavenumber==1592));DGR
atio(1)=DGR1(1)/DGR2(1);

DGR1(2)=C2(find(wavenumber==1357));DGR2(2)=C2(find(wavenumber==1592));DGR
atio(2)=DGR1(2)/DGR2(2);

DGR1(3)=C3(find(wavenumber==1357));DGR2(3)=C3(find(wavenumber==1592));DGR
atio(3)=DGR1(3)/DGR2(3);

DGR1(4)=C4(find(wavenumber==1357));DGR2(4)=C4(find(wavenumber==1592));DGR
atio(4)=DGR1(4)/DGR2(4);

DGR1(5)=C5(find(wavenumber==1357));DGR2(5)=C5(find(wavenumber==1592));DGR
atio(5)=DGR1(5)/DGR2(5);

DGR1(6)=C6(find(wavenumber==1357));DGR2(6)=C6(find(wavenumber==1592));DGR
atio(6)=DGR1(6)/DGR2(6);

DGR1(7)=C7(find(wavenumber==1357));DGR2(7)=C7(find(wavenumber==1592));DGR
atio(7)=DGR1(7)/DGR2(7);

map = makeColorMap([0 1 0],[1 0 0],[0 0 0],clus); %Creates user defined
colormap

indices=zeros(clus,7);indices(:,1)=GGR1;indices(:,2)=1:clus;indices(:,6)=
GGRatio;indices(:,7)=DGRatio;

indices=sortrows(indices,1); %Sort cluster indices
(ascending) on basis of G intensity

GGRatios1=indices(:,6);DGRatios1=indices(:,7);

indices(:,3:5)=map; %Attach colormap data

dat=sortrows(indices,2); %Now sort on basis of
cluster index

map=dat(:,3:5);

subplot(1,2,1)

```

```

imagesc(D(:, :, 1)) %Creates 2D visual image

colorbar

colormap(map) %Implements our colormap

xlabel('X ( $\mu$  M)')

ylabel('Y ( $\mu$  M)')


subplot(1,2,2)

[x y]=size(map);

plot(wavenumber,C1,'Color',map(1,:));hold on
plot(wavenumber,C2+0.1,'Color',map(2,:));
plot(wavenumber,C3+0.2,'Color',map(3,:));
plot(wavenumber,C4+0.3,'Color',map(4,:));
plot(wavenumber,C5+0.4,'Color',map(5,:));
plot(wavenumber,C6+0.5,'Color',map(6,:));
plot(wavenumber,C7+0.6,'Color',map(7,:));

hold off


legend('Cluster 1','Cluster 2','Cluster 3','Cluster 4','Cluster
5','Cluster 6','Cluster 7');

xlabel('Wavenumber cm^(Allen, Kotchey et al.)');

ylabel('Normalized Intensity');hold off

box off


..... the above code was repeated for each tissue sample....


GGRatio_MPOKO_28_Day=[GGRatios1 GGRatios2 GGRatios3 GGRatios4 GGRatios5
GGRatios6 GGRatios7 GGRatios8 GGRatios9 GGRatios10];

DGRatio_MPOKO_28_Day=[DGRatios1 DGRatios2 DGRatios3 DGRatios4 DGRatios5
DGRatios6 DGRatios7 DGRatios8 DGRatios9 DGRatios10];

```



```
%% Saving data
```

```
data=struct('GG_WT_PBS',GGRatio_WT_PBS,'GG_WT_1_Day',GGRatio_WT_1_Day,'GG_WT_7_Day',GGRatio_WT_7_Day,'GG_WT_28_Day',GGRatio_WT_28_Day,'GG_MPOKO_PBS',GGRatio_MPOKO_PBS,'GG_MPOKO_1_Day',GGRatio_MPOKO_1_Day,'GG_MPOKO_7_Day',GGRatio_MPOKO_7_Day,'GG_MPOKO_28_Day',GGRatio_MPOKO_28_Day,'DG_WT_PBS',DGRatio_WT_PBS,'DG_WT_1_Day',DGRatio_WT_1_Day,'DG_WT_7_Day',DGRatio_WT_7_Day,'DG_WT_28_Day',DGRatio_WT_28_Day,'DG_MPOKO_PBS',DGRatio_MPOKO_PBS,'DG_MPOKO_1_Day',DGRatio_MPOKO_1_Day,'DG_MPOKO_7_Day',DGRatio_MPOKO_7_Day,'DG_MPOKO_28_Day',DGRatio_MPOKO_28_Day);
```

```
save Peak_Ratio_data.mat data
```

```
toc
```

All of the data generated on the I_D/I_G ratios was then taken into Excel to calculate statistics and generate graphs.

Appendix 3

Peer reviewed publications