Imaging carbon nanoparticles and related cytotoxicity

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Abstract. Carbon-based nanoparticles have attracted significant attention due to their unique physical, chemical, and electrical properties. Numerous studies have been published on carbon nanoparticle toxicity; however, the results remain contradictory. An ideal approach is to combine a cell viability assay with nanometer scale imaging to elucidate the detailed physiological and structural effects of cellular exposure to nanoparticles. We have developed and applied a combination of advanced microscopy techniques to image carbon nanoparticles within cells. Specifically, we have used EFTEM, HAADF-STEM, and tomography and confocal microscopy to generate 3-D images enabling determination of nanoparticle spatial distribution in a cell. With these techniques, we can differentiate between the carbon nanoparticles and the cell in both stained and unstained sections. We found carbon nanoparticles ($C_{60}$, single-walled carbon nanotubes (SWNT), and multi-walled carbon nanotubes (MWNT)) within the cytoplasm, lysosomes, and nucleus of human monocye-derived macrophage cells (HMM). $C_{60}$ aggregated along the plasma and nuclear membrane while MWNTs and SWNTs were seen penetrating the plasma and nuclear membranes. Both the Neutral Red (NR) assay and ultra-structural analysis showed an increase in cell death after exposure to MWNTs and SWNTs. SWNTs were more toxic than MWNTs. For both MWNTs and SWNTs, we correlated uptake of the nanoparticles with a significant increase in necrosis. In conclusion, high resolution imaging studies provide us with significant insight into the localised interactions between carbon nanoparticles and cells. Viability assays alone only provide a broad toxicological picture of nanoparticle effects on cells whereas the high resolution images associate the spatial distributions of the nanoparticles within the cell with increased incidence of necrosis. This combined approach will enable us to probe the mechanisms of particle uptake and subsequent chemical changes within the cell, essential for identifying the toxicological profiles of carbon nanoparticles.

1. Introduction
Carbon-based nanoparticles (CNP) have attracted significant attention due to their unique physical, chemical, and electrical properties. Of specific interest are C60 fullerenes, single-walled (SWNT), and multi-walled carbon nanotubes (MWNT). Potential applications are extending into everyday settings: C60 as structural reinforcers [1], building blocks in photovoltaics and optoelectronics [2], chemotherapeutic agents [3], antioxidants, enzyme inhibitors, and imaging contrast agents [4]; SWNTs
as field-emission transistors [5], imaging probes [6], gene and drug delivery vehicles [7][8], and anti-tumour agents[9]; and MWNTs as fillers in composite materials to improve structural and electrical properties [10], sensors [11], microcatheters [12], tissues engineers [13], and drug carriers [14]. Consequently, attention has focused on their potential toxicity to human health.

Numerous studies have been published on CNP toxicity; however, the results remain contradictory. Table 1 highlights some of the recent toxicological findings for C60s, SWNTs, and MWNTs. Generally, C60s and SWNTs have been found to exhibit both cytotoxic and non-cytotoxic responses. Some studies have suggested unpurified SWNTs are more toxic than MWNTs and C60 [15] while others have found no inflammation [16] and fast systemic excretion [17]. MWNTs seem fairly cytotoxic, generating free radicals [18], inflammation [19], and DNA damage [20]. Behaving similarly to asbestos, longer nanotubes can cause frustrated phagocytosis, where digestive enzymes can leak from the phagosomes of incompletely phagocytosed nanoparticles [21], while shorter ones can yield little to no toxicity [22]. No clear uptake mechanism has been identified; however, some have suggested a mechanical nanoneedle piercing of the cell membrane [7].

Clearly, cell viability assays are not sufficient tools alone for assessing CNP toxicity as they are subject to variability and only reveal broad toxicological effects. Similarly, imaging alone provides a visual depiction that can be subjectively interpreted positively or negatively. An ideal approach is to combine the cell viability assays with nanometer scale imaging to elucidate the detailed physiological and structural effects of cellular exposure to nanoparticles. Imaging, however, remains a challenge due to the difficulty of attaining high spatial resolution with sufficient contrast to distinguish between nanoparticles and various cell organelles.

Transmission electron microscopy (TEM) is invaluable for imaging such small particles; however, the traditional techniques developed for inorganic imaging limit what can be seen when applied to organic systems. Standard bright-field imaging yields little contrast amongst cell structures; consequently, heavy stains are used. Yet potential problems arise when differentiating between the stains and nanoparticles. Additionally, conventional sample thicknesses of 70 nm or greater can cause misinformation as 3D data is projected into a 2D image [23].

We have developed and applied a combination of advanced microscopy techniques that address these issues and allow us to image CNPs within cells. One technique is energy filtered TEM (EFTEM), where images are created by filtering out inelastically-scattered electrons. Cell structures and CNPs have different energy loss spectrums, and as stains may alter these spectrums, EFTEM is ideal for mapping nanoparticles in cells unstained. Furthermore, electron energy loss spectroscopy (EELS) can be used to identify CNPs [24].

Another technique is high angle annular dark field scanning TEM (HAADF-STEM), where electrons scattered to high angles by differing carbon atom densities provide image contrast. As the intensity of scattering is proportional to the mass-density, HAADF-STEM is sensitive to the atomic number and is ideal for imaging SWNTs and MWNTs because of the stronger scattering from the iron catalysts. HAADF-STEM can be further combined with tomography to generate 3-D images enabling determination of nanoparticle spatial distribution in a cell without the effect of chromatic aberration as described in Porter et al [24].

As TEM is a non-viable technique, confocal microscopy offers the advantage of generating 3-D images from living cells. Cells are stained to visualise the plasma membrane and nucleus, while the CNPs are seen via reflectance from either the metal catalysts or post-filled heavy metals [25].

In this paper, we present data of C60, SWNT, and MWNT effects on human macrophage cells from cell viability assays and various imaging techniques in order to understand the potential toxic effects of CNPs on cells. We will also highlight the limitations of each tool when used alone and the benefits when combined.
Table 1. Summary of recent toxicity studies for C60, SWNTs, and MWNTs.

| SWNT                      | TOX | LENGTH | MODEL              | CITATION                     |
|---------------------------|-----|--------|--------------------|------------------------------|
| Altered nucleus, mitochondria, tonofilaments, organelles, F-actin | +   | Not specified | Human keratinocyte | Shvedova AA 2003 Free Rad. Oxid. Stress. 91-103 |
| Inhibit cell proliferation, increased apoptosis genes, and decreased cell adhesion time and dose dependently; caused chromatin condensation, nucleosomal DNA fragmentation | +   | Not specified | Human embryo kidney | Cui D 2005 Toxicol. Lett. 155 73-85 |
| More toxic than MWNTs and C60 | +   | ~1µm    | Rat alveolar macrophages | Jia G 2005 Environ. Sci. Technol. 39 1378-83 |

| C60                      | TOX | SIZE | MODEL              | CITATION                     |
|--------------------------|-----|------|--------------------|------------------------------|
| Inhibited cell proliferation and differentiation | +   | Not specified | Mice midbrain micromass | Tsujiya T 1996 FEBS Lett. 393 139-45 |
| Water derived nano-C60 caused cell membrane damage and generated O2* | +   | 60nm  | Human dermal fibroblast & liver carcinoma | Sayes CM 2004 Nano. Lett. 4 1881-7 |
| Caused no lethal, acute, or subacute toxicity; produced hypertrophy & hyperplasia but no fibrosis | -   | 200-1000 | Male Wistar rats | Gharbi N 2005 Nano. Lett. 5 2578-85 |
| Pristine C60: rapid ROS-dependent, caspase-independent necrotic cell death; C60(OH)2* delayed, ROS-independent, caspase-mediated apoptosis | +   | 96.3nm | Mouse fibrosarcoma, rat & human glioma | Isakovic A 2006 Toxicol. Sci. 91 173-83 |
| Water derived nano-C60 & C60(OH)24 yielded little to no toxic difference compared to control | -   | 160±50 nm | Rats: intratracheal instillation | Sayes CM 2007 Nano. Lett. 8 2399-406 |
| Minimal toxicity: no gross or microscopic lesions; C60 nanoparticle lung burden greater than microparticle; lung half-lives = 26-29 days | -   | C60 nano & micro particles | Male rats: aerosol inhalation | Baker GL 2008 Toxicol. Sci. 101 122-31 |
| Caused lipid peroxidation; damaged fish brain cell membranes; increased detoxification enzymes (ie. GSH, P450) | +   | 30-100 nm | Juvenile largemouth Bass | Oberdorster E 2004 Environ. Health Perspect. 112 1058-62 |
| Strongly bound to single-strand DNA and minor grooves of double-stranded DNA | +   | ~1nm | Simulation | Zhao X 2005 Biophys. J. 89 3856-62 |
| Found C60 along plasma and nuclear membranes and within cytoplasm, lysosomes, and nucleus | -   | 900±100 nm | Human macrophages | Porter AE 2006 Acta Biomater. 2 409-19 |
| Pristine C60 entered lipid bilayer easily and remained inside creating micropores; functionalised C60 (C60(OH)20) attached to bilayer surface only | +   | 1.2 nm | Simulation | Qiao R 2007 Nano. Lett. 7 614-9 |

Inflammation

| Iron                      | TOX | SIZE | MODEL              | CITATION                     |
|--------------------------|-----|------|--------------------|------------------------------|
| Both unpurified and purified did not generate O2* or NO | -   | Not specified | Rat macrophages | Kagan VE 2006 Toxicol. Lett. 165 88-100 |
| Induced ROS at higher extent than asbestos | +   | 0.5-1µm | Human mesothelioma | Wick P 2006 Phys. Stat. Sol. 243 3556-60 |
| Dose dependent ROS generation corresponds to Fe content | +   | Not specified | Mouse keratinocyte | Grabinski C 2007 Carbon 45 2828-35 |
| Process                          | Length     | TOX        | MODEL                  | CITATION          |
|---------------------------------|------------|------------|------------------------|-------------------|
| Estimated threshold length of uptake at 189±17 nm | 189±17 nm | Human lung fibroblasts | Becker ML 2007 Adv. Mater. 19 939-45 |
| Less toxic than unfunctionalised; distributed through body quickly, blood half-life = 3.5 hours | -          | Not specified | Male KM mouse          | Wang HF 2004 J. Nanosci. Nanotechnol. 4 1019-24 |
| Entered via active-uptake (phagocytosis) | ~1μm       | Mouse peritoneal macrophage | Cherukuri P 2004 J. Am. Chem. Soc. 126 15638-9 |
| Functionalised not enter via endocytosis | 300-1000 nm | Human & mouse fibroblast | Pantarotto D 2004 Chem. Commun. 1 16-17 |
| Found in cytoplasm, lysosomes, and nucleus with dose dependent toxicity | Not specified | Human macrophages | Porter AE 2007 Nature Nanotech. 2 713-7 |

### Toxins

- **Oxidized more toxic than pristine; time and dose dependent**
  - MWNT
  - Length: 1-5μm
  - Model: Human T lymphocyte
  - Citation: Bottini M 2006 Toxicol. Lett. 160 121-6

- **Agglomerates more toxic than well-dispersed**
  - MWNT
  - Length: Not specified
  - Model: Human mesothelioma
  - Citation: Wick P 2007 Toxicol. Lett. 168 121-31

- **Increased IL-8, macrophages, lymphocytes, neutrophils; multifocal granuloma around NTs; mild to no peribronchial fibrosis**
  - MWNT
  - Length: Not specified
  - Model: Guinea pigs; intratracheal instillation
  - Citation: Grubek-Jaworska H 2006 Carbon 44 1057-63

- **DNA damage by unpurified and purified; dose dependent**
  - MWNT
  - Length: Not specified
  - Model: Mouse stem embryonic
  - Citation: Zhu L 2007 Nano. Lett. 7 3592-7

- **Unground (5.9μm) harder to clear than ground (0.7μm); both increased inflammation**
  - SWNTs
  - Length: 0.7μm
  - Model: Sprague-Dawley rats
  - Citation: Muller J 2005 Tox. Appl. Pharmacol. 207 221-31

- **Ground (not unground) caused dose-dependent toxicity and increased TNF-α**
  - SWNTs
  - Length: 5.9μm
  - Model: Rat peritoneal macrophage
  - Citation: Sato Y 2005 Mol. BioSyst. 1 176-82

- **220nm caused less inflammation than 825nm; 220nm inflammation disappeared after 4 wks while 825nm remained same throughout**
  - SWNTs
  - Length: 220nm
  - Model: Human acute monocytic leukemia
  - Citation: Liu 2007 Nano. Lett. 7 3592-7

### Iron Uptake

- **Glucosamine functionalisation: No renal or severe acute toxicity; blood half-life longer than SWNTs**
  - Model: Tons of μm
  - Model: Kunming mice
  - Citation: Guo J 2007 Nucl. Med. Biol. 34 579-83

- **Functionalising with carbonyl (C=O), carboxyl (COOH), & hydroxyl (OH) increases toxicity**
  - Model: 160-180nm
  - Model: Lung cancer H596
  - Citation: Magrez A 2006 Nano. Lett. 6 1121-5

- **Dose dependent ROS generation corresponds to Fe content**
  - Model: Not specified
  - Model: Mouse keratinocyte
  - Citation: Grabinski C 2007 Carbon 45 2828-35

### Penetration

- **Penetrated cell by 2 hour, internalized by 6 and 24 hours**
  - Model: 70μm
  - Model: Mouse microglia
  - Citation: Kateb B 2007 NeuroImage 37 S9-S17

- **Found in vacuoles, cytoplasm, close to nucleus, pierce nuclear membrane**
  - Model: < 3.6μm
  - Model: Human epidermal keratinocytes
  - Citation: Monteiro-Riviere NA 2005 Tox. Lett. 155 377-84

- **Observed frustrated phagocytosis with dose dependent TNF-α and O2- production**
  - Model: > 50μm
  - Model: Human peripheral mononuclear
  - Citation: Brown DM 2007 Carbon 45 1743-56

- **Nanoneedle hypothesis, not via energy-dependent process; crossed membrane with no cell death**
  - Model: 20nm
  - Model: HeLa
  - Citation: Pantarotto D 2004 Angew. Chem. Int. Ed. 43 5242-6

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*a* Quick assessment of toxic potential: ‘+’ exhibits toxicity, ‘-’ exhibits little to no toxicity

*b* Average particle size in diameter

*c* Only the first author is listed

*d* Average length
2. Materials and Methods

2.1. CNPs
C60s were purchased from Aldrich, Dorset, UK, and were characterised via TEM. SWNTs produced via the HiPco process were purchased from Carbon Nanotechnologies, Inc., and were characterised by high-resolution TEM and EELS. MWNTs were synthesised via chemical vapour deposition (CVD) from a solution of 5.6 wt% ferrocene in toluene injected into a furnace at 760 °C under an argon atmosphere; they were characterised by scanning electron microscopy (SEM), TEM, energy dispersive x-ray microanalysis (EDX), and inductively coupled plasma atomic emission spectroscopy (ICP-AES).

2.2. In vitro study

2.2.1. Cell culture. Mature human monocyte-derived macrophage cells (HMM) were isolated from human buffy coat residues (National Blood Transfusion Service, Brentwood, UK). Buffy coat residues were washed once with phosphate-buffered saline (PBS), and the resulting cell sediment was mixed with an equal volume of fresh PBS. Thirty milliliters of diluted buffy coat residue were layered onto 15 ml of LymphoPrep (Axis-Shields, Oslo, Norway), and, after centrifugation at 20 °C for 30 min at 600g, the opaque interphase of mononuclear cells was removed and washed three times with PBS containing 4 mg/ml bovine serum albumin (BSA) to remove platelets. Monocytes were then enriched by an additional centrifugation step in a Percoll gradient (24). Mononuclear cells were re-suspended in 4 ml of PBS and mixed with 8 ml of Percoll: Hanks’ balanced salt solution (10× concentrate) (6:1, at pH 7.0). After centrifugation at 20 °C for 30 min at 450g, the monocytes were collected from the top of the gradient, washed in PBS/BSA, and seeded in 24 and 48-well tissue culture plates at 1–2 × 10^6 cells/well and 0.5–1 × 10^6 cells/well respectively using macrophage serum-free medium (MΦ-SFM, Invitrogen) unless otherwise stated. After incubation for 1 h at 37 °C, any remaining non-adherent cells were removed by washing twice with PBS. Adherent monocytes were cultured at 37 °C in humidified air/5% CO_2 using MΦ-SFM for at least 6–7 days prior to experiments unless stated otherwise, renewing the culture medium twice a week.

2.2.2. CNP treatment. C60 was dispersed in 10ml of freshly distilled, filtered, and de-gassed tetrahydrofuran (THF) and left in a vial with a screw cap to stir for 24 hours at ambient temperature; when added to cells, the final THF concentration was < 0.1% THF in MΦ-SFM. SWNTs were dispersed in 0.1% of THF and MΦ-SFM and water bath sonicated for 30 min. MWNTs were first sterilized in ethanol then dispersed in a final volume concentration of 250 μg/ml of BSA in MΦ-SFM; the solution was sonicated in a water bath for ~1 hour. Concentration for imaging was 5 μg/ml for all CNPs unless otherwise stated.

2.2.3. Cell assays. Cell viability was assessed via Neutral Red (NR), which measured lysosomal accumulation of NR dye in viable cells. For each treatment, three experiments each in triplicate were performed and statistically analysed using one-way ANOVA and least significance difference (LSD) with significance set at p < 0.05.

2.3. Imaging

2.3.1. Electron microscopy. For electron microscopy studies, samples were fixed in 4% gluteraldehyde in piperazine-1,4-bis(2-ethanesulphonic acid) (PIPES) buffer, dehydrated, embedded, and sectioned with an ultramicrotome at 40 nm for EELS and high-resolution STEM (HR-STEM), 70 nm for EFTEM studies, and 300 nm for 3D electron tomography. TEM sections were deposited on lacy carbon film grids and bare 600-mesh copper TEM grids. Selected sections were bulk stained with osmium tetroxide and post-stained with uranyl acetate and lead citrate for 5 min in each to enhance contrast from cell. For freeze-drying, HMMs seeded and treated on formvar coated gold grids were cryo-fixed by rapid immersion into liquid propane cooled by liquid nitrogen. Samples were freeze-
dried by raising the temperature to room temperature over 24 hours at a pressure of 5x10^{-6} bar [26].

EFTEM studies were performed on a Philips CM300 operated at 300 kV with a Gatan imaging filter (GIF) model 2002 using 10μm objective aperture. To optimize contrast, zero-loss images of unstained sections were taken using a 3eV slit while stained sections were taken using a 20eV slit. Low-loss energy filtered series were recorded from 0 to 30 eV using a 2 eV slit and 2 eV step size. EELS was extracted from the EFTEM series using IDL image processing software. HAADF-STEM of stained sections was performed on an FEI Tecnai F20 operated at 200 kV using a 30 nm condenser aperture and a camera length of 150-200 mm. EFTEM tomography was performed on the stained and unstained 150 nm thick sections and HAADF-STEM tomography on unstained freeze-dried whole cells. An image series was acquired using the FEI Tecnai F20 operated at 200kV. Datasets were acquired over a tilt range of −68° to +56° (EFTEM) and −70° to +70° (STEM) using a step size of 2°. 3D reconstruction was carried out using weighted back-projection [27] and a simultaneous iterative reconstruction technique (SIRT) [28].

Cell death was also quantitatively differentiated between necrosis and apoptosis via morphology [29]. Necrotic cells were characterised as electron-translucent with cytoplasmic and nuclear contents appearing to be leached out while apoptotic cells appeared shrunken with heavily capped chromatin. 100 cells were indexed for each group.

2.3.2. Confocal microscopy. HMMs were seeded and treated on coverslips and imaged on a Leica TCS SP2 confocal microscope. Hoerchst and FM-464 stained the nuclei and plasma membrane, respectively. CNPs were visualised via reflectance from the iron catalyst in the MWNTs and the AgI filled in the SWNTs. Z-plane stacks were taken to create a 3-D image.

3. Results

3.1. Characterisation
Average diameter of C60 clusters was 900±100 nm. HiPco-produced SWNTs have diameters from 0.5 to 3 nm with ~10 wt% ash content [30]. MWNTs had an average diameter of 68 nm and length of 26 μm with ~5 wt% Fe2O3.

3.2. Cell viability
No alteration in cell viability was found in the THF vehicle control; none was performed for BSA due to its non-toxic nature. At 2 days, the CNPs exhibited little or inconsistent toxicity: C60 was not toxic, SWNT was significantly toxic at 0.31, 1.25, 2.5, and 5 μg/ml, and MWNT at 1.25 and 2.5 μg/ml. At 4 days, the SWNTs were significantly toxic at 0.31, 0.625, and concentrations greater than 2.5 μg/ml, while MWNTs at 10 μg/ml (Figure 1).

![Figure 1](image_url)

**Figure 1.** Toxicity of CNPs after 2 and 4 day treatment as measured by NR assay. Values represent mean ± SE of three experiments each performed in triplicates (ANOVA with LSD, p < 0.05). Significance within each sample labelled with asterisk.
**Figure 2.** Zero-loss EFTEM of C60 in HMMs: (a) C60 clusters along plasma membrane, (b) C60s inside a lysosome. (m = membrane, mi = mitochondria, n = nucleus, nm = nuclear membrane)

**Figure 3.** Zero-loss dark-field EFTEM of SWNT aligned along plasma membrane in the cytoplasm. (n = nucleus)
3.2.1. **Figure 4.** Comparison between various imaging techniques revealing the limitations of HAADF-STEM and bright field STEM and benefit of plasmon mapping in identifying CNPs: (a) EELS of SWNT in the cell showing characteristic peaks differentiating graphitic and amorphorous carbons, (b) HAADF-STEM of SWNT penetrating the lysosomal membrane, (c) bright field STEM, (d) plasmon mapping between graphitic and amorphous carbon to differentiate SWNT from cell (e, reproduced from [25]).
Figure 5. HAADF-STEM of SWNT: (a) in the lysosome and pushing up against lysosomal membrane, (b) bright field STEM of boxed region from (a) showing SWNTs. (ly = lysosome, cy = cytoplasm)

Figure 6. TEM analysis of cells treated with SWNTs and MWNTs at 5 μg/ml for 4 days showing proportion of necrotic to apoptotic cells. Values represent mean ± SE (ANOVA with LSD, p < 0.05).

3.3. Imaging

3.3.1. 2D. With bright-field TEM, cells were stained with lead citrate and uranyl acetate stains in order obtain the contrast needed to visualise mitochondria, ribosomes, and nuclei. CNPs were found within cells; however, it was difficult to determine whether the CNPs were inside or on the surface. With zero-loss EFTEM, after 2 days, C60 clusters were found aligned along the plasma membrane Figure 2a) and inside lysosomes and nuclei (Figure 2b). SWNTs were found in lysosomes after 2 days, while after 4 days they were found with the long axes aligned parallel to the plasma membrane translocating the lysosomal membrane (Figure 3). MWNTs were found translocating the plasma
membrane into the cytoplasm and, at times, nuclei after 4 days. Low-loss EELS confirmed identification of the CNPs (Figure 4). HAADF-STEM images of stained cells were taken for SWNTs and MWNTs to further compare CNP internalisation and cell morphology alterations. SWNTs were found in lysosomes and nuclei (Figure 5). Cells remained healthy after 2 days; however, increased cell mortality was correlated with higher SWNT and MWNT concentration. Furthermore, cell death via necrosis or apoptosis were differentiated and found that both SWNTs and MWNTs were correlated with greater necrotic death (Figure 6).

3.3.2. 3D. Zero-loss EFTEM tomography of C60s confirmed alignment along the plasma and nuclear membrane as well as localisation in secondary lysosomes and the nucleus; HAADF-STEM tomography of a freeze-dried whole cell revealed C60 alignment along the nuclear membrane but no nuclear internalisation (Figure 7). EFTEM tomography of SWNT-treated cells confirmed alignment along and penetration of the nuclear membrane (Figure 8). HAADF-STEM of a whole cell revealed MWNT internalisation (Figure 9); confocal microscopy further confirmed membrane translocation into the cytoplasm and nucleus with live cells (Figure 10).

4. Discussion
After 2 days incubation of HMMs with C60s, SWNTs, and MWNTs, the NR assay revealed inconsistent toxicity patterns which may result from assay variability. Increased viability values may be due to increased dye uptake from lysosomal distension by particle accumulation or adsorption of the dye to the CNPs causing false increased viability [31]. At 4 days, results were more consistent with SWNT toxicity increasing significantly at concentrations greater than 2.5 µg/ml. The NR assay is widely used for its fast, inexpensive, and simple procedure for screening viability in large number of samples; however, the assay is subject to variability and does not discriminate between the routes of cell death. As a result, we wanted to use imaging to further understand the relationship between CNP localisation and cell mortality.

In traditional TEM, heavy metal stains are required to generate the contrast to differentiate various cell components; however, these metal salts can obscure CNP identification. As a result, we pursued EFTEM using the low loss region of the energy-loss spectrum because: (i) acquisition times and beam damage could be minimized from the considerably lower energy-scattering magnitudes for core-loss, and (ii) the (π + σ) volume plasmon excitation which dominates the low-loss spectrum is sensitive to any slight differences in the electronic structure of the carbon species. Graphite-like carbonaceous material such as C60 and SWNTs exhibit a bulk plasmon energy at ~26 eV while amorphous carbonaceous material such as those in a cell are ~23 eV. Furthermore, graphitic carbon has an interband π→π* transition at 6 eV which is weak to non-existent in cell carbon material. Using an energy filter slit of 2 eV, images surrounding 20 eV and 26 eV could be recorded and an image ratio of 26 / 20 eV used to generate images that differentiated CNPs from cell material [32]. As graphitic carbon material has the extra 6 eV π→π* transition, EELS showing 2 peaks—6 and 26 eV—could confirm CNP identification.

Two-dimensional zero-loss EFTEM can be extended to tomography for 3D images; similar ratio images taken at every tilt can then be reconstructed. EFTEM tomography can be further combined with EELS to characterise CNPs; however, the volume information is limited by a specimen thickness of less than 100 nm. HAADF-STEM tomography allows imaging of thicker specimens or even whole cells up to 300 nm thick due to no post-specimen imaging lens, but CNPs cannot be characterised. Confocal microscopy allows imaging of live whole cells with 3D image reconstruction; however, resolution limits visualisation of smaller CNPs and CNPs cannot be characterized. With such differing benefits, we used all three in parallel to determine and confirm CNP localisation in cells.

We were able to confirm the presence of CNPs within the cell cytoplasm, lysosomes, and nuclei. C60s were found clustered along the plasma and nuclear membranes, and SWNTs fused along the plasma membrane; this supports the implications that C60s and SWNTs can cause lipid peroxidation [33][34][35]. Furthermore, peroxidation of phagosomal and lysosomal membranes could release digestive enzymes into the cell itself and result in cell mortality.
Figure 7. HAADF-STEM tomography of a freeze-dried cell treated with C60 (bright spots in b-h): (a, d) voltex reconstructions with a horizontal (a) and vertical slice (d) bisecting the reconstruction (arrow indicates direction perpendicular to orthoslice), (b-c) horizontal slices at different heights through the reconstruction – (e-h) vertical slices through the reconstruction (m = membrane, nu = nucleus, l = lysosome, c = cytoplasm, mi = mitochondria); reprinted with permission from [24]
Figure 8. EFTEM tomography of SWNT: (a) in the cytoplasm and pushing against the nuclear membrane, (b) vertical orthoslice through tomographic reconstruction showing SWNT inclusion in cell. (nm = nuclear membrane, a = SWNTs aligned along the nuclear membrane, n = nucleus, nm = nuclear membrane p = SWNTs penetrating into nucleus)

Figure 9. HAADF-STEM tomography projection of a MWNT in the nucleus.

Figure 10. Confocal microscopy 3D reconstruction of a MWNT penetrating the plasma membrane of a living cell.
Some SWNTs and MWNTs were found translocating the plasma, lysosomal, and nuclear membranes supporting the nanoneedle hypothesis as well as suggesting potential cytotoxicity from damaged membrane integrity or undesired fluid and ion exchanges [7]. The similarity between MWNTs and asbestos also suggested the possibility of frustrated phagocytosis [21]. Additionally, CNP presence within the nucleus could potentially cause DNA damage [33][35][36].

We further elucidated CNP effects on cells by directly combining cell viability with imaging. The high resolution of TEM imaging allows visual differentiation between apoptotic and necrotic death. For both SWNTs and MWNTs, we found increased cell death with longer exposure time; additionally, there was a proportionally greater increase in necrotic than apoptotic cells, suggesting a preference for necrotic death from CNP exposure.

5. Conclusion
Clearly, further research is required; however, this study demonstrates that only a combination of cell viability assays with high resolution imaging will help to fully understand the uptake mechanism, internal trafficking, and toxicological profile of CNPs.

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