Effects of functionalization on the targeting site of carbon nanotubes inside cells

Alexandra E. Porter1*, James S. Bendall2, Mhairi Gass3, Karin Muller4, Jeremy Skepper4, Paul Midgley5 and Mark Welland2

1Dept. Materials, Imperial College London, South Kensington, London SW7 2AZ UK, 2UK SuperSTEM, Daresbury Laboratory, Daresbury, Cheshire WA4 4AD, UK, 3The Nanoscience Centre, University of Cambridge, 11 J. J. Thompson Avenue, Cambridge CB3 OFF, 4Multiimaging Centre, Dept. of PDN (Physiology, Development and Neuroscience, Anatomy Building, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK, 5Dept. of Materials Science and Metallurgy, University of Cambridge, Pembroke Street, Cambridge CB2 3QZ, UK

a.porter@imperial.ac.uk

Abstract. Functionalized single-walled carbon nanotubes (SWNTs) are currently being investigated for a variety of applications, including contrast agents for medical imaging1. However before they can be used commercially it is necessary to assess whether they enter cells, the site they target within the cell and whether they cause any cytotoxicity. Here we characterize uptake of unlabelled, acid-treated, COO- functionalized SWNTs by human monocyte derived macrophage cells using both low-loss energy loss spectroscopy and compare our findings to previous work on unpurified SWNTs. The acid-treated SWNTs were less aggregated within cells than unpurified SWNTs. Acid treatment was found to affect the distribution of intracellular SWNTs. Bundles, and also individual acid treated SWNTs, were found frequently inside lysosomes, cytoplasm and also inserting into the plasma membrane whereas unpurified non-functionalised SWNTs entered lysosomes and occasionally the nucleus.

1. Introduction

Single walled carbon nanotubes (SWNTs) are currently being explored in medical applications due to their unique structure and properties which make them ideal candidates for drug delivery systems [1]. To be suitable for medical applications, it is necessary first to water solubilise the SWNTs so they are presented to the cell in individual form and second to purify the SWNTs to minimise any cytotoxicity arising from the catalyst nanoparticles. One simple technique to solubilise SWNTs is to reflux and dissolve them via a multi-step acid treatment using both hydrochloric and nitric acid [2]. Nitric acid treatment will alter the surface properties of the SWNTs by generating defects which break the end caps apart and introducing hydrophilic carboxylic acid groups at the tube ends and possibly, at defects on the sidewalls [3]. Therefore it is important to assess the uptake and cytotoxicity of acid-treated SWNTs as they are the precursors for the synthesis of a number of other functionalizations of SWNTs.

Direct imaging of unlabelled, individual SWNTs by electron microscopy is very challenging, because it is difficult to distinguish carbon based nanotubes from carbon-rich organelles due to...
similarities in their chemical composition and dimension. Visualizing SWNTs inside cells will help us understand how SWNTs enter cells, where they migrate to, and their fate after uptake. Previously we exposed human monocyte-macrophages to unpurified HiPco SWNTs and demonstrated that a combination of low-loss energy-filtered transmission electron microscopy (EFTEM) combined with electron energy loss (EEL) spectrum imaging—a method that yields characteristic energy-loss information—enables clear differentiation of SWNTs in the cell [4]. These techniques gave a good image contrast of unstained sections that cannot be achieved using conventional imaging techniques. These images confirmed that unpurified SWNTs localise primarily as bundles within lysosomes, but were also seen to enter the cytoplasm and localize within the cell nucleus. They were seen to cause cell mortality in a dose dependent manner. Visualizing acid treated COO\textsuperscript{-} functionalised SWNTs within cells will be particularly challenging as the SWNTs will be more disperse than pristine untreated SWNTs.

The goal of this study will be to characterize and visualise acid-treated water soluble SWNTs alone and within human monocyte derived macrophage cells (HMMs). HMMs were chosen as a model as this cell type comprises the first line of defence against foreign organisms or particles and are central to the body’s immune responses. Specifically we aim to establish the sites they target within the cell and the uptake mechanism, relating the difference in uptake to changes in the chemistry of the SWNT walls.

2. Materials and Methods

2.1. Materials

SWNTs were purchased from CNT@Rice (HiPco as-prepared). The nanotubes underwent a purification procedure to remove any unwanted non-tubular carbon species and catalyst particles. The nanotubes were initially refluxed in concentrated nitric acid (70\%) for 3 hours, filtered through a PTFE membrane with a pore size of 200nm, washed and dried and heated in air for 40 minutes at 380\(^\circ\)C. These steps were effective at releasing the catalyst particles from their carbon shells and oxidising them. The sample was then refluxed for 6 hours in concentrated HCl (33\%) after which they were filtered through a 200nm pore PTFE membrane, washed and dried. TEM characterisation of the sample showed that the purification procedure was effective at removing the catalyst particles and amorphous carbon material, leaving the nanotubes themselves intact. Raman spectroscopy confirmed that the acid treated SWNTs had a greater number of functional groups on the carbon walls than non-treated SWNT.

2.2. Transmission electron microscopy

TEM studies of SWNTs nanotubes in cells were performed at a concentration of 5 \(\mu\)g/ml for 4 days in accordance with previous work [4]. Purified SWNT were non-cytotoxic after 4 days exposures to HMMs at concentrations ranging from 0-20\(\mu\)g/ml. To establish the mode of cellular uptake, cells were exposed to acid treated SWNTs at both 4\(^\circ\)C and 37 \(^\circ\)C for 4 hours. High-resolution imaging, high angle annular dark field scanning transmission electron microscopy (HAADF-STEM) and EELS were performed at the UK SuperSTEM laboratory on a 100kV VG HB501 dedicated STEM fitted with a Nion second generation spherical aberration corrector and a Gatan Enfina electron-energy loss spectrometer. The convergence semiangle of the electron probe was 24 mrad for both imaging and spectroscopy. The collection semiangle for the EELS was 4 mrad and 70 – 210 mrad for HAADF imaging. SuperSTEM enables both BF and HAADF images to be acquired simultaneously. Low-loss EELS spectrum images were acquired at every pixel over a used-defined area with an energy dispersion of 0.1eV per channel, covering an energy range of \(\sim0\)eV to \(\sim100\)eV.
3. Results and Discussion

High magnification lattice imaging confirmed the graphitic structure of the carbon nanotubes had been preserved by acid treatment and that the iron catalyst had been removed. To confirm the intracellular localisation of individual SWNTs, EEL spectra were acquired from HMMs exposed to SWNTs using unstained cell sections [2]. Medium resolution HAADF-STEM appeared to show SWNTs bundles within the cell (Fig. 1a). Low loss EEL spectrum images were taken from the boxed-out region in Fig. 1c.

**Figure 1** (a) HAADF-STEM image of SWNT bundles within the cytoplasm of an unstained cell after 4 days exposure. The white box shows area analyzed by low-loss EELS spectroscopy. The inset shows an intensity profile taken along the direction of the arrow, the increase in intensity at the location of the SWNTs is evident. Low-loss EELS spectra for SWNTs in the cell (green/light) and the cell (grey) are shown in (b). The π to π* transition at ~6eV is seen clearly only in the spectrum taken from the SWNT bundle, it also exhibits a higher bulk plasmon energy. c) Map showing the change in the plasmon peak position. Range from 17.5eV (blue/ dark) to 21.5eV (white). Contrast due to SWNTs can be seen that is not identified in conventional imaging. d) High resolution lattice image of intracellular SWNTs. Some of the SWNTs have been indicated with a white dotted line as the contrast is weak.

A low-loss EELS spectra for SWNTs in the cell and the cell are shown in Fig. 1b. The map of plasmon energy was created by fitting a Gaussian to the (σ+π) bulk plasmon peak [5] in the aligned spectrum image using Digital Micrograph. The energy of the peak was extracted from the Gaussian fit. The position of the plasmon maximum ranged from 17.5eV from the cell to 21.5eV from the intracellular SWNTs. Hence, by mapping the peak position (Fig. 1c), enhanced contrast due to SWNTs was possible. The observed energy shift is small compared to the individual values because the nanotubes
are within the cellular structure resulting in a plasmon containing information from both the SWNTs and cell. Although the measured shift is small, the stability of the cold-FEG tip combined with the high energy dispersion means that it is still possible to extract such information. The resolution of such a fit has previously been studied by Sanchez et al [6] using an Enfina spectrometer on a VG 601 STEM where the standard deviation of the plasmon energy from 800 spectra (GaAs material) was 7meV. As the same spectrometer is used in this work, and the Cs corrected VG 501 has a very high stability it is concluded that a shift in plasmon energy in the order of 1eV can confidently be observed. High resolution lattice imaging also confirmed the presence of SWNTs within the cell (Fig. 1d).

The mechanisms by which SWNTs enter cells are not fully understood. Confocal microscopy studies and computer simulations have indicated that SWNTs could either traverse the cellular membrane via endocytosis [7] or insert into and diffuse through the lipid bilayer [8]. Low loss EEL spectrum imaging confirmed that SWNTs interacted with, and possibly inserted into, the plasma membrane of macrophage cells (Fig. 2).

**Figure 2** Bundles of SWNTs fusing with the plasma membrane of a cell in unstained cell sections after 4 days exposure. (a) A bundle of SWNTs fusing with the plasma membrane of the cell causing membrane disruption (HAADF STEM, stained section). (b) Plasmon HAADF image of SWNT fusing the plasma membrane of unstained cell. Range from 17.5eV (blue/dark) to 21.5eV (white).

Imaging intracellular SWNTs after 4 hours at 4°C was found to be very challenging. One simple method to enhance contrast from SWNTs inside cells is to fill them with heavy metal halides as described in ref.9. This method completely encapsulates the GdCl$_3$ inside the SWNTs, does not introduce functional groups onto the surface and also removes toxicity issues [10, 11]. Therefore cells were also exposed to GdCl$_3$ filled SWNTs (Gd-SWNTs [12]. After 4 hours at 37°C, individual Gd-SWNTs inserted into the plasma membrane of the cell (Fig. 3a,b). Notably, the Gd-SWNTs entered the cell parallel to the plasma membrane and then entered the cytoplasm with a cork-screw morphology (Fig. 3b). No evidence of cell penetration by Gd-SWNTs was observed at 4°C, suggesting that cellular uptake of the Gd-SWNTs was only active and not passive.
Figure 3 Human monocyte derived macrophages exposed to Gd-SWNTs at 37°C for 4 hours. a) Individual Gd-SWNTs inserting into the plasma membrane of unstained cells. b) Individual Gd-SWNTs inserting parallel to the plasma membrane of an unstained macrophage cell then entering the cytoplasm with a “cork-screw” morphology.

In summary, we were able to directly visualise acid treated SWNTs entering different intracellular sites than untreated SWNTs [4], where negatively charged SWNTs localise more frequently within the cytoplasm of the cell. SWNTs entered both cell lysosomes and also inserted into the plasma membrane. This platform will be used in future investigations to guide studies to assess uptake of more medically relevant classes of functionalized SWNTs being developed as vehicles for the delivery of therapeutic and diagnostic agents.
4. References

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