Cellular Uptake and Cytotoxic Impact of Chemically Functionalized and Polymer-Coated Carbon Nanotubes

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The impact of nanomaterials such as carbon nanotubes on biological matter is a topic of increasing interest and concern and requires a multifaceted approach to be resolved. A modified cytotoxic (lactate dehydrogenase (LDH)) assay is developed in an attempt to offer a valid and reliable methodology for screening carbon nanotube toxicity in vitro. Two of the most widely used types of surface-modified multiwalled carbon nanotubes (MWNTs) are tested: ammonium-functionalized MWNTs (MWNT-NH_3^+) and Pluronic F127 coated MWNTs (MWNT:F127). Chemically functionalized MWNTs show significantly greater cellular uptake into lung epithelial A549 cells compared to the non-covalently Pluronic F127-coated MWNTs. In spite of this, MWNT:F127 exhibit enhanced cytotoxicity according to the modified LDH assay. The validity of the modified LDH assay is further validated by direct comparison with other less reliable or accurate cytotoxicity assays. These findings indicate the reliability of the modified LDH assay as a screening tool to assess carbon nanotube cytotoxicity and illustrate that high levels of carbon nanotube cellular internalization do not necessarily lead to adverse responses.

1. Introduction

Due to their unique physicochemical and biological properties, carbon nanotubes (CNTs) have attracted particular attention especially in gene delivery and silencing, cancer therapy, and diagnostic purposes.[1–6] One of the most fascinating properties of CNTs to date is their ability to directly translocate biological membranes in what has been described by many as "the nanoneedle hypothesis".[7] However, the

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implication of such behavior creates the need for a careful toxicological assessment of carbon nanotubes and a better understanding of their biological impact to allow further exploration in biomedical applications. Most of the studies undertaken to date have focused mainly on the evaluation of pristine non-functionalized CNTs from a health and environmental toxicology point of view, while little has been done using surface-modified CNTs that are the most promising material for biomedical applications. For this reason a systematic evaluation of different types of surface-modified CNTs should attain more attention both in terms of cellular uptake and cytotoxicity.

It is now well-accepted that carbon nanotubes can interact not only with polymers, proteins, nucleic acids, and small molecules through supramolecular self-assembly but also with a range of fluorochromes used as probes in a series of cytotoxicity assays. In a systematic study, Worle-Knirsch et al.[8] reported that the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay can be unreliable for use with carbon nanotubes due to the association between CNTs and the insoluble formazan crystals leading to false-positive results. It was suggested that use of other cytotoxicity assays such as lactate dehydrogenase (LDH), flow cytometry-based Annexin V/PI (propidium iodide) staining, and other water soluble tetrazolium salt-based assays such as WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate), INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride), and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assays should be preferred. In addition, Monteiro-Riviere et al. identified similar problems when assessing the toxicity of carbon black using MTT, Neutral Red, and cytokine-based assays.[9] Casey et al. confirmed in a spectroscopic analysis the interactions between single-walled carbon nanotubes (SWNTs) and various dyes (Commassie Blue, Almar Blue, Neutral Red, MTT, and WST-1) used for cytotoxicity assessments and concluded that these assays were not suitable for the quantitative toxicity evaluation of carbon nanotubes.[10] In an attempt to find a reliable, non-absorbance-based cytotoxicity assay for use with CNTs, the same group described a novel approach using the clonogenic assay.[11] Although reliable, this assay is difficult to implement for routine use and is time consuming. Moreover, most cytotoxicity assays are used as high-throughput, rapid screening tools for toxicity evaluation. Ironically, since the work by Herzog et al.[11] was published no other group has used the clonogenic assay, yet most nanotube toxicity evaluations are predominantly carried out using the absorbance- or fluorescence-based assays. Monteiro-Riviere et al.[12] have recently screened a range of widely used viability and cytotoxicity assays with different nanoparticles including SWNTs. They found that SWNTs interfered to a different extent with most of the classical toxicity assays used.[12] Interestingly, Guo et al.[13] also reported that SWNTs cause the depletion of amino acids and vitamins from the media leading to reduced viability of cultured liver hepatocellular carcinoma (HepG2) cells. Therefore, the need for a rapid and reliable screening cytotoxicity assay has become indispensable for the valid assessment of the myriad carbon nanotube types fabricated and used today.

Here, we have developed a modified version of the LDH protocol as a valid assay for screening carbon nanotube cytotoxicity by attempting to eliminate all interactions between the small probe molecules used and the carbon nanomaterial, thus minimizing all possible artifacts. Two types of surface-modified MWNTs were tested: ammonium functionalized MWNTs (MWNT:NH_3^+) and Pluronic F127-coated MWNTs (MWNT:-F127). Cellular internalization within lung epithelial A549 cells of the two types of MWNTs were comparatively determined by optical microscopy, electron microscopy, and flow cytometry and were then correlated with their ensuing cytotoxicity by the modified LDH assay and a panel of conventional and widely used cytotoxic assays of various biological endpoints.

2. Results

2.1. Aqueous Dispersibility and Individualization of Surface-Modified MWNTs

The dispersibility in aqueous media is a key requirement if CNTs are to be used for biomedical applications. In an attempt to find a correlation between the type of CNT surface modification and their toxicological profile, we performed a direct comparison between two types of widely used surface-modified CNTs. The two types of MWNTs (**Figure 1**) were prepared either by chemical functionalization via conversion of the carboxylic groups into amino functionalities by an amidation reaction generating ammonium functionalized MWNTs (MWNT-NH_3^+) or by non-covalently coating the MWNT with the block copolymer Pluronic F127, which exhibit surfactant-like properties, affording F127-coated MWNTs (MWNT:F127). The two MWNT types were chosen not only because of their different surface-modification approaches that have been repeatedly used in the literature, but also because they showed similar characteristics in terms of aqueous dispersibility and individualization properties as observed by transmission electron microscopy (TEM) (Figure 1, see CNT alone).

2.2. Assessment of MWNTs Uptake in A549 cells by Flow Cytometry and Light Microscopy

Flow cytometry analysis was used to determine the degree of uptake of the two different types of MWNTs as has been described earlier.[14,15] Such an assay has been initially proposed to assess the interaction (adsorption and uptake) between nanoparticles and cells.[16,17] Uptake of CNT by cells has also been assessed using the same technique by our laboratories[14,15] and those of others[18] by measuring the increase in cell granularity or light scatter by cells incubated with CNTs. As shown in Figure 1a, there was a dose-dependent increase in the side scatter (90 ° degree) of laser light (at 488 nm) by cells incubated with increasing concentrations of MWNT-NH_3^+. On the other hand, a minimal change in side scatter of light was obtained with cells incubated with MWNT:F127 even at concentrations as high as 125 μg mL^{-1} suggesting minimal

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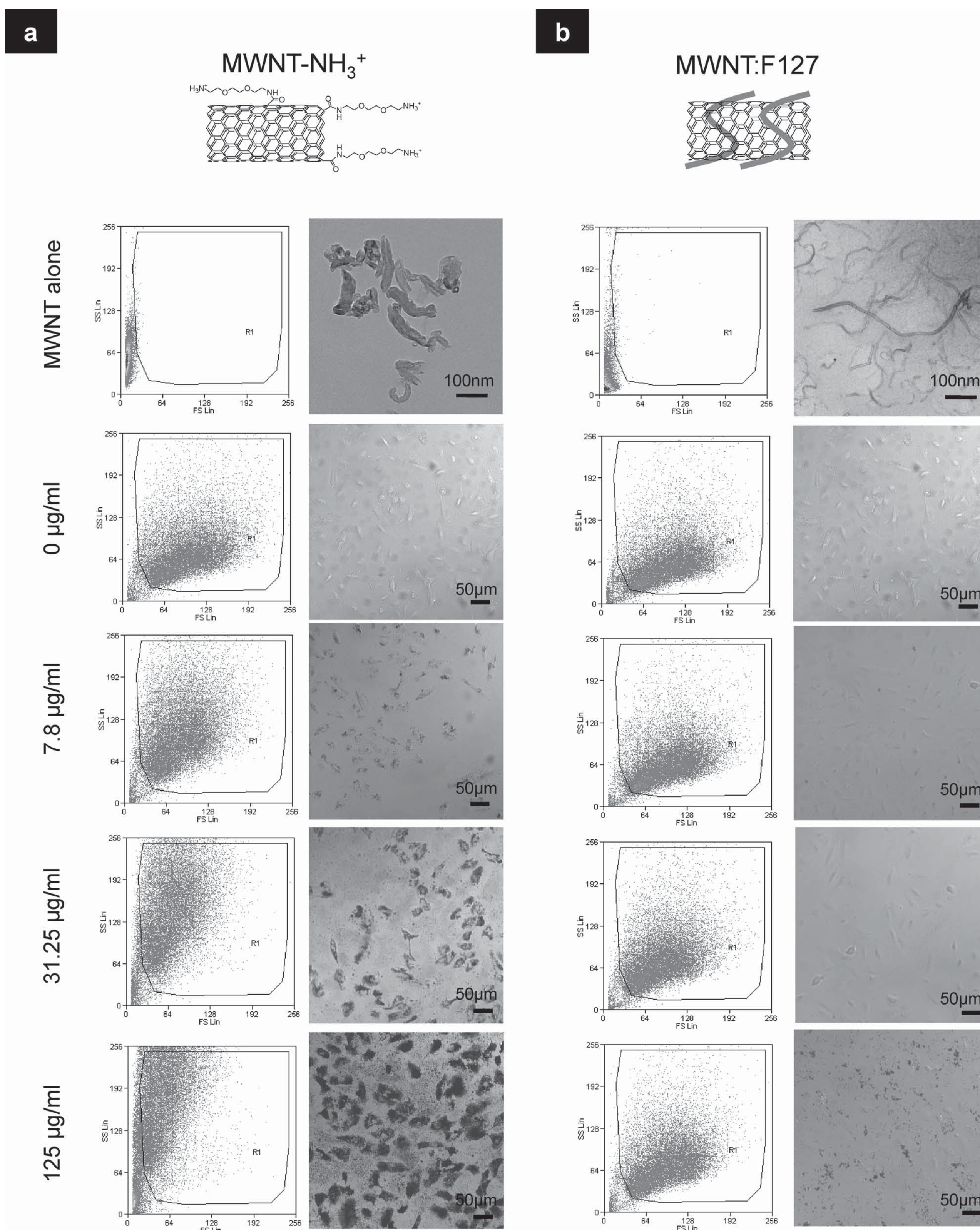

Figure 1. Analysis of cellular uptake of surface-modified MWNTs using flow cytometry and light microscopy. Human lung carcinoma A549 cells were incubated for 24 h with surface-modified MWNTs (0–125 µg mL^{-1}). Bivariate scatter histograms of the sideward-scattering (*Y*-axis) versus forward-scattering (*X*-axis) were recorded by flow cytometry for a) MWNT-NH_3^+ and b) MWNT:F127 and are shown along side light microscopy images of the treated cells. Cellular association or uptake of MWNT-NH_3^+ was confirmed by the increase in granularity (sideward scattering) as a function of concentration. The bivariate scatter histograms of CNT alone (MWNT-NH_3^+ and MWNT:F127) at 125 µg mL^{-1} without cells were also assessed (top panel) and are excluded from the analysis gate. TEM images of MWNT-NH_3^+ (a) and MWNT:F127 dispersion (b) show the good dispersibility of both samples (scale bar of 100 nm). Light microscopy images (20× magnification lens) were representative of three wells.

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cellular uptake compared to MWNT-NH_3^+ (Figure 1b). These results were in complete agreement with the light microscopy images shown in parallel to the dot-plot flow cytometry (FACS) graphs in Figure 1a,b (also Supporting Information Figure S1). Although light microscopy imaging is only qualitative and does not differentiate between membrane bound or internalized CNTs, in combination with the FACS data there is little doubt that MWNT-NH_3^+ showed significantly higher internalization within A549 cells. Almost the entire cell culture seemed to be completely stained in black (color of CNTs) in a concentration-dependent manner, while much less interaction was observed with MWNT:F127 after 24 h (Figure 1a,b). In addition, in order to further confirm the cell internalization of nanotubes, transmission electron microscopy data of A549 cell sections (**Figure 2**a,b) correlated well with the flow cytometry and light microscopy, showing overwhelming MWNT-NH_3^+ uptake inside A549 cells compared to fewer MWNT:F127 observed intracellularly (Figure 2c,d; see arrows).

2.3. Toxicity Assessment of MWNTs in A549 Cells

The cytotoxicity of MWNT-NH_3^+ and MWNT:F127 were then assessed using the conventional MTT, LDH, and Annexin V-FITC/PI assays and compared to the modified LDH assay in order to elucidate any interference between CNT and the molecules that are used as probes in the cytotoxicity assays mentioned above. It is noted that throughout these dose-dependent investigations, a dose of cationic (DOTAP:cholesterol) known to be cytotoxic to cells or dimethyl sulfoxide (DMSO) was also used as a positive control.

2.3.1. MTT Assay

The MTT assay showed false-negative cytotoxicity readings with MWNT-NH_3^+, indicated by a dose-dependent increase in cell viability as the concentration of MWNT-NH_3^+ increased from 1.9 to 125 μg mL^{-1} (Supporting Information Figure S2a). On the other hand, MWNT:F127 showed a reduction in A549 cell viability particularly at 125 μg mL^{-1} (Supporting Information Figure S2b). To mimic the interactions between MWNT and the chemicals used in an MTT assay, formazan (the product of MTT reduction) was spiked with increasing concentrations of both MWNT used in the study. Increased absorbance at 570 nm was observed at MWNT concentrations higher than 15 μg mL^{-1} in a concentration dependent manner for both types of MWNT (Supporting Information Figure S2c). This was thought to indicate that the intrinsic absorbance of MWNT could interfere with the reagents used in an MTT assay. Higher degree of interference with MWNT-NH_3^+ compared to MWNT:F127 was observed in the MTT spiking experiment (Supporting Information Figure S2c) that could be due to their better aqueous dispersibility. Therefore, it remained unclear based on the MTT assay data, whether the cytotoxicity observed with exposure of cells to MWNT:F127 at 125 μg mL^{-1} (Supporting Information Figure S2b) was genuine or due to any interactions between MWNT and the insoluble formazan as reported previously by others.[8]

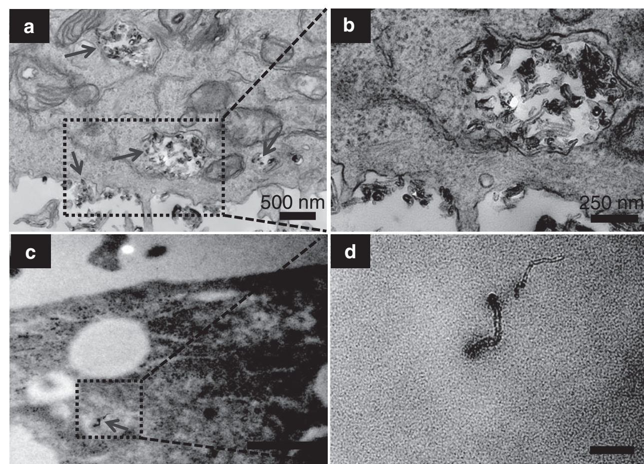

Figure 2. Cellular internalization of surface-modified MWNTs inside an ultrathin A549 cell section observed by TEM. a,b) MWNT-NH_3^+ observed inside intracellular vesicles and c,d) MWNT:F127 inside A549 unstained cell sections. Panels (b,d) are high-magnification images of MWNT containing sections from (a,c). Blue arrows indicate MWNTs.

2.3.2. Annexin V-FITC/PI Assay

The second assay studied was the flow cytometry-based Annexin V-FITC/PI staining. This is a well-established in vitro cytotoxicity assay in which the differentiation between the early and late stages of cell apoptosis and necrosis can be determined following exposure to test material. In the early stages of apoptosis, phosphatidylserine flip-flops to the outer layer of the plasma membrane, to which the Annexin V-FITC, a calcium-dependent phospholipid binding protein, binds giving rise to green fluorescence signals. At later stages of apoptosis or during necrosis, the membrane impermeable dye propidium iodide crosses defective plasma membranes and reaches the nucleus so cells stains in red fluorescence. Figure S3 (Supporting Information) shows the flow cytometry dot-plots representing the live cells (bottom left quadrant, FITC-/PI-), early apotopic cells (bottom right quadrant, FITC+/PI-), late apoptotic/necrotic cells (top right quadrant, FITC+/PI +), and cell debris (top left quadrant, FITC-/PI +). Annexin V/PI staining assay showed no induced apoptosis or necrosis after 24 h of incubation at a range of concentrations (1.9–125 μg mL^{-1}) of both types of MWNT or the Pluronic F127 alone at equivalent concentrations used (Supporting Information Figure S3). In comparison, the cationic liposome positive control, showed both apoptosis and necrosis of the cells at a toxic concentration (0.48 mM) (Supporting Information Figure S3). As the Annexin V/PI assay indicated, no considerable toxicity with either MWNT types and for the dose escalation regime chosen could be determined, possibly due to quenching of the fluorescent probes by CNT.[19] However, it was difficult from these results alone to establish the validity of Annexin V/PI staining as a screening tool to assess the cytotoxicity of MWNTs, so a third assay was still required.

2.3.3. Conventional and Modified LDH Assays

Worle-Knirsch and co-workers[8] have suggested that the LDH assay can be appropriate to study CNT-mediated cytotoxicity. The original LDH assay is based on the indirect

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determination of the number of damaged or lysed cells by measuring the amount of LDH secreted into the culture media following cell damage. Figure S4 (Supporting Information) showed an apparent increase in LDH release after incubation with both MWNT types indicating signs of toxicity induced by MWNT. However, according to the original LDH assay protocol, the cell media is incubated with the LDH substrate to calculate the percentage of LDH released from damaged cells, corresponding to the percentage of cell death. Herein, there is a risk of interference from the intrinsic absorbance of CNT at 490 nm and such interference was confirmed by the similar absorbance readings obtained from the CNT containing (spiked) media (without incubation with cells) shown as black bars in Figure S4a,b (Supporting Information). The interference was more striking with the MWNT:F127 (Supporting Information Figure S4b). Moreover, the positive control (cells treated with 10% DMSO) did not cause an increase in LDH release, instead showed an apparent decrease compared to untreated control cells which could be due to the inhibition of the enzyme activity by the organic solvent DMSO (Supporting Information Figure S4).

We then modified the LDH assay to completely exclude all MWNT from the reaction medium in an attempt to eliminate the risks of interference. This was achieved by initially lysing cells that were not damaged by MWNT followed by centrifugation of the cell lysate to precipitate and remove the pelleted CNT before incubation with the LDH substrate. It is important to note that centrifugation of the cell media alone used in the original LDH assay could not lead to precipitation and removal of all MWNT (since both types used were highly dispersed) prior to the addition of the LDH reagents (data not shown). In addition, the time points assessed by the original LDH assay cannot be very long because the half life of the LDH is only 9 h once released from the cells. The modified LDH proposed here can also allow for longer time points of assessment.[12]

The modified LDH assay showed that cell exposures with the chemically functionalized MWNT-NH_3^+ from both manufacturers (Nanocyl and Nanostructured & Amorphous Materials Inc.) were not cytotoxic at the used concentration range (1.9–125 μg mL^{-1}) after 24 or 48 h incubation periods (**Figure 3** and Supporting Information Figure S5). In comparison, cell treatment with the polymer-coated MWNT:F127 led to a dose-dependent toxicity with 60% and 40% viability at 125 μg mL^{-1} concentration after 24 and 48 h, respectively. Treatment with Pluronic-F127 block copolymer alone did not exhibit any signs of toxicity with almost 100% cell viability after 24 h, even though cytotoxicity was observed (60% cell viability) after 48 h at the equivalent highest dose used.

2.3.4. Ultrastructural Changes in A549 Cells by TEM

To confirm the validity of the modified LDH assay as a screening tool for CNT cytotoxicity, the assay was combined with extensive monitoring of any ultrastructural changes of the intracellular compartments in A549 cells after incubation with both types of MWNT using TEM. Despite the significantly higher degree of cellular uptake observed by light microscopy, flow cytometry (Figure 1) and TEM (Figure 2) in the case of MWNT-NH_3^+, only MWNT:F127 treated cells (with 50 μg mL^{-1}) exhibited mitochondrial abnormalities. Mitochondria in those cells appeared swollen and with loss of inner invaginations and folds of their membrane (cristae) (**Figure 4**). Interestingly, Pluronic F127 alone at an equivalent concentration (500 μg mL^{-1}) did not cause any mitochondrial damage or any other characteristic structural abnormalities, as was also the case with MWNT-NH_3^+. The average length of mitochondria in untreated cells and those treated with F127 alone was found to be around 500–600 μm as shown in Figure 4a. Cells treated with MWNT:F127 showed enlarged mitochondria with average lengths between 1000 and 1200 μm and only 10% of the mitochondria (number of mitochondria, $n = 29$) appearing to be morphologically healthy (H). These results were considered as validation of the cytotoxicity data observed with the modified LDH assay, indicating that the observed cytotoxicity from treatment of A549 cells with Pluronic-coated MWNTs was due to significant mitochondrial damage.

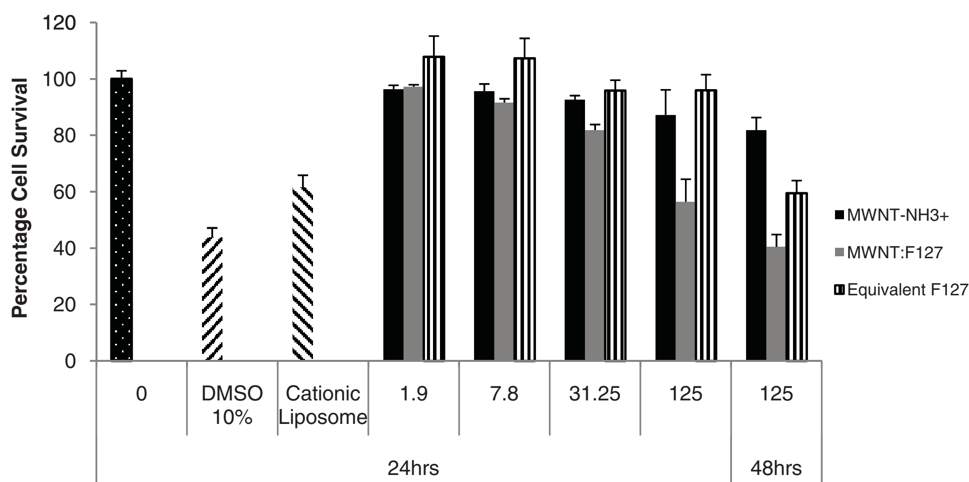

Figure 3. Modified LDH assay of A549 cells. A549 cells were incubated for 24 h with MWNT-NH_3^+ (0–125 μg mL^{-1}), MWNT:F127 (0–125 μg mL^{-1}), or Pluronic F127 alone (0–1250 μg mL^{-1}). DMSO (10%) was used as a positive control. A dose dependent cytotoxicity was observed with MWNT:F127 after 24 h incubation.

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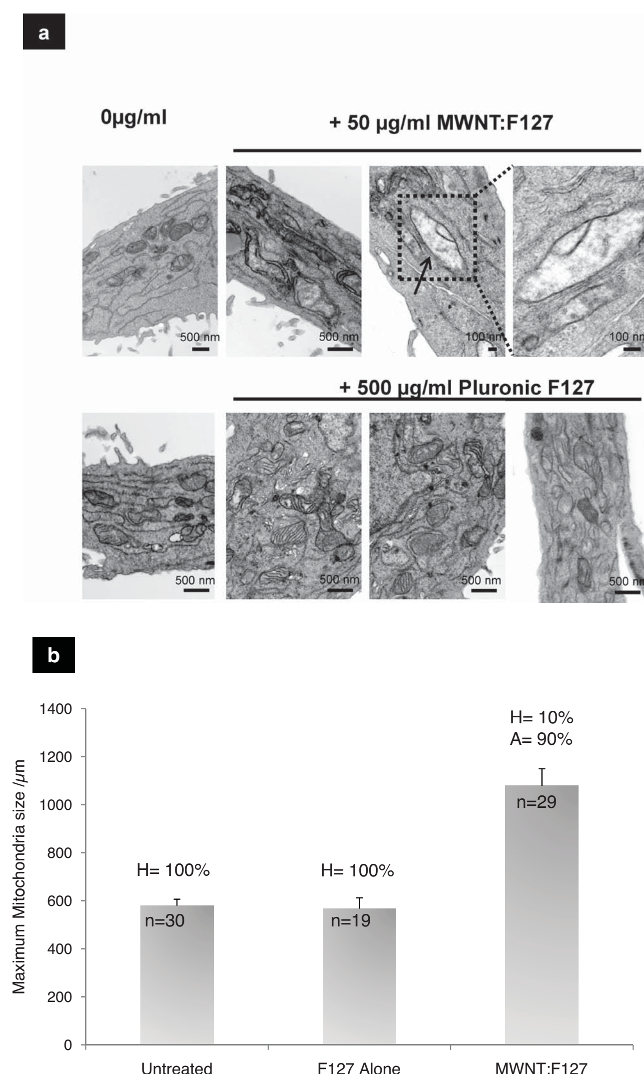

Figure 4. Mitochondrial damage by MWNT: F127. a) TEM of ultrathin A549 cell sections treated with MWNT:F127 (50 µg mL^{-1}) or Pluronic F127 alone (500 µg mL^{-1}) for 24 h. Mitochondrial damage (shown by arrows) was observed in MWNT:F127 treated cells but not with Pluronic F127 alone. b) Quantitative analysis of mitochondrial changes expressed as average mitochondria length (µm) ± standard deviation (SD) ($n = 19–30$), as measured using Image J. The percentages of healthy (H) versus abnormal mitochondria (A) are shown above the bars.

3. Discussion

As the use of CNT is expanding, comparison between the published studies is very hard to make due to differences in most measured parameters. On the one hand non-functionalized CNTs are being tested while their dispersibility is considered poor for use in biomedical applications. On the other hand, different assays, tissue cultures, incubation times, and dose ranges have been reported. Hence, it was thought that a systematic comparison of the cellular uptake and cytotoxicity of CNTs can only be made if materials have similar physical properties and are compared using the same assays under the same conditions. In view of this, two types of surface modification methodologies that render the CNTs water dispersible were explored. The first was based on the chemical functionalization of purified MWNT by oxidation and subsequent conversion of the carboxylic groups into ammonium functionalities, while the second strategy was based on the non-covalent coating of purified MWNT with Pluronic F127 block copolymer.

First, the cellular uptake of MWNT-NH_3^+ and MWNT:F127 was investigated based on changes in the side scatter of laser light by cells incubated with MWNT in addition to light microscopy imaging. It was found that MWNT-NH_3^+, which did not contain any macromolecules on their surface, were taken up in large quantities by cells and were not toxic to A549 cells, MWNT:F127 were internalized to a much lesser extent but were found to be cytotoxic particularly at the highest doses studied.

The dose-dependent cytotoxicity observed here with MWNT:F127 using the MTT assay (Supporting Information Figure S1) can either be attributed to a real biological activity leading to cell damage or due to the strong interaction between the MWNT and the insoluble formazan as suggested by Krug and co-workers.[8] However, false negative results may also be obtained (indicating higher cell viability), which can be due to the intrinsic absorbance of CNT at 570 nm particularly if higher cellular uptake of CNT occurs as was the case with the MWNT-NH_3^+ (Supporting Information Figure S2). Similar results have been described by Monteiro-Riviere et al. stating the possibility of false indications of cell viability when carbon black (CB) toxicity was studied using the Neutral Red staining, MTT, and interleukin release assays due to strong van der Waals interactions.[20] Due to such possible interactions associated with the MTT assay (the CNT intrinsic absorbance and CNT-formazan interaction) it is considered greatly unreliably and should be avoided, especially in cases of high cellular uptake of CNT.

Assays with different biological endpoints such as the Annexin V/PI are very useful in providing information about the mechanism of cell death (i.e., apoptosis or necrosis) in addition to the time frame during which apoptosis occurs. However, this assay was not found to be sensitive enough in the dose regime studied here to assess CNT cytotoxicity and, especially in cases when the cell damage expected, is not apparent. This may also be due to the fact that the fluorescent probes used (FITC (fluorescein) and PI) can be easily quenched on interaction with the CNT[19] or due the logistics of the protocol in which the apoptotic cell population can be easily underestimated because of removal of the incubation medium required. Monteiro-Riviere et al.[12] have also recently mentioned the latter issue in relation to the Annexin V/PI assay.

The third cytotoxicity assay studied was the LDH assay which is based on the indirect assessment of the number of lysed cells based on the amount of released LDH following cellular damage as the LDH enzyme is cytoplasmic in nature. This assay needed to be modified because it is also colorimetry-based and could give rise to false negatives (an untrue high cell viability) due to the intrinsic absorbance of CNTs at 490 nm as was also observed with MTT assay (Supporting Information Figure S2). In the modified LDH assay protocol developed, all cells were lysed at given time points and the whole cell lysate was centrifuged to remove and discard all

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CNTs associated with the cells. In this way we eliminated any subsequent direct or indirect interference between the CNTs and the assay reagents, hence the assessed LDH content measured directly reflected the number of viable cells after treatment.

Based on the data obtained, we concluded that the polymer-coated MWNT:F127 were more prone to lead to cytotoxic responses compared to chemically functionalized MWNT-NH_3^+ (from two different nanotube manufacturers) that showed high cell viability even at the highest exposure doses. The block copolymer F127 alone was found to be non-cytotoxic at concentrations equivalent to those in MWNT:F127 as shown by the MTT, original LDH, and modified LDH assays (Supporting Information Figure S2,S3 and Figure 3). However, it can be speculated that more copolymer molecules may possibly be internalized within A549 cells in the presence of CNTs (via the interaction of nanotubes with the plasma membrane). In that case, the observed cytotoxicity of the MWNT:F127 may be attributed to higher concentrations of the copolymer inside cells in comparison to cells treated with F127 alone. It is worth mentioning that Kabanov et al.[21] have previously suggested that block copolymers can act as biological modulators, even though generally regarded as biologically inert entities. Pluronic copolymers have also been described to affect the microviscosity of membranes. In addition, Pluronic copolymers (mainly the unimers) have been found to partition intracellularly with the mitochondrial membranes leading to subsequent release of cytochrome C and increase in the level of reactive oxygen species,[21] which can lead to the inhibition of cellular respiration. Lastly, one cannot also completely exclude any direct damage to the mitochondria caused by MWNTs similar to the report by Narita et al.,[22] who observed MWNTs inside these organelles even though our TEM studies did not offer any such evidence.

Overall, our studies advised that the MTT assay should be avoided because of high risk of unreliable data collection, primarily due to molecular interactions between CNTs and the small molecules used. Moreover, the Annexin V/PI assay, although reliable and biologically useful, it was found to be less sensitive than the modified LDH assay to assess the possible minute or early-stage cytotoxic responses from exposure to CNTs and should be used with caution, always in combination with other assays. The combination of the modified LDH assay with the intracellular structural studies by TEM were found to be the most powerful and reliable tools for the accurate assessment of the biological impact and any adverse effects of CNTs in contact with living cells.

4. Experimental Section

4.1. Materials

Purified MWNTs (pMWNTs), the precursor of ammonium functionalized MWNTs (MWNT-NH_3^+), were purchased from Nanostructured & Amorphous Materials Inc. (Houston, USA) at 94% purity (stock no. 1240XH). The pMWNTs used to prepare Pluronic-coated MWNT were obtained from Nanocyl (Belgium) at ≥95% purity (stock no. NC3150). Chemicals and solvents were obtained from Sigma-Aldrich (UK) and used as received. F12 Ham media, fetal bovine serum (FBS), penicillin/streptomycin, and phosphate buffered saline (PBS) were purchased from Gibco (Invitrogen, UK). The Annexin V/PI kit and the Promega Cytotox 96 non-radioactive cytotoxicity assay (LDH kit) were purchased from Roche (UK) and Promega (UK) Ltd., respectively.

4.2. Surface-Modified MWNT Dispersions

Pluronic-coated MWNTs (MWNT:F127) were prepared by dispersion in 1% Pluronic F127 at a final concentration of 1 mg mL^{-1} by bath sonication for 30 min as previously described.[4] Functionalized MWNT-NH_3^+ (from both Nanstructured & Amorphous Materials Inc. and Nanocyl) were synthesized as follows: oxidized MWNTs were obtained following the procedure previously reported;[23] 200 mg were heated in 10 mL of neat oxalyl chloride at 62 °C for 24 h. After evaporation in vacuo the resulting nanotubes were dispersed in a solution of Boc-monoprotected diamino-triethyleneglycol (TEG) (530 mg) in distilled tetrahydrofuran (THF; 15 mL) and heated at reflux for 48 h.[24] The nanotubes were re-precipitated several times from methanol/diethyl ether by successive sonication and centrifugation. The Boc protecting groups were removed overnight using 4 M HCl in dioxane (20 mL) to afford ammonium functionalized MWNTs (MWNT-NH_3^+) (185 mg) following evaporation of the acid solution and re-precipitation in diethyl ether. The Kaiser test showed a loading of 320 μmol of amino groups per gram nanotube material. The MWNT-NH_3^+ were dispersed in 5% dextrose for toxicological assessment.

4.2.1. Transmission Electron Microscopy (TEM) of Surface-Modified MWNTs Dispersions

A drop of MWNT dispersion was placed on a grid with a support film of Formvar/carbon, excess material was blotted off with a filter paper and the MWNT were examined under FEI CMI 20 BioTwin Electron Microscope (Einhoven, Netherlands) using a Lab6 emitters. Images were captured using AMT Digital Camera.

4.3. Culture of A549 Monolayers

Epithelial lung carcinoma cells (A549; ATCC, CCL-185) were maintained and passaged in F12 Ham media supplemented with 10% FBS, 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, and 1% L-glutamine at 37 °C in 5% CO_2. Cells were passaged twice a week using trypsin-EDTA 0.05% when reaching 80% confluency.

4.4. Cellular Uptake of Surface Modified MWNTs by Flow Cytometry (Side-Scatter Analysis) and Light Microscopy

MWNT-:NH_3^+ and MWNT:-F127 were used as sterile stock solutions at 0.5 mg mL^{-1} (in 5% dextrose) and 1 mg mL^{-1} (in water), respectively. Stock dispersions were bath sonicated

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for 15 min prior use in cell culture studies. A549 cells were seeded into 24-well plates (50 000 cells per well) and left to attach overnight before incubation with both types of MWNTs. Cells were then incubated for 24 h with MWNT-NH_3^+, MWNT:F127 (0–125 μg mL^{-1}) or the equivalent Pluronic F127 concentration (0–1250 μg mL^{-1}) in complete media at 37 °C in a humidified atmosphere (5% CO_2). Monolayers were photographed before trypsinization to compare the results obtained from light microscopy with sideward-scattering light analysis. For light scatter analysis by flow cytometry, cells were washed with PBS to remove unbound MWNT, trypsinized, centrifuged at 240 × g for 5 min at 4 °C, and resuspended in PBS then transferred to 1.5 mL microcentrifuge tubes and kept on ice for immediate analysis by flow cytometry.

4.5. Transmission Electron Microscopy of Cell Sections

In TEM studies, cells were incubated with MWNT at 50 μg mL^{-1} for 24 h. Following the incubations, cells were rinsed briefly in saline (0.9% NaCl) to remove any non-ingested particles and were then fixed in 4% glutaraldehyde in 0.1% piperazine-*N*,*N'*-bis(2-ethanesulfonic acid (PIPES) buffer, pH 7.2 for 1 h at 4 °C. After several rinses in deionized water (DIW), the samples were osmicated (1% OsO_4, 0.15% potassium ferricyanide; 2 mM $CaCl_2$ in DIW) for 1 h at room temperature (RT). This osmication process also acts to enhance contrast from lipids inside the cells. Again, the samples were washed several times with DIW and then bulk stained for 1 h at RT in the dark using uranyl acetate. Following two washes in DIW, the sample was dehydrated in graded solutions of ethanol (70%, 95%, 100%), three times in each solution for 5 min. After an additional two washes in 100% acetonitrile, the sample was infiltrated with Quetol 651 resin over 4 d using fresh resin each day. Resin was cured at 60 °C for 24 h. Ultrathin sections (≈70 nm) were cut using a Leica Ultracut ultramicrotome mounted on 300 mesh bare copper grids and post-stained with uranyl acetate and lead citrate for 2 min each. TEM images were captured using a Gatan 2k × 2k charge-coupled device (CCD) camera US1000.

Quantitative analysis of mitochondrial damage was performed based on the average length (μm) of all counted mitochondria (n = 19–30) in the TEM cell sections using Image J analysis. The percentage of healthy (H) versus abnormal mitochondria (A) was also given.

4.6. Cytotoxicity Assessment of Surface-Modified MWNTs

4.6.1. MTT Assay

MTT solution was prepared as 5 mg mL^{-1} stock in sterile PBS, 0.2 μm filtered and was stored as 2 mL aliquots at –20 °C. DMSO was used as formazan solubilization solution. A549 cells were seeded at a density of 10 000 cells per well in flat bottomed 96-well plates and left to adhere overnight at 37 °C and 5% CO_2. Cells were then treated with the MWNT-NH_3^+ or MWNT:F127 dispersions diluted in complete media at a concentration range from 0–125 μg mL^{-1} for 24 h. Healthy control cells were incubated with complete media while positive controls were 10% DMSO treated. After 24 h, the medium was removed and replaced by 120 μL of MTT solution (20 μL MTT stock solution diluted with 100 μL complete media) for 3.5 h. The MTT solution was then replaced by 100 μL of DMSO to each well and left for 10 to 15 min at 37 °C to solubilize the formazan crystals. The absorbance was measured at 570 nm using a plate reader to determine the cell viability as a percentage of healthy control cells. In a formazan spiking experiment, healthy control cells were incubated with MTT solution under the same conditions for 3.5 h and then the produced formazan was dissolved in DMSO and allowed to interact with a range of MWNT (MWNT-NH_3^+ and MWNT:F127)concentrations (0–250 μg mL^{-1}) and absorbance read at 570 nm.

4.6.2. Annexin V-FITC/PI Assay

The Annexin-V-FITC/PI staining assay was performed according to the instructions of the manufacturer. In brief, cells were trypsinized, centrifuged at 240 × g for 5 min, washed twice with PBS, and stained with Annexin V/PI solution for 15 min at 15–25 °C. Cationic liposomes (DOTAP(N-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate) :cholesterol 2: 1) were used as positive cytotoxic control at 0.48 mM final concentration. 20 000 cells were analyzed on a CyAn ADP flow cytometer (DakoCytomation) using 488 nm excitation and 515 nm and 615 nm bandpass filters for FITC and PI detection, respectively. Electronic compensation of the instrument was performed to exclude overlapping of the two emission spectra. Percentage cells stained with Annexin V, PI, or both was calculated.

4.6.3. Original Lactate Dehydrogenase (LDH) Assay

LDH assay was performed using the Promega Cytotox 96 non-radioactive cytotoxicity assay according to the manufacturer instructions. After incubation with MWNT (as described in MTT assay), 50 μL cell media was mixed with 50 μL of the substrate mix in a new microtiter plate and incubated for 30 min at room temperature. The absorbance at 490 nm was measured using a plate reader. The amount of LDH released was an indication of the number of damaged or lysed cells. The percentage cell death or LDH released was calculated using the following equation:

$$\%\text{LDH released} = \frac{A_{490\,\text{nm of treated and untreated cells}} - A_{490\,\text{nm of media alone}}}{A_{490\,\text{nm of maximum of untreated cells}} - A_{490\,\text{nm of media alone}}} \times 100$$

Where $A_{490\,\text{nm}}$ is the absorbance at the wavelength 490 nm; $A_{490\,\text{nm of media alone}}$ is the absorbance of the media alone at 490 nm; and $A_{490\,\text{nm of maximum untreated cells}}$ is the absorbance of the maximum LDH induced from the untreated cells. In an attempt to evaluate the interference from the intrinsic absorbance of MWNTs on the original LDH assay protocol, the absorbance of the MWNT dispersions was also read at 490 nm. This control was referred to as “CNT containing media (no cells)” in comparison to the actual assay, which was referred to as “LDH cell media.”

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4.6.4. Modified LDH Assay

LDH assay was subsequently modified to avoid any interference from MWNTs. The LDH content was assessed in intact cells that survived the treatment, instead of detecting the amount of LDH released in the media upon MWNT induced-cell death. In brief, media was aspirated and cells were lysed with 10 μL of lysis buffer (0.9% Triton X100) mixed with 100 μL serum and phenol free media for 45–60 min at 37 °C to obtain cell lysate, which was then centrifuged at 16 060 × g for 5 min in order to pellet down the MWNTs. 50 μL of the supernatant of the cell lysate was mixed with 50 μL of LDH substrate mix in a new microtiter plate and incubated for 15 min at room temperature. The absorbance was read at 490 nm using a plate reader. The amount of LDH detected represented the number of cells that survived the treatment. The percentage cell survival was calculated using the following equation:

$$\%\,\text{Cell Survival} = \frac{A_{490\,\text{nm of treated cells}}}{A_{490\,\text{nm of untreated cells}}} \times 100$$

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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