Published in final edited form as:

Nanotoxicology. 2013 November ; 7: 1211–1224. doi:10.3109/17435390.2012.729274.

Combination of small size and carboxyl functionalisation causes cytotoxicity of short carbon nanotubes

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Abstract

The use of carbon nanotubes (CNTs) could improve medical diagnosis and treatment provided they show no adverse effects in the organism. In this study, short CNTs with different diameters with and without carboxyl surface functionalisation were assessed. After physicochemical characterisation, cytotoxicity in phagocytic and non-phagocytic cells was determined. The role of oxidative stress was evaluated according to the intracellular glutathione levels and protection by *N*-acetyl cysteine (NAC). In addition to this, the mode of cell death was also investigated. CNTs <8 nm acted more cytotoxic than CNTs 20 nm and carboxylated CNTs more than pristine CNTs. Protection by NAC was maximal for large diameter pristine CNTs and minimal for small diameter carboxylated CNTs. Thin (<8 nm) CNTs acted mainly by disruption of membrane integrity and CNTs with larger diameter induced mainly apoptotic changes. It is concluded that cytotoxicity of small carboxylated CNTs occurs by necrosis and cannot be prevented by antioxidants.

Keywords

single-walled carbon nanotubes; multi-walled carbon nanotubes; oxidative stress; apoptosis; necrosis

Introduction

Carbon nanotubes (CNTs) are used in a variety of technical applications, electronics and consumer products to improve their physical and chemical properties, and are also candidates for anti-cancer treatments, implants and advanced wound dressings (Zhang et al. 2010). Before these products are introduced to the market, those parameters which may cause toxicity have to be identified in order to develop CNTs devoid of cytotoxicity, immunogenicity, thrombogenicity and genotoxicity.

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Declaration of interest

The authors declare that there are no competing interests.

Supplementary material available online

Supplementary Figures 1s, 2s.

CNTs are made of graphene sheets arranged either in single sheets or in multiple layers. The number of layers/walls, purity, aspect ratio and surface functionalisation of the CNTs may determine their biocompatibility, however the previous reports on their biocompatibility show highly disparate effects. These disparate effects are displayed in the following: typical single-walled CNTs (SCNTs) are 1–2 nm in diameter, and the diameters of multi-walled CNTs (MCNTs) range from 2 to 100 nm. SCNTs are generally believed to act more cytotoxic than MCNTs (Cveticanin et al. 2010; Hu et al. 2010; Tian et al. 2006) but Yamashita et al. (2010) and Murr et al. (2005) reported correspondingly low cytotoxicity when SCNTs and MCNTs of similar size were compared. Nygaard et al. (2009), by contrast, described higher cytotoxicity of MCNTs. The differences in cytotoxicity between MCNTs and SCNTs are reported to be due to a lower purity and a higher tendency for aggregation of the SCNTs (Pulskamp et al. 2007; Wick et al. 2007). Metal contamination of the CNTs, especially iron, increases the generation of cytotoxic reactive oxygen species (ROS). However, it also appears that relatively pure CNTs act cytotoxic by generation of ROS (Di Giorgio et al. 2011; Srivastava et al. 2011). In addition to contamination, the aspect ratio (ratio of diameter to length) is believed to be an important determinant for cytotoxicity. In most reports, greater cytotoxicity was seen for long and thin CNTs (Kalbacova et al. 2006; Kim et al. 2010; Magrez et al. 2006; Poland et al. 2008), but other data did not show a dependency from the aspect ratio (Simon-Deckers et al. 2008; Wang et al. 2009). The low degree of hydrophilicity of the CNTs induced the formation of aggregates once the CNTs were suspended in physiological solutions. This effect was unwanted because it decreased the bioavailability of the tubes, hampered the dosing and made handling of CNTs difficult. Acid treatment was included to increase hydrophilicity and improve dispensability but this also changed the cytotoxicity. Acid-treated, carboxyl-functionalised SCNTs and MCNTs were found to act more cytotoxic than their pristine counterparts in several studies (Dong et al. 2011; Liu et al. 2010a; Patlolla et al. 2010; Saxena et al. 2007; Vittorio et al. 2009; Wang et al. 2011), which has been attributed to the higher number of structural defects generated during the functionalisation (Muller et al. 2008). Other authors, however, did not report a clear indication for an increased toxicity nor showed any difference in the cytotoxic action between carboxylated and pristine CNTs (Albini et al. 2010; Lee & Geckeler 2010).

Despite numerous studies on the toxicity of CNTs, no relation between certain particle parameters and cytotoxic action of CNTs are available because disparate effects were reported, as demonstrated in the previous section.

The reasons for these disparate findings are manifold. One main cause consists of the purity and quality of the CNTs; CNTs are mostly produced by arc discharge, laser ablation or chemical vapour deposition. These techniques differ in yield, costs, range of tube lengths, quality of the tubes and control over parameters (Duncan et al. 2007). CNTs produced by arc discharge need further purification but show quality. The CNTs, which are obtained by laser ablation, are well defined and have high quality but the technique is very expensive. CNTs obtained by chemical vapour deposition are mainly used for industrial production, the product is pure but defects in the tubes are common. These defects are believed to increase cytotoxicity due to the generation of ROS (Vittorio et al. 2009). Irrespective of the fact that CNTs are produced by the same mode of synthesis, the products from different producers may differ in terms of diameters and polydispersity. As aggregation influences the biological activity of the tubes, different results are expected. Differences in dispersion are also caused by different mechanical pre-treatment and preparation of the dispersions. Ultrasound treatment increases the dispersion of the tubes but it also breaks down these tubes (Lu 1996), thereby increasing cytotoxicity (Vankoningsloo et al. 2010). The presence or absence of natural and synthetic dispersants in the medium is linked to additional differences. Some of the synthetic dispersants, for instance, Triton X-100, cause adverse cellular effects independent from the effect of the CNTs. Such effects caused by the dispersant were

reported by some studies although not by others (Alpatova et al. 2010). An additional problem shows the interference of CNTs with conventional cytotoxicity screening assays described in several studies (Monteiro-Riviere et al. 2009; Worle-Knirsch et al. 2006; Belyanskaya et al. 2007; Davoren et al. 2007). As interference of CNTs and screening essays have not been mentioned, it is not clear if this effect was simply overlooked. It could be that in those studies, where no interference was reported, also no controls were included to account for such interference. Particularly for nanoparticles, the assay conditions play an important role for the outcome of cytotoxicity testing (Geys et al. 2010). In addition to this, biological differences of the cell lines used for the testing play an important role. Such differences in the sensitivity were identified by the testing of more than one cell line (Herzog et al. 2007; Hu et al. 2010; Sohaebuddin et al. 2010; Soto et al. 2007; Thurnherr et al. 2011).

On one hand, production, contamination and differences in purity of the CNTs influence the result. On the other hand, there are problems in the preparation of the samples for the biological testing, choice of the assay and the cell lines. Therefore, CNTs with different aspect ratios and surface functionalisation, but similar content of metal catalysts, should be studied. With the exception of a few examples, such studies are currently unavailable. Through the use of CNTs from the same commercial provider, Sohaebuddin et al. (2010) identified higher cytotoxicity of thin MCNTs in non-phagocytic cells and cytotoxicity of thicker MCNTs in phagocytic cells. Fenoglio et al. (2012) identified the diameter as a decisive parameter for the toxic effects of CNTs *in vitro* and *in vivo*.

For biomedical applications, particularly for imaging, short and ultra-short CNTs are preferred because many target cells (e.g. cancer cells) are non-phagocytic cells, and they take up short CNTs more easily than long CNTs (Sitharaman et al. 2010). The most promising applications for CNTs are for vaccinations and cancer treatments. The advantage of CNTs for cancer treatment is their high loading capacity, 35% mitoxantrone (w/w) and 20% doxorubicin (w/w) for SCNTs (Heister et al. 2012), 38% paclitaxel (w/w) for MCNTs and 36% for pegylated gMCNTs (Lay et al. 2010; Sobhani et al. 2011) combined with their ability of infrared light absorption. Absorption of infrared light by cellular chromophores and normal tissues in general is minimal and leads to the selective destruction of cancer cells targeted by CNTs. MCNTs showed a higher efficacy of gene delivery than liposomes (Madani et al. 2011).

Although numerous studies on the toxicity of CNTs have been published, the role of specific parameters, especially diameter and surface charge, is not clear because different preparations, exposure conditions and cell systems were used. This study investigates the role of diameter and surface functionalisation in the cytotoxic effects of CNTs by using short MCNTs and SCNTs of different diameters and dependent on carboxyl functionalisation. Through the use of CNTs from the same supplier and demonstrating the absence of metal contamination, the authors studied the influence of diameter and surface functionalisation on different cell types. The panel of investigated cell types represents target organs for nanoparticle accumulation, blood and reticulo-endothelial system (EAhy926 cells for endothelial cells, DMBM-2 cells for macrophages and TK-6 cells for lymphocytes), liver (HepG2 cells for hepatocytes), lung (A459 for alveolar cells) and the ubiquitously present connective tissue (V79 fibroblasts). Cytotoxicity and mode of action (necrosis vs. apoptosis, intracellular oxidative stress) were analysed. Fetal bovine serum (FBS) was used as dispersant due to the fact that all nanomaterials are coated with protein and other macromolecules once they come into contact with body fluids (Cedervall et al. 2007).

Materials and methods

Particles

Short CNTs (0.5–2 μm) with and without COOH-functionalisation were purchased from CheapTubes Inc. (Brattleboro, VT, USA). These CNTs are synthesised by catalytic chemical vapour deposition and are purified with dilute nitric acid. MCNTs are functionalised through repeated reductions, and extractions in KMnO_4 (unpublished information from the provider) and show a low amount of contaminants (ash <1.5 wt%). SCNTs contain <3 wt% amorphous carbon and 5–6 wt% double-walled CNTs (DWCNTs)/MCNTs (CheapTubes Inc., product information, 2011). SCNTs are functionalised with air oxidation (unpublished information from the provider).

SCNTs (termed SCNT and SCNTc) with 1–2 μm diameter, purity >90% and MCNTs in the diameters <8, 20–30 and >50 nm, purity >95%, termed as MCNT8 and MCNT8c, MCNT20 and MCNT20c, MCNT50 and MCNT50c were used.

Physicochemical characterisation of particles

All CNTs were characterised immediately after ultrasound treatment. All dilutions were prepared from a freshly prepared stock solution. Loose aggregates that started to form after 5 min were re-suspended by vortexing.

Characterisation by transmission electron microscopy—The CNTs were dispersed in DMEM (Dulbecco's modified Eagle's medium) cell culture medium at 1 mg/ml dilution and treated with ultrasound for 20 min; 5 μl of this solution was placed on a carbon-coated copper grid that had previously been treated with a Pelco easiGlowTM glow discharge device. After 1 min of incubation, the solution was withdrawn using non-hardened microscopic filter paper (Whatman, GE Healthcare Life Sciences, Dassel, Germany).

Images were taken using a FEI Tecnai G2 20 transmission electron microscope (FEI Co., Hillsboro, OR, USA) with a Gatan UltraScan[®] 1000 CCD camera. Acceleration voltage was 80 kV. Sizes of CNTs were measured from the transmission electron microscopy (TEM) images.

For energy-dispersive X-ray spectroscopy (EDX), the specimens were diluted in double distilled water, sonicated and placed on pioloform-coated nickel grids. Scanning TEM (STEM) micrographs were made with a FEI Tecnai 20 electron microscope at 20,000 $\times$ magnification and 120 kV acceleration voltage with a high angle annular dark field detector. EDX spectra were made with a standard SUTW detector (EDAX) in rectangular selections within the field of view of these micrographs and each spectrum was recorded for 2 min. Data on the elemental composition were attained using Peak ID and Quantify functions of TEM imaging and analysis (TIA) software (FEI Co.).

The authors chose to use EDX in combination with STEM rather than with scanning EM. In STEM, the electron beam penetrates through the sample and thus would have recorded entrapped metals even if they were situated under-neath the surface of the CNTs. In addition, the authors made sure that each EDX spectrum was made from a single CNT rather than from an area where the CNTs aggregated. Images recorded with a high-angle annular dark field detector (Fischione Inc., Export, PA, USA) showed that the beam penetrated through these areas. A total of 12 spectra were recorded from the specimens and no metal contaminants were visible in any of these 12 spectra.

Characterisation by dynamic light scattering—Particles were characterised regarding size, and zeta potential was measured by dynamic light scattering (DLS) and laser

Doppler velocimetry (LDV) using a ZetaSizer Nano-ZS (Malvern Instruments, Malvern, UK). Particles were diluted with DMEM + 10% FBS to a concentration of 1 µg/ml and sonicated in an Elmasonic S40 water bath (ultrasonic frequency: 37 kHz, 40W, Elma, Singen, Germany) for 20 min. After equilibration of the sample solution to 25 °C, size and zeta potential was measured at 633 nm and a detection angle of 90°. NNLS software was used for the sample analysis.

Characterisation by light microscopy—During the incubation of CNTs with the cells, aggregates were formed. The sizes of these aggregates, which were bound to the cells, were determined from brightfield images taken with IX51 microscope (Olympus, Munich, Germany) and with LSM510 Meta microscope (Zeiss, Oberkochen, Germany).

Cell culture

EAhy926 (kind gift from Dr. C. J. Edgell), A549, HepG2, DMBM-2 and V79 (all obtained via Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and TK-6 cells (Cell lines service, Eppelheim, Germany) were studied. With the exception of the TK-6 cells, all cells were adherent cells.

Exposures for cytotoxicity screening

Adherent cells were cultured and seeded 24 h prior to the particle treatment in the medium recommended by the provider. CNTs were suspended in DMEM with 10% FBS and the suspensions were put into an Elmasonic S40 water bath (ultrasonic frequency: 37 kHz, 40 W, Elma, Singen) for 20 min before being added to the cells. Cells were exposed to CNTs in concentrations of 0–500 µg/ml. Routinely, CNT suspensions were applied directly to the cells. In order to study the effect of single particles and aggregates separately, suspensions were centrifuged $2465 \times g$ for 20 min at room temperature (RT) to remove large aggregates according to Raja et al. (2007) and only the supernatant was applied to the cells.

To assess the role of oxidative stress, cellular exposures were also performed in the presence of *N*-acetyl cysteine (NAC; Roth, Karlsruhe, Germany) using two protocols: 1) 500 µM of NAC during the entire incubation period and 2) pre-incubation of the cells with 1 mM NAC for 2 h and addition of the CNT suspension in the fresh medium.

Exposures were performed at 37 °C in a 95% air/5% CO₂ atmosphere and two time points (4 and 24 h) were evaluated. The effect of the interference was estimated by inclusion of additional controls, colour control (cells + CNT) and interference control (CNT + assay compounds). Plain polystyrene particles (20 nm; Thermo Scientific, Particle Technology, Fremont, CA, USA) at concentrations of 400 µg/ml (4 h exposure) and 200 µg/ml (24 h exposure) suspended in DMEM + 10% FBS served as positive particle control. As negative control, 500 µg/ml 200 nm plain polystyrene particles (Thermo Scientific) for both time points were used. The reaction to the controls varied between the cell lines from 0% to 8% viable cells for the positive controls and from 85% to 118% viable cells for the negative controls.

Adenosine triphosphate content—CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used according to the manufacturer's instructions. Plates were equilibrated to RT for approximately 30 min and reconstituted CellTiter-Glo Reagent was added 1:1 to the amount of cell culture medium present in each well. Plates were shaken for 2 min and incubated for additional 10 min at RT before well contents were transferred to a luminescence compatible 96-well plate and luminescence was read on a Lumistar (BMG Labtech, Offenburg, Germany).

Formazan bio reduction—CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Mannheim, Germany) was used according to the manufacturer's instructions. CNTs were removed by repeated washing with phosphate buffered saline (PBS) from the adherent cells; 20 µl of the combined MTS/PMS solution was added to 100 µl of each well and plates were incubated for 2 h at 37 °C in the cell incubator. Absorbance was read at 450 nm on a plate reader (SPECTRA MAX plus 384, Molecular Devices, Wals, Austria). Two different protocols were used for adherent cells. In one protocol, the formazan product was detected in the same plate. In order to correct for the absorbance of the CNTs, a control containing only cells + CNTs (without MTS reagent) was included for each concentration. From the absorbance of the formazan bio reduction, the absorbance of the cells + CNTs was then subtracted. This assay with control for each concentration is termed MTSco in the following.

In the other protocol, the supernatant after formation of the formazan product was transferred to a new plate to ensure that the signal was not influenced by absorbance of CNTs incorporated into cells. This assay is termed MTS in the following.

Cells in suspension were centrifuged after the reaction with the assay and the supernatant measured.

Quantification of DNA—The CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR, USA) was used for the quantification. After incubation and washing of the cells, plates were deep-frozen at −70 °C. Plates were then thawed at RT, and 200 µl of the CyQUANT GR dye/cell lysis buffer was added. Fluorescence was read at 485/520 nm using a fluorescence plate reader (FLUOstar Optima, BMG Labtech).

Mode of action

YoPro-1/PI labelling—Cells (1.2×10^5) were seeded per well and treated with CNTs suspended in DMEM with 10% FBS for 24 h. Cells were rinsed three times in PBS. The Vybrant Apoptosis Assay Kit #4 (Invitrogen, Lofer, Austria) composed of 100 µl YoPro-1 and 1.5 mM propidium iodide (PI) was used according to the instructions in the manual. Each dye of 0.5 µl was added to 1 ml DMEM, mixed and incubated for 30 min at 4 °C in the dark. Fluorescence was read at excitation/emission (Ex/Em) wavelength of 485/520 nm for YoPro-1 and 544/612 nm for PI using a fluorescence plate reader (FLUOstar Optima, BMG Labortechnik) and cells were viewed with a LSM510 Meta microscope (Zeiss) with the following settings: 488 nm/BP 505–550 nm for the green channel (YoPro-1) and 543 nm/LP 560 for the red channel (PI). The dose-dependent changes in YoPro-1 and PI signals on incubation with 50–10–200 µM hydrogen peroxide (H₂O₂) served as positive control.

Membrane integrity—The CytoTox-ONE® Homogeneous Membrane Integrity Assay (Promega) was used according to the instructions given by the producer. In brief, 2 µl of lysis solution was added to each lysis control well and all wells received a volume of CytoTox-ONE Reagent equal to the volume of cell culture medium present in each well. Incubation for 10 min at RT started after mixing for 30 s and was stopped by addition of 50 µl of Stop solution (per 100 µl of CytoTox-ONE Reagent added). Finally, the plate was shaken for 10 s and fluorescence recorded at a fluorometer (FLUOstar Optima, BMG Labtech) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. After the subtraction of the blank value, the average fluorescence from the samples was normalised to the maximum lactate dehydrogenase (LDH) release (lysis control).

Quantification of reduced glutathione—Cells were seeded in 75 cm² flasks and pre-cultured for 24 h and treated for 4 h with CNTs and positive control (*tert*-butylperoxide;

Sigma-Aldrich, Vienna, Austria). GSH was determined according to the manual of the Bioxtech GSH-400-colorimetric assay for glutathione (GSH; Oxis International Inc., Foster City, CA, USA). In short, cells were first rinsed three times with PBS to remove adhering CNTs and then scraped off from the plastic, suspended in PBS and collected by centrifugation. The cell pellet was suspended in 500 µl of 5% ice cold metaphosphoric acid in distilled water and centrifuged again; 300 µl from this supernatant was added to 600 µl of PBS and, upon mixing, 50 µl of each R1 reagent and R2 reagent were also added. After an incubation for 10 min at 25 °C in the dark, absorbance was read at 400 nm with a photometer (SPECTRA MAX plus 384, Molecular Devices). GSH standards were prepared and assessed in parallel to the samples.

Statistical analysis

Data from three to four independent experiments were subjected to statistical analysis. These data are represented as means $\pm$ SD. Data were analysed with one-way analysis of variance (ANOVA), followed by a Tukey's HSD *post hoc* test for multiple comparisons (SPSS 19 software, SPSS Inc., Chicago, IL, USA). The differences between two samples were analysed by independent t-test and Levine's Test for Equality of Variances. The results with *p*-values of less than 0.05 were considered to be statistically significant.

Results

Since the aim of the study was to correlate physicochemical parameters to cytotoxicity, CNTs were characterised by DLS, TEM and EDX analysis. For the comparison of cytotoxicity, the most suited screening assay was identified and the cell lines grouped according to their GSH content. The protective effect of NAC was determined and the mode of cytotoxic action was evaluated.

Physicochemical characterisation

The sizes of the CNTs were determined by several techniques including DLS, TEM and light microscopy. DLS is not an appropriate method for size determination of non-spherical particles, but it is a good way to measure the surface charge. All CNTs with nominal diameters of <50 nm showed larger hydrodynamic diameters in DLS. The surface charge for both types of CNTs, carboxyl-functionalised and pristine CNTs, was slightly negative.

With TEM, data similar to the nominal diameters have been assessed. No general differences in the diameters of carboxylated and pristine CNTs were seen. The only exception is the measurement for SCNTs, which were seen exclusively in bundles. The thickness of single SCNTs, sticking out from these bundles, could not be reported exactly. Carboxylated SCNTs formed bundles with larger calliper than pristine SCNTs (Figure 1, Table I). MCNTs, except MCNT8c, formed aggregates but no bundles. Although all CNTs should measure 0.5–2 µm in length according to the producer, non-aggregated CNTs (on average) measured between 217 and 556 nm and bundles (on average) between 543 and 816 nm.

During the incubation with cells, CNTs formed large and differently shaped aggregates, which could be seen in the light microscope and measured up to 100 µm length in one direction. No obvious relation of the size of the primary CNTs and/or the surface functionalisation to the size and frequency of these aggregates was seen.

To identify metal contaminants from residues of catalysts, X-ray energy-dispersive spectrum data were obtained (examples of TEM images and EDX spectra are seen in Figure 1s of the Supplementary Material). As Table II shows, in addition to carbon, only O, Si and Ni were detected. The presence of Si in the samples is due to the dispersion of CNTs in water, where

it dissolved from glass bottles (Jedlicka & Clare 2001) and Ni originates from the grid, on which the CNTs were viewed (Li et al. 2011). Despite the different functionalisation methods of SCNTs and MCNTs, the purity did not differ significantly between all CNTs and, most importantly, metals from catalysts were below threshold.

Cytotoxicity screening

Comparison between different screening assays—To study the suitability of the screening assays, the exposures included a colour control and an interference control. Formazan bioreduction with the MTS assay was used as the gold standard since this assay (out of a panel of six screening assays) was evaluated as the best performing one for SCNTs (Monteiro-Riviere et al. 2009). Additionally in this study, the following assays (which were not evaluated in the study of Monteiro-Riviere et al.) were carried out: quantification of cellular adenosine triphosphate (ATP) content, of DNA, and of protein. Microscopical evaluation was performed, in parallel to the screening assays, for verification of the results.

Quantification of ATP by chemoluminescence and fluorescent detection of DNA content produced similar data for all CNTs at 4 and 24 h and did not discriminate between the different CNTs. In addition, these findings did not match with the microscopical assessment.

Fluorescent detection of LDH release and detection of total protein by sulforhodamine B staining identified the SCNTc as the most cytotoxic CNTs. Both assays, however, showed only very small differences between the CNTs, and the fluorescence data were contradicted for several CNTs by data from the MTS assay (Figure 2).

The formazan bioreduction assay was performed using two protocols to avoid interference of the CNTs. In one protocol, after the incubation with the tetrazolium salt, the supernatant was transferred to another plate for measurement (MTS). In the other protocol, the reaction was measured in the plate but for each sample the absorbance of cells + CNTs without MTS reagent was subtracted (MTSco). Both protocols produced a similar classification of the CNTs into cytotoxic and non-cytotoxic tubes, and were consistent with the microscopical assessment. Viability data, however, were significantly different for all CNTs, with the exception of SCNT and MCNT50c. Therefore, for the comparisons between cell lines, only the MTS assay was used.

Cytotoxicity screening in different cell lines—Cell lines differing in growth pattern (adherent/suspension) and in phagocytosis were used and viabilities compared after 4 and 24 h of exposure. At concentrations of 50 µg/ml CNTs no significant decrease in viability was seen for all CNTs for both time points. At higher concentrations decreases in viability were most pronounced in EAhy926 cells; 100 µg/ml of all CNTs significantly decreased viability after 4 h of exposure. Most epithelial cell lines, however, were less sensitive and significant decreases in viability at 100 µg/ml were seen after 24 h for carboxylated and pristine SCNTs and MCNT8s in 16 out of 24 tests and for carboxylated and pristine MCNT20s and MCNT50s in 9 out of 24 incubations (Figure 3). Carboxylated CNTs significantly reduced viability in 14 out of 24 incubations and pristine CNTs in 11 out of 24 incubations. At 500 µg/ml significant loss in viability was seen for all CNTs except for MCNT20 and MCNT50 in TK-6 cells (Figure 2s, Supplementary Material).

To identify the role of aggregates in the cytotoxic action of CNTs, supernatants after centrifugation were assessed in EAhy926 cells. Even at 500 µg/ml, only minimal decreases (viability: 85–100%) in viability were seen for pristine and carboxylated CNTs. The lack of cytotoxicity was mostly due to a reduction in the concentration of CNTs in the sample because (according to the absorbance of the supernatant at 560 nm) samples of 500 µg/ml

MCNT8 and MCNT8c contained only 40–50 µg/ml after the centrifugation. At these concentrations, the non-centrifuged CNTs reduced cell viability to 65–75% of the controls.

Role of oxidative stress

To identify the role of oxidative stress in the cytotoxic action of the CNTs, different approaches were used. First, GSH levels were determined, and the cell lines classified into high and low GSH-containing ones. On this basis, cytotoxicity of CNTs in low and high GSH-containing cell lines was compared. Second, the changes of GSH levels upon incubation with non-cytotoxic concentrations of CNTs were determined. Lastly, cytotoxicity was determined in the presence and absence of NAC. This radical scavenger may protect against extracellular and intracellular ROS, in the event that it is added together with the CNTs. When it is used as pre-incubation prior to the addition of the CNTs, it can improve the intracellular antioxidant status by replenishment of the intracellular sulfhydryl pool and reduction of intracellular ROS.

EAhy926, DMBM-2 and V79 contained significantly lower GSH levels than A549, HepG2, TK-6 cells, and these cells were classified into a low GSH (<2 nmol/10⁶ cells) and a high GSH (>2 nmol/10⁶ cells) group. Non-cytotoxic concentrations (25 µg/ml) CNTs caused only slight effects on the GSH content in cells with high GSH content (A549 and HepG2 cells). Only the positive control and SCNTc caused a significant decrease in GSH content compared with the untreated controls (Figure 4A). However, significant differences were detected when GSH decreases by carboxyl-functionalized CNTs and pristine CNTs were compared in these cells. Decreases in GSH were significantly higher for SCNTc, MCNT20c and MCNT50c in HepG2 cells and for SCNTc in A549 cells than for the pristine counterparts.

Cytotoxicity was higher in the low GSH cell lines than in the high GSH cell lines for all CNTs. After 24 h the difference between these groups was significant for 100 µg/ml SCNTc and MCNT50c, 200 µg/ml SCNTc, MCNT8c and MCNT50c and for all CNTs at 400 µg/ml (Figure 4B, carboxylated CNTs shown). Pristine and carboxylated CNTs reacted similarly.

To find out if an externally added antioxidant has the same protective effect as intracellular GSH, co-incubations and pre-incubation with NAC in EAhy926 cells with low GSH levels (1.12 ± 0.31 nmol/10⁶ cells) were used. Significant protection was seen upon both incubation protocols for all CNT concentrations after 4 h and for 100 µg/ml at 24 h. Both NAC applications protected against pristine CNTs to a similar degree but pre-incubation protected slightly better than simultaneous incubation against cell damage by carboxylated CNTs. A significant gain in viability upon simultaneous incubation of NAC is seen for all CNTs at 100 µg/ml. Protection at 200 µg/ml is absent for SCNTc, MCNT8c and MCNT50c, for all carboxylated CNTs at 400 µg/ml (Figure 4C) and 500 µg/ml (not shown). The higher protective effect of NAC for pristine CNTs than for carboxylated ones and for 20 nm diameter CNTs than for <8 nm CNTs was not significant. When combinations of diameter and functionalisation were compared, significant effects were seen. The protection by NAC for damage by pristine CNTs 20 nm was significantly higher than that against carboxylated CNTs <8 nm at 200 and 400 µg/ml after 4 h of incubation and at 100, 200, 400 and 500 µg/ml after 24 h of incubation (Table III).

In order to identify parameters, which may identify higher cytotoxicity in specific cell lines, Figure 5 summarizes significant differences between CNTs. This includes reduction of intracellular GSH levels in HepG2 cells, protection by high intracellular GSH levels, protection by incubation with NAC and number of cell lines, where significant decreases in viability were seen for the respective CNT. SCNTc and MCNT50c reacted cytotoxic in more cell lines than the pristine counterparts whereas no difference was seen for MCNT8s

and MCNT20s regarding functionalisation. Combination of small size and carboxylation strongly increased cytotoxicity; <8 nm carboxylated CNTs reacted cytotoxic in nine cell lines versus 20 nm pristine CNTs in four cell lines. The higher cytotoxicity of carboxylated CNTs versus pristine ones was linked to decreases in GSH levels for SCNTs and MCNT50s but decrease of GSH levels in cells treated with MCNT20c was not correlated to a higher cytotoxicity. NAC protected EAhy926 cells against cytotoxicity by pristine CNTs 20 nm significantly better than against that of carboxylated CNTs <8 nm.

Mode of action

Due to interference with various membrane integrity and apoptosis assays (e.g. LDH, caspase 3/7), the mode of cell damage was assessed by uptake of the fluorescent dyes: YoPro-1 and PI. This assay has two major advantages: first, interference and quenching of the signal by direct interaction is less likely because the fluorescent read-out is located in the nucleus and agglomerated CNTs are located at the plasma membrane and in the cytoplasm. Second, potential quenching of the fluorescent signal affects both dyes in the same way. Therefore, even in the case of interference, the ratio can still be analysed. The YoPro-1 dye is taken up exclusively by apoptotic cells; necrotic cells preferentially take up PI. Secondary apoptotic cells are double-stained. By microscopical evaluation, different staining pattern of the CNTs can be discerned (Figure 6). Cells exposed to CNTs with larger diameter, like MCNT50, showed predominantly staining with YoPro-1, whereas those exposed to CNTs with small diameter, like SCNTc, were mainly stained with PI. These findings were supported by fluorometric measurements. Significant differences in the YoPro-1 fluorescence compared with untreated controls as 100% were seen for CNTs with a larger diameter (Figure 7A). At the same concentration, MCNT20 and MCNT50 caused significantly more apoptosis than the carboxylated counterparts. When the ratio of PI/YoPro-1 fluorescence was analysed, the differences that were most prominent were those at 150 µg/ml. While small pristine CNTs (SCNT, MCNT8) showed a high ratio, indicating necrosis, the large CNTs (MCNT20, MCNT50) showed a low ratio, indicating apoptosis (Figure 7B). Lastly, the carboxylated CNTs showed PI/YoPro-1 ratios >1 indicating that apoptosis was not the predominant mode of action.

Discussion

In this study, a panel of small CNTs with different thickness and different surface modifications was tested in various cell lines. In all cell lines, thin (<8 nm) and carboxyl-functionalised tubes were found to be more cytotoxic than thick (20 nm) and pristine CNTs. The involvement of oxidative stress in the toxic action of all CNTs was evidenced by the protective action of NAC and the lower cytotoxicity of CNTs in cell lines with higher GSH content. It was also found that exogenously added antioxidants did not have the same effect as intracellular GSH levels. Thin CNTs acted predominantly by necrosis, whereas apoptosis appeared to be the main mode of action for thicker tubes.

Physicochemical characterisation of the nanoparticles in the culture medium is important because often particle sizes differ from that indicated by the producer. Although the length of the small CNTs was indicated as 0.5–2 µm, most single CNTs measured between 200 and 400 nm and CNTs longer than 500 nm were only rarely seen. The most likely cause for the short lengths appears to be the destruction of CNTs by ultrasound treatment, which has also been reported by other groups (Lu 1996; Mu et al. 2009). Thin tubes were rarely seen as single tubes but were arranged predominantly as bundles. Evaluation by electron microscopy, however, cannot exclude the presence of single tubes because first, only a small fraction of the total suspension can be analysed by this method and second, electron beam irradiation destructs CNTs preventing the detection of single tubes (Molhave et al. 2007).

It is also important for the interpretation that data are generated with a reliable screening assay since nanoparticles interfere with screening assays by multiple mechanisms (Fröhlich et al. 2010; Fröhlich et al. 2012). As high amounts of CNT aggregates were bound to the cells, interference of CNTs with several conventional screening assays may occur. Such interference has been repeatedly reported in the literature and includes absorbance, adsorption of the molecules used for detection (cytokines, LDH), and catalytic activity (Davoren et al. 2007; Zhang et al. 2007). In this study, independent of the mode of detection (fluorescence, luminescence or absorbance), most interference was seen for assays where the signal had to be determined after lysis of the cells: both chemoluminescence (ATP content) and fluorescent detection (DNA content) did not discriminate between high and low cytotoxic CNTs. Similar to previous reports, the detection by LDH was less sensitive due to the binding of the enzyme to the CNTs. MTS assay showed the best performance and best correlation with the microscopical evaluation, as has also been reported by Monteiro-Riviere et al. (2009).

The choice of the cells used for the cytotoxicity screening is important and in this study the SCNTs and thin (<8 nm) MCNTs showed the highest cytotoxicity in non-phagocytic cells. CNTs >50 nm caused only cytotoxicity in phagocytes, which is consistent with the data by Sohaebuddin et al. (2010), who also reported a selective cytotoxicity of the thick (>50 nm) CNTs for phagocytic cells.

The toxicity of the CNTs has often been attributed to their fibrous structure. Fibres, defined as length >5 µm and diameter ≤ 3 µm and aspect ratio ≥ 1:3 (WHO 1997), act more harmful than spherical particles (Oberdorster et al. 2005). The CNTs, which were used in this study, cannot be regarded as fibres according to this definition due to the fact that, when suspended in cell culture medium, single tubes measured less than 0.5 µm and CNT bundles less than 1 µm. Short CNTs, like the ones used in this study, were developed because of their lower cytotoxicity and higher clearance. Pristine, hydrophobic CNTs are not well excreted (Yang et al. 2007). However, functionalised and hydrophilic CNTs of 100–200 nm length can be excreted by the kidney and have very short half-life times in blood (Liu et al. 2007; Ruggiero et al. 2010). The link of cytotoxicity and small diameter, observed in this study, appears to indicate that not the aspect ratio but small diameter *per se* determines the cytotoxicity of CNTs.

In this study, CNT concentrations of 50 µg/ml acted cytotoxic only in the endothelial cell lines EAhy926. Blood plasma levels of nanocarriers for drug delivery can also reach higher concentrations. In the cytostatic drug, Abraxane®, the drug load accounts for 10% of the total nanoparticle mass. Maximum serum concentrations for paclitaxel are 23 µg/ml (Abraxane, product information, 2012), corresponding to nanoparticle concentrations of 230 µg/ml. Liposome concentrations in Doxil® at peak plasma levels of 4.12 µg/ml doxorubicin are 34.5 µg/ml (Soundararajan et al. 2009). Liposome concentrations in Ambisome® at peak plasma levels of 7.3–83.7 µg/ml of amphotericin B range between 24.3 and 276.2 µg/ml (Ambisome, product information, 2012) and liposomes in Daunoxome® at peak plasma levels of 33.4–52.3 µg/ml daunorubicin can even reach 585–917 µg/ml (Feingold et al. 2010).

As a potential mode of toxic action, the authors focused on ROS generation, apoptosis and necrosis. An involvement of ROS in the cytotoxic action of CNTs has been reported in the majority of the studies on SCNTs (Di Giorgio et al. 2011; Manna et al. 2005; Sarkar et al. 2007; Sharma et al. 2007; Zhang et al. 2011; Zhiqing et al. 2010) and on MCNTs (e.g. Brown et al. 2010; Guo et al. 2011; Liu et al. 2008; Rama Narsimha Reddy et al. 2011; Srivastava et al. 2011; Ye et al. 2011). In other studies, no correlation of oxidative stress and cytotoxicity was shown for both types of CNTs (Liu et al. 2010b; Tabet et al. 2009;

Tsukahara & Haniu 2011). As the comparison of data between studies is complicated by testing of CNTs from different sources, different purity, etc. and assessment on different cell types, the role of oxidative stress should only be compared between CNTs with similar physicochemical parameters, purity and in the same experimental setting. In the study by Sohaebuddin et al. (2010), where fibroblasts, bronchial epithelial cells and macrophages were exposed to pristine MCNTs in diameters of <8, 20–30 and >50 nm, all CNTs induced ROS generation by 2,7-dichlorodihydrofluorescein diacetate fluorescence in fibroblasts but only the thin CNTs induced ROS generation in all cell types.

The different degree of ROS production may be linked to different intracellular GSH levels. To reveal a potential role of GSH in the cytotoxic action of all CNTs, the authors correlated basal GSH levels to the loss in viability caused by CNTs in the respective cell line. These data suggest that high basal GSH levels decrease the sensitivity of cells to damage by CNTs. However, it is possible that the GSH content is not the main reason for the different sensitivities. The TK-6 cell line (showing the highest GSH content and the lowest cytotoxicity) is the only cell line growing in suspension and it might be suspected that these cells are not exposed to the same concentration of CNTs doses. Differences in cellular doses were reported between cells that were exposed to gold nanoparticles in upright and inversed culture (Cho et al. 2011), and demonstrated the importance of the location of the cells in the culture well. In addition to this, particle concentration and medium composition change sedimentation, as shown for titanium dioxide nanoparticles by Allouni et al. (2009). Other reasons for a higher resistance to toxic agents in cells with high GSH levels include higher bcl-2 levels, higher expression of multiple drug resistance protein and resistance to apoptosis. This is due to the fact that all these parameters are positively correlated to GSH content (Li et al. 2010; Wang et al. 1994). Although GSH levels are usually linked to oxidative stress, depletion of intracellular GSH is an independent mode of cytotoxic action. Cytotoxicity of arsenic trioxide, for instance, is independent from the generation of ROS (Han et al. 2008).

Muller et al. (2008) supposed that the higher cytotoxicity of carboxylated CNTs is due to the generation of oxidative stress caused by defects in the carbon sheets in combination with not fully oxidized areas in the sheet. Other authors claim that abundant small carbon fragments, which originate during the oxidation process, cause the higher cytotoxicity of these CNTs (Wang et al. 2011). According to Liu et al. (2010a), impairment in cell signalling, inducing for instance apoptosis, is more important than generation of ROS. The results on the partial rescue of cells by incubation with NAC also indicate that generation of ROS can explain the cytotoxicity of CNTs only in part.

CNTs may act by disruption of membrane integrity, induction of apoptosis and inhibition of proliferation (e.g. Di Giorgio et al. 2011). To identify a potential relation between the thickness of the investigated CNTs and the predominant mode of action, necrosis and apoptosis were compared. In the same non-cytotoxic concentration, the CNTs with small diameter tested in this study predominantly caused necrosis, whereas those with larger diameter caused more apoptosis. Effects on the plasma membrane causing membrane disruption mainly cause death by necrosis, and membrane damage has been reported as a major mode of action for MCNTs with 67 nm of diameter (Hirano et al. 2008). Although nanopenetration is thought to be a mode of entry for CNTs by some groups (e.g. Firme & Bandaru 2010), other authors doubt that thinner CNTs have sufficient energy to pierce the plasma membrane (Pogodin & Baulin 2010). The lack of protection by NAC in this study suggests mechanical damage of the plasma membrane as potential mode of action. CNTs could remove lipids from pre-existing holes in the plasma membrane, cause focal dissolution of the plasma membrane or create hydrophilic pores (Lin et al. 2010; Mecke et al. 2005;

Panessa-Warren et al. 2006). Preliminary studies of CNT-exposed cells by TEM did not show obvious defects in the plasma membrane (data not shown).

Conclusion

Independent from the surface functionalisation and from the cell type, thin CNTs in this large comparative study acted more cytotoxic than thick CNTs. Carboxylation of the CNTs increased their cytotoxicity, suggesting that shielding by other functional groups is advantageous. Neither high intracellular GSH levels nor exogenous addition of antioxidants prevent cytotoxicity by CNT with small diameter and carboxyl functionalisation suggesting that these particles mainly act by ROS-independent plasma membrane damage or by membrane receptor signalling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Austrian Science Fund grant P 22576-B18. The authors would like to thank Gertrud Havlik for preparing the CNTs for TEM and Verena Pfeifer for quantification of GSH levels.

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Figure 1. Morphology of CNTs by transmission electron microscopy. SCNT and SCNTc were seen only in bundles. All other CNTs were also discerned as single tubes.

Figure 2.

Cytotoxicity of CNTs. a: comparison of the effect of 100 µg/ml CNTs on EAhy926 cells after 24 h of exposure assessed by various screening assays. Viability was higher when assessed by sulforhodamine B assay (protein) and by LDH release. Formazan bioreduction by MTS with different protocols (MTSco and MTS) indicated greater decreases in viability. For better comparison with the other assays, 100% minus the percentage of LDH release (no LDH) is indicated.

Figure 3.

Comparison of cytotoxicity in different cell lines at CNT concentrations of 100 µg/ml after 24 h of exposure assessed with MTS. Except for EAhy926 cells, viability was decreased only for small CNTs (SCNTs, CNT8s) for most cell lines and for thick CNTs (CNT50, CNT50c) in phagocytic cells. TK-6 cells showed no clear pattern: only very small decreases in viability were seen for all CNTs; they were significant for CNT8, CNT20c and CNT50c. Significant decreases are indicated by asterisk ($p < 0.05$).

Figure 4.

Role of oxidative stress in CNT cytotoxicity according to GSH levels and reaction to the radical scavenger *N*-acetyl cysteine. (A) Changes in GSH levels in HepG2 and A549 cells after exposure to non-cytotoxic concentrations (25 µg/ml) of various CNTs for 24 h show significant decreases compared with untreated controls only for SCNTc and the positive control *tert*-butylperoxide. When cells treated with carboxylated CNTs are compared with those treated with pristine CNTs significant greater decreases in GSH content are seen in the former group. Significant ($p < 0.05$) differences to the untreated control are indicated by asterisks and significant differences between groups are linked by brackets. (B) Viabilities after incubation for 24 h with different concentrations of carboxylated CNTs in cell lines with low ($< 2 \text{ nmol}/10^6 \text{ cells}$, EAhy926, DMBM-2 and V79) and high ($> 2 \text{ nmol}/10^6 \text{ cells}$, A549, HepG2 and TK-6 cells GSH content) are shown. With increasing concentration significant differences are seen for more CNTs. Brackets link columns with significant differences. (C) Differences in viability in the presence and absence of 500 µM *N*-acetyl cysteine for 24 h assessed by MTS. A significant protective effect is seen for more concentrations of the pristine CNTs than for carboxylated CNTs.

	SCNT	SCNTc	CNT8	CNT8c	CNT20	CNT20c	CNT50	CNT50c
SCNT	Prot GSH, 2	GSH↓,						
SCNTc	GSH↓,	4			Prot NAC		Prot NAC	
CNT8			5					
CNT8c				5	Prot NAC		Prot NAC	
CNT20		Prot NAC		Prot NAC	Prot GSH, 2	GSH↓,		
CNT20c					GSH↓,	2		
CNT50		Prot NAC		Prot NAC			Prot GSH, 2	GSH↓
CNT50c							GSH↓	3

Figure 5.

Summary of significant differences between CNTs regarding reduction of GSH levels in HepG2 cells (GSH), protection by intracellular GSH levels in cell line with $>2 \text{ nmol}/10^6$ cells at $200 \text{ }\mu\text{g}/\text{ml}$ (Prot GSH) and protection by incubation with NAC in EAhy926 cells at $100\text{--}500 \text{ }\mu\text{g}/\text{ml}$ (Prot NAC).

The number of cell lines with significant decrease in viability at $100 \text{ }\mu\text{g}/\text{ml}$ is given as number in red; CNTs, carbon nanotubes; GSH, glutathione; MCNTs, multi-walled carbon nanotubes; NAC, *N*-acetyl cysteine; SCNTs, single-walled carbon nanotubes.

Figure 6.

Microscopic images of A549 cells exposed to CNTs for 24 h and stained with YoPro-1 (green)/PI (red) for differentiation between apoptosis and necrosis; 100 μM H_2O_2 , which induced apoptosis and necrosis to similar degree, is used as positive control overlay of the red and green channel signal is shown in yellow. Cells exposed to 100 $\mu\text{g}/\text{ml}$ CNTs stain with YoPro-1 and PI to similar degree for CNT20c and show predominantly PI staining for SCNTc exposure and mainly YoPro-1 staining for CNT50.

Figure 7.

Changes in the amount of apoptotic and necrotic cells according to staining with propidium iodide (PI) and YoPro-1 in A549 cells after exposure to CNTs for 24 h assessed by fluorometry. (A) YoPro-1 signals were increased for incubations with all CNTs 20 nm as indication of increased apoptosis. Low doses of CNTs produced a higher increase than high doses. Incubation with small CNTs induced a relative decrease in the fluorescent signal. Changes in fluorescence are normalised to untreated controls. Significant changes ($p < 0.05$) are indicated by asterisk. (B) Dose-related changes in the fluorescence ratio of the red and the green channel (PI/YoPro-1). Small CNTs show higher ratios indicating a predominance of necrosis over apoptotic cell death. The ratio is normalised to the ratio in untreated cells.

Table I

Characterisation of CNTs suspended in DMEM + 10% FBS by DLS/LDV and TEM.

Sample	DLS/DLV data		TEM data			
	Hydrodynamic size (nm)	(mV)	Diameter of single CNTs (nm)	Length of single CNTs (nm)	Diameter of CNT bundles (nm)	Length of CNT bundles (nm)
SCNT	16.4	−9.72	~2 nm	n.d.	28.3 ± 10.6	543 ± 60.8
SCNTc	15.7	−8.1	~2 nm	n.d.	62.5 ± 41.9	816 ± 275.4
MCNT8	26.8	−6.96	4.7 ± 0.48	222 ± 126.2	n.a.	n.a.
MCNT8c	16.3	−9.64	4.2 ± 0.8	217 ± 117.9	24.3 ± 5.1	600 ± 282.8
MCNT20	124.4	−9.78	18.9 ± 0.9	446 ± 77.9	n.a.	n.a.
MCNT20c	38.8	−10.3	15.3 ± 2.5	251 ± 94.4	n.a.	n.a.
MCNT50	51.9	−7.28	62.8 ± 5.7	355 ± 96.4	n.a.	n.a.
MCNT50c	50.4	−11.0	63.6 ± 11.3	392 ± 195.3	n.a.	n.a.

CNTs, carbon nanotubes; DLS, dynamic light scattering; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; LDV, laser Doppler velocimetry; MCNT, multi-walled CNT; n.a., not analysed; SCNT, single-walled CNT; TEM, transmission electron microscopy.

Table II

EDX data for carboxylated and pristine SCNTs and MCNTs.

	C	O	Si	Ni
SCNT	90.97 ± 0.27	4.37 ± 0.95	4.32 ± 0.64	0.33 ± 0.06
SCNTc	90.58 ± 1.49	5.30 ± 1.25	3.17 ± 0.32	0.95 ± 0.06
MCNT	91.3 ± 2.71	4.95 ± 1.25	1.04 ± 0.95	1.3 ± 1.4
MCNTc	92.52 ± 1.63	4.18 ± 1.02	2.32 ± 0.59	0.96 ± 0.12

Data are indicated as atomic % and the mean ± SD is given; EDX, energy-dispersive X-ray spectroscopy; MCNTs, multi-walled carbon nanotubes; SCNTs, single-walled carbon nanotubes; SD, standard deviation.

Table III

Gain in viability by simultaneous incubation of EAhy926 cells with CNTs and NAC.

Group	Time point (h)	Concentration of CNTs			
		100 µg/ml	200 µg/ml	400 µg/ml	500 µg/ml
	4				
Pristine, large		19 ± 3%	24 ± 9%	29 ± 8%	22 ± 14%
Carboxylated, small		18 ± 9%	7 ± 6%	7 ± 4%	7 ± 7%
	24				
Pristine, large		37 ± 10%	39 ± 11%	46 ± 6%	36 ± 2%
Carboxylated, small		10 ± 6%	-2 ± 2%	-3 ± 1%	-1 ± 4%

CNTs, carbon nanotubes; NAC, N-acetyl cysteine.