Surface modification of multiwall carbon nanotubes determines the pro-inflammatory outcome in macrophage

Ting Zhang, Meng Tang, Lu Kong, Han Li, Tao Zhang, Yuying Xue, Yuepu Pu

Key Laboratory of Environmental Medicine Engineering, Ministry of Education, School of Public Health, Southeast University, Nanjing 210009, China
Jiangsu key Laboratory for Biomaterials and Devices, Southeast University, Nanjing 210009, China
Department of Material Science and Engineering, National Key Laboratory of Solid State Microstructures, Nanjing University, Nanjing 210032, China

Abstract

Carbon nanotubes (CNTs) are widely used in industry and biomedicine. While several studies have focused on biological matters, attempts to systematically elucidate the toxicity mechanisms of CNTs are limited. The aim of the present study was to evaluate and compare the cytotoxicity of raw multi-walled carbon nanotubes (MWCNTs) and MWCNTs functionalized with carboxylation (MWCNTs–COOH) or polyethylene glycol (MWCNTs–PEG) in murine macrophages. Our results show that only MWCNTs–COOH and raw MWCNTs alter the oxidative potential of macrophages by increasing reactive oxygen species and the expression of pro-inflammatory factors in both a concentration- and surface coating-dependent manner. The data suggest that compare with raw MWCNTs and MWCNTs–PEG, the MWCNTs–COOH produces a significant increase in ROS generation, interruption of ATP synthesis, and activation of the MAPK and NF-κB signaling pathways, which in turn upregulates IL-1β, IL-6, TNF-α, and iNOS to trigger cell death. These findings suggest that contributory cell uptake caused by physicochemical factors rather than residual metal catalysts plays a role in ROS-mediated pro-inflammatory responses in vitro.

Keywords: Multi-walled carbon nanotubes; Surface-functionalized; Macrophages; Pro-inflammatory; Toxic mechanisms

1. Introduction

Carbon nanotubes (CNTs) have attracted a great deal of attention in the nanotechnological development field. Raw CNTs have an enduring metallic nature and are highly hydrophobic. Therefore, the search for novel surface functionalization methods, including covalent and noncovalent functionalization, has recently increased.

Functionalized CNTs (f-CNTs) are generally considered to be more biocompatible because of an improved hydrophilicity and dispersion in aqueous solutions; in addition, f-CNTs can be utilized for subcutaneous glucose sensors [1], tissue engineering [2], cancer therapy and drug carriers [3]. Previous studies indicated that raw CNTs are more toxic than f-CNTs [4–5], for example, carboxylated- [6], phosphorylcholine-[7], and polystyrene-functionalized CNTs [8] induce less cytokine production, pulmonary inflammation, and fibrosis when compared with raw CNTs.

Based on their wide range of biomedical applications, each permutation of surface-modified CNTs should be systematically evaluated for potential toxic effects on biological systems. Over the last decade, research has focused on understanding the side effects of CNTs. At the cellular level, in vitro studies have found that CNTs induced lower cell viability and altered cell adhesion and cell...
Studies also showed that CNTs physically damage the cell membrane [11], altered the mitochondrial membrane potential, increased intracellular reactive oxygen species [12], activated apoptosis [13,14], and triggered inflammation [15]. In addition, several lines of evidence showed that CNTs impaired the phagocytic function of monocytes/macrophages [16–18].

Macrophages play an important role in orchestrating immune and inflammatory responses; however, little is known about the fate of MWCNTs inside macrophages. Investigations of CNTs and other needle-like materials suggest that longer materials induce frustrated phagocytosis while shorter materials translocate into the nucleus causing irreparable nuclear damage, oxidative stress, and inflammation [19–21]. Furthermore, the intracellular reactive oxygen species (ROS) induced by CNTs that contain transition element contaminants (such as Fe, Ni, and Cu) depends on the concentration of the metal impurities [22]. Under normal physiological conditions, cells control ROS levels by balancing ROS generation and elimination using a scavenging system. However, excessive ROS can damage cellular proteins, lipids, and DNA, which can lead to cell apoptosis, inhibition of cell proliferation and changes in gene expression. Additionally, ROS play a major role in the cell signaling processes by triggering mitogen-activated protein kinases (MAPKs). These enzymes react to extracellular stimuli and control various cell proliferation activities, including gene expression, mitosis, differentiation, and cell survival/apoptosis [23]. Some MAPKs (ERK, p38, and JNK) regulate inflammatory and immune responses [24], and their signaling pathways are involved in LPS-induced pro-inflammatory gene expression in macrophages [25]. ROS may also act on upstream regulators of specific transcription factors [26]. For example, ROS activate NF-κB while simultaneously reducing its DNA binding capacity and thus, its ability to mediate transcription of pro-inflammatory molecules. The resulting free NF-κB translocates to the nucleus, binds to κB-binding sites in the promoter regions of target genes and induces the transcription of pro-inflammatory mediators, such as iNOS, COX-2, TNF-α, IL-1β, IL-6, and IL-8 [27]. Oxidative stress has been frequently reported as the toxic mechanism for CNTs-induced apoptosis and inflammation in various cell types. For example, Manna et al. [28] found that single-walled carbon nanotubes (SWCNTs) induce oxidative stress in human keratinocytes (HaCaT). The excessive ROS in HaCaT cells activates the transcription factor NF-κB which consequently induces cell death by apoptosis/necrosis or inflammatory responses. A study reported that a decrease in the size of carbon black particles from 250 nm to 14 nm caused an increase in the concentration of cytosolic Ca2+ ions that led to activation of NF-κB and enhanced ROS generation. Previous studies showed that CNTs generate an inflammatory response in macrophages; however, the underlying mechanisms have not been well described.

Here, we evaluated the cytotoxicity of three types of MWCNTs, including raw and functionalized with -COOH or -PEG, in RAW 264.7 macrophages. We found that MWCNTs–PEG were less cytotoxic and produced less inflammation when compared with MWCNTs–COOH and raw MWCNTs. These data demonstrate the importance of surface properties for MWCNT toxicity. The mechanism of these effects in macrophages is likely due to differences in the cellular uptake of MWCNTs and the subsequent activation of inflammation pathways.

2. Materials and methods

2.1. Surface modification of nanotubes

Raw multi-walled carbon nanotubes (MWCNTs; purity > 95%; outer diameter 10–20 nm; length 5–15 μm) were purchased from Shenzhen Nano–Technologies Port Company (Shenzhen, China). The synthesis of MWCNTs functionalized with carboxylation or polyethylene glycol was completed using procedures previously described (see Table 1 for the three nanotube structures) [29].

Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Raw MWCNTs</th>
<th>MWCNTs–PEG</th>
<th>MWCNTs–COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (μm)</td>
<td>5–15</td>
<td>0.3–0.6</td>
<td>0.3–0.6</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td>10–20</td>
<td>10–20</td>
<td>10–20</td>
</tr>
<tr>
<td>Carbon content (wt%)</td>
<td>&gt;98.9</td>
<td>&gt;99.0</td>
<td>&gt;98.8</td>
</tr>
<tr>
<td>Residual catalyst metal (Fe, Ni) content</td>
<td>&lt;0.10 wt%</td>
<td>&lt;0.07 wt%</td>
<td>&lt;0.16 wt%</td>
</tr>
</tbody>
</table>

TEM morphology (Measure bar 50 nm)

TEM images show the nanotube morphology and size distribution. The dry powder of MWCNTs (raw MWCNTs, MWCNTs–COOH, MWCNTs–PEG) was weighed and suspended in complete DMEM (10% FBS in 90% DMEM media) and diluted to final concentrations of 25, 50, 100, and 200 μg/ml. To study the effects of toxicity, RAW264.7 cells were cultured at 50,000 cells/ml in 96-well plates (Corning Inc., NY, USA) for 12 h prior to nanotube exposure (from 25 to 200 μg/ml). Untreated cells cultured in complete DMEM containing 10% PBS was used as the negative control.

2.4. Cytotoxicity assay

The assay for cytotoxicity involved the reduction of a water-soluble tetrazolium salt and was completed using a Cell Counting Kit-8 (CCK-8, Keygen Biotech Company, Nanjing, China) according to the manufacturer’s instructions. CNTs can interfere with classical end-point methods which are dependent on colorimetric
measures. Therefore, a modified procedure was used in our studies to attempt to avoid the interference. In brief, an additional step was involved in the CCK testing procedure. After the incubation period with CCK–8 solution, the supernatant was transferred to a new centrifuge tube and centrifuged 12,000 rpm, centrifuged at high speed, and then transferred to another plate for O.D. measurements (450 nm). Cell viability was expressed as the ratio between the amount of formazan from cells treated with the different MWCNTs and untreated control cells. Samples were assayed in quadruplicate.

2.5. Determination of cellular ATP levels

The ATP level was measured by the luciferin–luciferase method following the protocol of ATP assay kit (CellTiter-Glo luminescent cell viability assay, Promega). After 12 h incubation with MWCNTs with different concentrations, cells were washed twice and then resolved with ATP lysis buffer on ice. ATP in cell lysate was measured using a POLARstar OPTIMA microplate reader (Mithras LB940, Berthold, Germany).

2.6. Measurement of intracellular ROS

The concentration of intracellular reactive oxygen species (ROS) was detected using DCFH-DA (Keygen Biotech Company, Nanjing, China) according to the manufacturer’s instructions. After exposure to the MWCNTs, cells in 6-well plates were washed twice with PBS and incubated in 2 ml of a working solution of DCFH-DA at 37 °C in the dark for 20 min. Next, cells were trypsinized, washed twice with cold PBS, and resuspended in PBS for analysis of intracellular ROS using a FACScan flow cytometer (FACScalibur, BD Becton Dickinson, San Jose, CA, USA). The DCFH fluorescence emission was determined using a 525 nm band-pass filter. The mean fluorescence intensity (MFI) of 10^4 cells was quantified using Cell Quest Software.

2.7. Transmission electron microscopy of cellular ultrastructures

The ultrastructural alterations of RAW264.7 cells induced by three types of MWCNTs were observed using TEM at an accelerating voltage of 80 kV. After incubation with 200 μg/ml of MWCNTs for 12 h, cells were harvested, washed with PBS, prefixed with 2.5% glutaraldehyde at 4 °C for 1 h, dehydrated in ascending grades of ethanol, and subsequently embedded in epoxy resin. The prepared ultra-thin sections (40 nm) were contrasted with 0.3% lead citrate (Merck, Germany) and imaged with a JEM 1010 electron microscope (JEOL, Peabody, MA) at an accelerating voltage 80 kV.

2.8. Total RNA isolation and analysis using quantitative real-time PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA). RT-PCR analysis was carried out as previously described [29]. GAPDH was used as an internal control. The primer sequences for PCR analysis are referred to in our previous study [29].

2.9. Western blotting analysis

Total cellular protein extracts were prepared as previously described [26]. Briefly, RAW264.7 cells were exposed to MWCNTs for 12 h, washed once with ice-cold PBS, and lysed in an ice-cold RIPA buffer (Beyotime Institute of Biotechnology, Jiangsu, China) containing a cocktail of protease inhibitors (complete mini; Roche Diagnostics, Mannheim, Germany) for 30 min. After centrifugation of the lysates at 12,000 rpm at 4 °C for 10 min, the supernatants were collected and stored at −80 °C until use. The protein concentrations of the extracts were determined with a bicinchoninic acid (BCA) protein assay (Beyotime Institute of Biotechnology, Jiangsu, China). An equal amount (50 mg) of protein was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) for 1 h at room temperature. Next, the membrane was incubated with rabbit polyclonal antibodies for IκB-α, p-IκB-α, P65, p-P65, P-ERK, ERK, p-JNK, JNK, p-p38, and p38 (1:1000, CST, MA, USA) overnight at 4 °C, washed with TBST, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG/anti-goat IgG secondary antibodies for 1 h at 37 °C (1:2000, CST, MA, USA). Protein bands were visualized using the West Pico chemiluminescence reagent (Pierce, Rockford, IL, USA). Western blot quantification was performed using Image Station 440CF (Kodak Digital Science).

2.10. Statistical analysis

All experiments were repeated a minimum of three times. Data are presented as the mean ± standard deviation for the indicated number of independently performed experiments. Student’s t-test was used to determine statistical significance. A P < 0.05 was considered significant.

3. Results and discussion

3.1. Characterization of f-MWCNTs

Prior to toxicity assays, the three types of MWCNTs were characterized based on morphological shape, chemical composition, and size distribution of the nanotubes (Table 1). After oxidation, the length and diameter of 50 tubes were randomly measured using TEM images. The results showed the length of functionalized MWCNTs was shortened to 300–600 nm. In addition, TEM images revealed that the skeleton structure of MWCNTs–COOH and MWCNTs–PEG remained intact. DLS measurements indicated the presence of functionalized MWCNT aggregates in the 80–200 nm range.

Under our experimental conditions, we used PBS as an agent to help suspend unfractionalized multiwall nanotubes in culture medium. MWCNTs–PEG and MWCNTs–COOH were easily dispersible in medium with negligible tendency to form agglomerates. In addition, ultrasonication was used as an important procedure to improve dispersibility of the MWCNTs. Significant agglomeration occurred in cells incubated with raw MWCNTs. Raw MWCNTs showed adherence to each other, forming dense micron sized assemblies completely covering the cell surface. In contrast, MWCNTs–COOH and MWCNTs–PEG agglomerated slightly in medium and remained well dispersed after incubation.

3.2. Cell viability and ATP synthesis

The cell viability after exposure to MWCNTs was determined in vitro using RAW264.7 cells. Macrophages protect the body via phagocytosis against foreign material, dead cells, and debris through a variety of mechanisms. These processes are implicated in both protective and adverse roles for macrophages in the regulation of the immune response during various pathogenic processes, including inflammation, and fibrosis [30]. Regarding particular matter, macrophages are vital for uptake and clearance processes [31–33]. There are methodological problems to consider when assessing CNT toxicity. For example, CNTs can interfere with classical end-point methods that are dependent on colorimetric measurements. Therefore, a modified procedure was used in our studies to avoid this interference. An additional step was used
in the CCK test. After incubation with the CCK-8 solution, the supernatant was transferred to a new centrifuge tube and centrifuged at 12,000 rpm and transferred to another plate for reading. As shown in Fig. 1, the cell viability of macrophages showed a MWCNT concentration-dependent decrease. Considering the viability assays are vital steps in toxicology that explain the cellular response to a toxicant. Also, they give information on cell death, survival, and metabolic activities. We have exploited the high sensitivity of luminescence-based assay and fluorescent based assay to study the cytotoxicity of MWCNTs. The ATP concentration is described as the product of the viable cell number and the average amount of ATP produced by each viable cell. The result showed that the ATP concentration in RAW264.7 decreased heavily with the concentration of the three types of MWCNTs treatment increased, especially the MWCNTs–COOH and raw MWCNTs (Fig. 2). These results indicated that the bioenergy crisis (ATP depletion) should have great interference with the massive death of RAW264.7 cells [34].

![Cell Viability Graph](image1)

**Fig. 1.** The viability of RAW264.7 cells after a 12-h exposure to MWCNTs (25–200 μg/ml) was assessed using a CCK-8 assay. The results are expressed as % mitochondrial activity. Values are shown as the mean ± SD. Significant differences from the control are marked with asterisks (* for P-value < 0.05, ** for P-value < 0.01).

![ATP Content Graph](image2)

**Fig. 2.** ATP content in RAW264.7 cells after MWCNT exposure after a 12-h exposure to MWCNTs (25–200 μg/ml) was assessed using luminescent assay. The results are expressed as % intracellular ATP content. Values are shown as the mean ± SD. Significant differences from the control are marked with asterisks (* for P-value < 0.05, ** for P-value < 0.01).

![ROS Levels Graph](image3)

**Fig. 3.** ROS levels in RAW264.7 cells after MWCNT exposure. ROS formation after MWCNT exposure was investigated using a DCFH-DA assay. Cells were incubated with MWCNTs (25, 50, 100, and 200 μg/ml) for 12 h and then with 20 μM DCF-DA for 40 min. H2O2 (10 μM) was used as a positive control. Values are shown as the mean ± SD. Significant differences from the control are marked with asterisks (* for P-value < 0.05, ** for P-value < 0.01).
Interestingly, the cell viability after exposure to MWCNTs–PEG was slightly higher when compared to cells exposed to MWCNTs–COOH or raw MWCNTs. Previously, we quantitatively measured the uptake of MWCNTs by RAW264.7 cells using a simple high-throughput method [29]. The results showed that cellular uptake of MWCNTs is positively correlated with their surface charge. In contrast, small MWCNTs–PEG were observed in the cellular interior after 12 h of incubation, suggesting that MWCNTs–PEG were unable to traverse cell membranes independently. The uptake efficiency of the functionalized MWCNTs correlated with the observed decrease in cell viability. Thus, the cytotoxicity of MWCNTs is based on their cellular uptake. These results are similar to previous in vitro results that showed functionalization- and density-dependent enhanced cellular uptake was accompanied by an increased intracellular stress response, cytotoxicity, and cell death [35]. However, the question remains as to how MWCNTs influence important intracellular events and what mechanisms are used to exert their cytotoxic properties.

3.3. Intracellular reactive oxygen species (ROS)

It is well known that ROS levels are a critical parameter in cellular processes. Low levels of ROS regulate various cell signaling processes, such as growth, proliferation, and differentiation. Generally, cells exposed to nanomaterials show increased ROS levels. Accumulation of intracellular ROS is the main characteristic of oxidative stress; therefore, the detection of ROS reflects the intracellular oxidative stress status, which is an important indicator of cell health. In the present investigation, we measured the intracellular ROS levels in RAW264.7 cells after exposure to MWCNTs. ROS generation was monitored via an increase in fluorescence intensity of dichlorofluorescein (DCF) over time. As shown in Fig. 3, MWCNTs induced a functionalized- and concentration-dependent increase in ROS. The largest change in ROS observed was an 11-fold increase after 12 h of exposure to 200 μg/ml MWCNTs when compared with untreated controls.

The dual role of ROS is particularly evident with regards to the function of phagocytic cells of the innate immune system. A small amount of ROS triggers some of the key mechanisms involved in cellular defense after particle uptake. However, phagocyte-derived excesses in ROS are known to injure human tissues and contribute to inflammation. Many previous reports found that certain nanoparticles (NPs), such as TiO₂, silver, silica, cerium oxide, and quantum dots (QDs), can induce the generation of ROS and cytotoxicity. On the contrary, other NPs, such as fullerene and its derivatives, possess a unique capacity for scavenging ROS and are categorized as a radical sponge because they react with free radicals. Thus, NPs can influence intracellular ROS levels by disturbing the balance between oxidant and antioxidant processes. In the present investigation, MWCNTs–COOH induced higher ROS values when compared with neutral surface MWCNTs (raw MWCNTs and MWCNTs–PEG). Remarkably, the ROS production induced by MWCNTs–COOH was more pronounced when compared with the other MWCNTs, and this ROS generation correlated with the different MWCNT modifications, cellular uptake and viability results from our previous studies [29]. It is known that ROS production and the subsequent oxidative stress causes mitochondrial dysfunction, which could contribute to apoptosis [36]. Therefore, apoptosis in cells exposed to MWCNTs should be evaluated for the relationship between oxidative stress and apoptosis. In addition, the ROS production induced by nanomaterials can damage cells externally (membrane) or internally (after nanomaterial uptake), and thus contribute to inflammation [37].
3.4. Apoptosis and necrosis caused by MWCNTs

We demonstrated that specific functionalized MWCNTs induced an increase in intracellular ROS and a decrease in cell viability and ATP content in RAW264.7 cells. Next, we quantified the percentage of cells exposed different concentrations of MWCNTs in early apoptotic, apoptotic, or late apoptotic (necrotic) stages using flow cytometry (Supplementary information). The mechanisms of apoptosis and necrosis are highly studied to investigate cell death. As shown in Fig. S1, exposure of cells to MWCNTs caused a slight, non-significant increase in the proportion of apoptosis and necrosis as a function of incubation time when compared with control group. MWCNT-induced apoptosis in RAW264.7 cells was confirmed using morphological criteria. Fig. 4 shows the typical TEM ultrastructural images of untreated cells (A and a) and cells exposed for 12 h to the three types of MWCNTs (B–E). Large phagocytic vesicles were observed in MWCNT-treated cells, suggesting that MWCNTs translocate into the murine macrophage-like RAW264.7 cells via phagocytosis. These data need further confirmation using other techniques. No apoptotic bodies were observed in any of the cells. The absence of significant apoptosis confirms that the ROS concentration was not high enough to induce RAW264.7 cell death after exposure to raw or oxidized MWCNTs. Additionally, the nanotubes that induced a lower level of ROS were unable to cross the cell membrane and instead provided support for cell proliferation.

The nanotubes that induced a higher level of ROS were cytotoxic because they were taken up by cells and damaged cellular organelles. TEM was employed to directly investigate mitochondrial morphology and damage before and after MWCNT exposure. As shown in Fig. 4a, normal morphological mitochondria were observed in RAW264.7 cells from the control group. However, after a 12-h incubation with MWCNTs–COOH, the mitochondria of RAW264.7 cells showed abnormal swelling and obscure cristae (Fig. 4E and e). In addition, at high magnification, agglomerated and individual MWCNTs were observed in the cytoplasm (Fig. 4B and D).

3.5. Activation of pro-inflammation responses by MWCNTs

Macrophages are a major source of cytokines and chemokines that are involved in immune responses. Upon stimulation by exogenous or endogenous factors, macrophages can synthesize and release a large variety of cytokines and cell signaling molecules, and chemokines contribute to the recruitment of circulating monocytes within tissues. Therefore, the evaluation of the pro-inflammatory response in macrophages exposed to MWCNTs is important for nanotoxicological assessments. As shown in Fig. 5, a significant increase in IL-1β and TNF-α mRNA expression was found after exposure to doses of raw MWCNTs and MWCNTs–COOH up to 50 μg/ml, but not MWCNTs–PEG. IL-6 and iNOS mRNA was prominently increased after exposure to a dose of 100 μg/ml of raw MWCNTs and MWCNTs–COOH. All doses of MWCNTs–COOH caused considerable increases in IL-1β, IL-6, TNF-α, and iNOS gene expression when compared with the control group and the MWCNTs–PEG and raw MWCNTs groups. These results indicate that MWCNTs enhance the transcription and promote the secretion of pro-inflammatory factors in macrophages. Notably, these results are consistent with the effects of the MWCNTs on cell viability, uptake, and ROS generation.

3.6. Activation of NF-κB signaling pathways

Studies have shown that ROS and inflammation are involved in many of the processes underlying macrophage activation. Many
of the key signal transduction molecules involved in macrophage activation, such as the transcription factor NF-kB, are known to be redox sensitive [38]; NF-kB is localized in the cytoplasm in its inactive form with the protein heterodimer p50/p65 and the inhibitor protein IkB. In the classical activation pathway, activation of NF-kB is dependent on IkB kinase and phosphorylation of IkB leads to its degradation with subsequent liberation and translocation to the nucleus [39].

We assessed the phosphorylation and degradation of IkB and p65 in RAW264.7 cells exposed to 25–100 μg/ml MWCNTs using Western blotting. A 12-h exposure of macrophages to MWCNTs was sufficient to trigger the phosphorylation and degradation of IkB and the translocation of p65 from the cytosol to the nucleus (Fig. 6). These data indicate that the NF-kB signaling pathway was activated after MWCNT exposure. Based on our additional results showing the production of ROS and pro-inflammatory genes, we suggest that MWCNTs, especially MWCNTs–COOH, enhance immune function through the NF-kB signaling pathway. Exposure to MWCNTs–COOH induced the degradation of IkB-α, and the nuclear translocation of p65 NF-kB. These results indicate NF-kB may play an important role in the induction of ROS and cytokines after MWCNT exposure. It has been previously shown that SWCNTs can induce pro-inflammatory cytokines and chemokines in THP-1-derived macrophages via the activation of NF-kB [40]. Manna et al. [28] demonstrated that the increased oxidative stress, inhibition of cell proliferation, and activation of NF-kB in SWCNT-treated keratinocytes was due to the activation of stress-related kinases. Our present study found that RAW264.7 cells exposed to MWCNTs showed a decrease in NF-kB expression in the cytoplasm and an increase in nuclei, indicating that NF-kB was activated via translocation.

3.7. Activation of MAPK signaling pathways

During inflammation, a broad array of cell signaling pathways are activated, and numerous genes and proteins are involved in these processes. ROS are involved in the inflammatory response and proliferation of cells via the activation of mitogen-activated protein kinase (MAPK) cascades. MAPKs are regarded as stress-sensitive kinases and they play a critical role in the regulation of cell growth and differentiation and control cellular responses to cytokines and stress [41]. In addition, MAPKs play a critical role in the modulation of NF-kB activity [42].

MAPKs are serine-threonine protein kinases that consist of extracellular signal-related kinases (ERK), c-Jun NH2-terminal kinases (JNK), and p38 MAPK. Once ROS production exceeds the capacity of the antioxidant proteins, free radicals can induce the oxidative modification and activation of MAPK signaling proteins [43,44]. In addition, the phosphorylation and activation of three major MAPKs (p38, ERK1/2, and JNK) can initiate inflammatory gene expression in LPS-induced macrophages [41], and activated p38 induces TNF-α and iNOS production by modulating NF-kB [41].

It has been well established that the phosphorylation and activation of MAPKs, especially p38, ERK, and JNK, is crucial for LPS-induced NF-kB activation and the subsequent activation of pro-inflammatory mediators in macrophages [41]. To determine the potential signaling pathways involved in MWCNT exposure, we measured ERK, p38, and JNK protein levels (and their phosphorylated form) in RAW264.7 cells exposed to 25–100 μg/ml of three different MWCNTs. Fig. 7 shows that the total ERK, JNK, and p38 protein levels were similar before and after MWCNT exposure. However, MWCNT exposure activated the LPS-induced phosphorylation of p38, ERK, and JNK in a dose-dependent manner. Moreover, JNK showed the largest difference between non-activated and activated (phosphorylated) states. Not surprisingly, the JNK pathway is typically activated by external “death” stimuli, such as TNF-α, that trigger inflammatory pathways. The p38 protein is activated by ROS and interacts with downstream transcription factors, such as NF-kB, following stimulation by TNF-α and IL-1β to induce cellular inflammatory injuries [45,46]. In addition, ERK pathway activation is involved in IKK activation and TNF-α production [47]. ERK and p38 induce iNOS and COX-2 following LPS stimulation [48,49]. Therefore, we confirmed that the recruited MAPKs induce a decrease in cell viability and an increase in the expression of inflammatory genes.

3.8. Crosstalk within the inflammatory pathway

MWCNT exposure activates macrophages and triggers the production of ROS and inflammatory mediators via the activation of MAPK and NF-kB signaling pathway; thus, it is important to explore the underlying mechanisms involved. The present study addressed
Fig. 8. Hypothetical scheme depicting the MWCNT-induced intracellular signaling in RAW264.7 cells that leads to inflammation through ROS-activated MAPK and NF-κB pathways: (1) extracellular MWCNTs are engulfed and cleared by macrophages upon contact; (2) reactive oxygen species produced inside the macrophages lead to activation of stress-dependent MAPK and NF-κB signaling pathways that regulate the production of pro-inflammatory genes.

how modified MWCNTs can cause pro-inflammatory effects but not induce apoptosis in RAW264.7 cells. These hypotheses are outlined in the schematic diagram shown in Fig. 8.

Based on our results, ROS and inflammation are interdependent responses to MWCNT exposure. Macrophages induce high levels of ROS release in an attempt to remove MWCNTs. However, MWCNT-mediated oxidative stress leads to activation of MAPK and NF-κB and contributes to the pro-inflammatory cascade [50]. One of the most frequently reported toxicity endpoints for CNTs is the formation of ROS that can be either protective or harmful during biological interactions. Nanoparticles are self-oxidative and react with phagocytic cells, including neutrophils and alveolar macrophages (AM), of the immune system to induce pro-oxidant effects via intracellular ROS generation, changes in mitochondrial respiration, and activation of NADPH-like enzyme systems [51,52]. When ROS are generated excess, lipid peroxides, and GSH depletion cause the phosphorylation and degradation of the NF-κB inhibitor, which is a critical step for NF-κB activation [53,54]. The in vitro activation of NF-κB following MWCNT exposure has been previously demonstrated [55]. NF-κB regulates the expression of many pro-inflammatory cytokines, including TNF-α and IL-1β [56,57]. MAPKs are the upstream kinases responsible for NF-κB activation. We speculate that oxidative stress and inflammation is a cause of cytotoxicity even at small levels.

3.9. Relationship between MWCNT characteristics and their toxic effects

One possible explanation as to why the different types of the carbon nanotubes produced varying inflammatory effects is that phagocytic cells attempt to engulf the fiber-like structures, which leads to activation of NADPH oxidase and production of ROS with or without internalization of the MWCNTs. Although, the role of NADPH oxidase-derived ROS for inflammasome activation has been called into question [58], an alternative hypothesis is that nanotubes are recognized by phagocytes not as single fibers but as entangled particles. This identification leads to their internalization via endocytosis with subsequent fusion with lysosomes inside the cell. The ensuing disruption of lysosomes may lead to the activation of the inflammasome. We previously reported that physicochemical modifications of MWCNTs may cause an increase in inflammation, and physicochemical modifications determine the state and stability of MWCNT dispersions, cellular uptake, and their cytotoxic effects [29]. Therefore, we speculate that the differing inflammatory responses caused by MWCNTs are due to their cell uptake and physicochemical properties.

4. Conclusion

Here, we evaluated the cytotoxicity and related signaling pathways of raw and surface-functionalized MWCNTs in RAW264.7 cells. Increased intracellular ROS, inflammatory mediators, and cytotoxicity indicates direct effects after cellular uptake. A possible mechanism of toxicity is proposed which involves a significant increase in ROS generation, interruption of ATP synthesis and the activation of the MAPK and NF-κB signaling pathways, which in turn cause the upregulation of IL-1β, IL-6, TNF-α, and iNOS in RAW264.7 cells. This work provides essential information for the development of novel biomedical applications for functionalized MWCNTs. However, a better understanding of the long-term toxicology of modified MWCNTs and their in vitro effects must be further investigated.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2014.11.013.

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