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Cytotoxicity, cytocompatibility, cell-labeling efficiency, and *in vitro* cellular magnetic resonance imaging of gadolinium-catalyzed single-walled carbon nanotubes

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Abstract

Cell tracking by magnetic resonance imaging (MRI) is an emerging technique that typically requires the use of MRI contrast agents (CAs). A MRI CA for cellular imaging should label cells efficiently at potentially safe concentrations, have high relaxivity, and not affect the cellular machinery. In this article, we report the cytotoxicity, cytocompatibility, and cell labeling efficiency in NIH/3T3 fibroblasts of novel, single-walled carbon nanotubes synthesized using gadolinium nano-particles as catalysts (Gd-SWCNTs). Cells incubated with the Gd-SWCNT showed a dose- (50–100 µg/mL nanotube concentration) and time- (12–48 h) dependent decrease in viability. 30% cell death was observed for cells incubated with Gd-SWCNTs at the maximum dose of 100 µg/mL for 48 h. Cells incubated with the Gd-SWCNTs at concentrations between 1–10 µg/mL for 48 h showed no change in viability or proliferation compared to untreated controls. Additionally, at these potentially safe concentrations, up to 48 h, the cells showed no phosphatidyl serine externalization (pre-apoptotic condition), caspase-3 activity (point of no return for apoptosis), genetic damage, or changes in their division cycle. Localization of Gd-SWCNTs within the cells was confirmed by transmission electron microscopy (TEM) and Raman microscopy, and these results show 100% cell labeling efficiency. Elemental analysis also indicates significant uptake of Gd-SWCNTs by the cells (10^8 – 10^9 Gd³⁺ ions per cell). Finally, T_1 -weighted MRI at 3 T of Gd-SWCNT-labelled cells show up to a four-fold increase in MR signal intensities as compared to untreated cells. These results indicate that Gd-SWCNTs label cells efficiently at potentially safe concentrations, and enhance MRI contrast without any structural damage to the cells.

Keywords

gadolinium; single walled carbon nanotubes; MRI contrast agent; Cytotoxicity; labeling efficiencies

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INTRODUCTION

Contrast-enhanced cellular magnetic resonance imaging (MRI) is an emerging area of research that involves cell labeling with MRI contrast agents (CAs) to improve non-invasive visualization and tracking of cells *in vivo*.¹ Efficient detection of labeled cells depends on a number of factors, including the physico-chemical properties of CAs, amount of CA uptake into cells, resolution of acquired images, and strength of the magnetic field. Iron oxide-based micro- and nano-particles are most widely used for cellular MRI.² T_2 or T_2^* -weighted MRI is typically performed on cells labeled with iron-oxide particles, and the resultant images show hypo-intense regions due to susceptibility artifacts created by these super paramagnetic MRI CAs.² Additionally, it is difficult to identify cells labeled with iron-oxide nanoparticles in T_2^* -weighted images due to poor inherent signal-to-noise ratio, partial volume effects, and similar signal intensity between labeled cells and hemorrhages, blood vessels or cells that accumulate due to immune response.³ Thus, there have been recent efforts to label cells with paramagnetic manganese (Mn)- or gadolinium (Gd)-based CAs that allow T_1 -weighted MRI with hyper intensity regions, resulting in brighter contrast between labeled cells and background tissue.^{4,5}

Carbon nanotubes are currently being explored for drug delivery, therapeutics, and diagnostic applications.^{6,7} Specifically for cellular MRI, single-walled carbon nanotube (SWCNT) Gd complexes, called gadonanotubes,^{8–10} have been reported as magnetic labels for cellular MRI.¹¹ These gadonanotubes are prepared by a “top-down” synthesis method, wherein pristine commercial iron or cobalt–nickel catalyzed single-walled carbon nanotubes (SWCNTs, length > 1 μm) are chemically cut into ultra-short tubes (US-tubes, length 20–80 nm); Gd^{3+} ions are subsequently loaded within the US-tubes through defects along the sidewalls of the US-tubes.¹²

Recently, we have developed a novel “bottom-up” chemical strategy to synthesize SWCNT lanthanoid [e.g., Gd or europium (Eu)] complexes. This synthetic strategy utilizes lanthanoid nanoparticles (average size ~ 1.8 nm) as catalysts to grow SWCNTs (average diameter 2.05 nm, length 500 nm–1.5 μm).¹³ The lanthanoid nanoparticles are embedded into and located at one end of the SWCNT.¹⁴ Physico-chemical characterization of water-solubilized, gadolinium-catalyzed SWCNTs (Gd-SWCNTs) show that they are paramagnetic, have high r_1 and r_2 relaxivities at clinically relevant (1.5 and 3 T) magnetic fields ($r_1 \sim 130 \text{ mM}^{-1}\text{s}^{-1}$, $r_2 \sim 160 \text{ mM}^{-1}\text{s}^{-1}$) with MRI signal intensity 14 times greater than clinical Gd-based MRI contrast agents. These studies indicate that the Gd-SWCNTs should be suitable as novel high performance T_1 MRI contrast agents for advanced applications such as molecular and cellular MRI.¹⁵

Multiple factors such as size (aspect ratio) of SWCNTs, the catalysts used in their growth, the water-solubilizing groups, and the method of administration affect *in vitro* and *in vivo* toxicity of SWCNTs.⁷ Therefore, it is prudent and necessary to assess safety of new SWCNT-based complex such as Gd-SWCNTs as a different compound. Specifically for cellular MRI applications, such as *in vivo* cell tracking,¹ cells will be labeled *ex vivo* with Gd-SWCNTs. To establish the optimal *ex vivo* cell labeling protocols with the Gd-SWCNTs, their cytotoxicity needs to be assessed to identify potentially safe dosages. Additionally the *in vitro* labeling efficiencies, and MRI of Gd-SWCNT-labeled cells needs to be determined to obtain insights into their efficacy and sensitivity (minimum detectable cells). This information will provide guidance in designing future *in vivo* cellular MRI experiments. Thus, in this article, we report the cytotoxicity, cytocompatibility (bioinert), cell labeling efficiencies, and *in vitro* cellular MRI of Gd-SWCNTs to establish the optimal *ex vivo* cell labeling protocols, and assess, their efficacy for cellular MRI *in vitro*.

MATERIALS AND METHODS

Gd-SWCNT synthesis and functionalization

The Gd-SWCNTs were synthesized using a previously developed chemical vapor deposition (CVD)-based method.¹³ The percent weight of Gadolinium (grams of Gd³⁺ ion in 100 g Gd-SWCNT solid) was 6.2%. A well-established literature method was used to non-covalently functionalize the hydrophobic SWCNTs with PEG₅₄₀₀-DSPE (where PEG = polyethylene glycol with average molecular weight of 5400 Da, and DSPE = phospholipid, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine) to render them water-soluble.¹⁶ Briefly, Gd-SWCNTs were sonicated in an aqueous solution of DSPE-PEG₅₄₀₀ for 6 h, and centrifuged at 26,000×g for 1 h to remove unreacted Gd-SWCNTs, producing Gd-SWCNT-DSPE-PEG₅₄₀₀. The hydrophobic DSPE wraps around SWCNTs via non-covalent interactions, and the hydrophilic PEG-water solubilizes the Gd-SWCNTs. PEG-DSPE has a positive charge, and thus, the overall charge of Gd-SWCNT-DSPE-PEG formulations was positive. The unreacted DSPE-PEG was removed by dialysis and filtration. The SWCNT concentration of functionalized Gd-SWCNT solution was determined by optical absorbance and used for *in vitro* studies.¹⁵

Cell culture and viability/cytotoxicity assessment

NIH/3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (v/v) and 1% penicillin/streptomycin under standard culture conditions. For cytotoxicity assays, 2×10^4 cells/well cultured overnight in 96-well plates were replaced with fresh medium, treated with Gd-SWCNTs at concentrations between 0.1–100 µg/mL, and incubated for 12, 24, or 48 h durations. Cell viability was assessed by Trypan blue exclusion method and LIVE/DEAD cell assay. For the Trypan blue exclusion method, at the three different time points, cells were detached by 0.05% trypsin-EDTA solution and stained with Trypan blue. The dead cells take up Trypan blue dye, and live cells exclude the dye. The number of viable and dead cells was then counted manually using a hemocytometer. For the LIVE/DEAD cell assay, a kit supplied by the manufacturer (Invitrogen, Eugene, OR) was used as per the manufacturer's protocol. The fluorescence emission obtained from live (green emission, ex/em ~495/~515 nm), and dead (red emission, ex/em ~495/~635 nm) cells was used to calculate the percentage of viable cells.

Lactate dehydrogenase (LDH) assay for membrane destabilization

Leaky membranes of damaged or dead cells release the cytoplasmic enzyme, lactate dehydrogenase (LDH), into the surrounding media.¹⁷ For the LDH analysis, 2×10^4 NIH/3T3 cells/well seeded in 24 well-plates were treated with Gd-SWCNTs at concentrations between 0.1–100 µg/mL. After 12, 24, or 48 h of incubation, 100 µL of the media in which the cells were incubated was collected and analyzed for the presence of any released lactate dehydrogenase (LDH) using a TOX-7 kit (Sigma-Aldrich). The LDH data is presented relative to the basal LDH released by control cells that were not treated with the Gd-SWCNTs.

Phosphatidyl serine externalization by annexin-V/propidium iodide (PI) counter staining

The amount of cells undergoing apoptosis or necrosis was quantified with the annexin-V-FITC/propidium iodide (PI) counter staining method. Cells were plated at a density of 1×10^6 mL⁻¹ in 24-well plates and treated with the various (0.1–100 µg/mL) Gd-SWCNT concentrations. After incubation for 24 and 48 h, the cells were washed in PBS and trypsinized. These cells were resuspended in 200 µL binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl₂, pH 7.4) and again incubated for 30 min at 4°C with 5 µL each of

annexin-V-FITC and PI. Next, the cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ) for the percentage of live cells (% of cells not stained by either of the fluorochromes), pre-apoptotic cells (stained with annexin-V only) and necrotic cells (stained with PI only). The data of the total analyzed cells is presented as the percentage of apoptotic and necrotic cells.

Apoptotic late stage assessment by caspase-3 activation

Caspase-3 activity was determined based on the hydrolysis of the peptide substrate, acetyl-Asp-Glu-Val-Asp *p*-nitroaniline (Ac-DEVD-pNA), by caspase-3, resulting in the release of *p*-nitroaniline (pNA).¹⁸ The released pNA has a maximum absorption at 405 nm. For the caspase-3 activation assay, cells ($\sim 0.2 \times 10^6$) treated with or without the different concentrations of Gd-SWCNTs for various time periods were trypsinized, collected, and centrifuged at $750 \times g$ for 5 min at 4°C. The pellet obtained was suspended in 200 μ L lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT) and left on ice for 20 min. Suspension was then centrifuged at $15,000 \times g$ for 15 min at 4°C and the supernatant was collected. Approximately 100 μ L of the supernatant was added to the 96-well microplate containing 90 μ L of caspase assay buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT) and 10 μ L of 2 mM Ac-DEVD-pNA substrate in DMSO. The reaction mixture was incubated for 4 h at 37°C. Different concentrations of the pNA were used to prepare the standard curve. Cleavage of the dye (pNA) from the substrate by the sample produces yellow color that has a maximum absorption at 405 nm. The measurements were made using a BIO-TEK Instruments FLx800 plate reader (Winooski, VT).

Cell cycle kinetics by flow cytometry

The cell samples for the cell cycle kinetics were prepared as follows. NIH/3T3 cells were first incubated in serum free media for 24 h. These serum-starved NIH/3T3 cells were treated with or without Gd-SWCNTs at various concentrations for 24 and 48 h. Next, at the end of the various time points, the cells were washed with PBS, trypsinized, suspended in 70% ethanol/PBS on ice, and centrifuged at $1000 \times g$ for 30 min. The cell pellets were resuspended in PBS, and treated with RNase (DNase free, 100 μ g/mL in PBS) and propidium iodide (40 μ g/mL in PBS) at 37°C for 30 min. The cells were centrifuged again at $1000 \times g$, and the pellet obtained was resuspended in PBS. This aggregated cell suspension was passed through a 19-gauge needle to obtain an individual cell suspension, and kept on ice until analysis. The number of cells in different phases of the cell cycle was analyzed using a FACScan Flow cytometer with Cell-FIT software (Becton Dickinson Instruments, NJ).

Genotoxic damage assessment by micronuclei formation

The micronuclei assay was performed to assess the genotoxic damage caused by Gd-SWCNTs to the cells.¹⁹ The samples were prepared and assayed as follows. Cells cultured in 24-well plates were treated with different concentrations of Gd-SWCNTs (0–100 μ g/mL), and incubated for 24 or 48 h. At the end of the treatments, cells were fixed in 4% formaldehyde for 30–45 min, and stained with 100 μ L of 0.1 % w/v of acridine orange (AO) in distilled water for 10 min. Next, the cells were washed three times with PBS, and scanned for micronucleated cells using a Zeiss fluorescent microscope (Carl Zeiss, Jena, Germany).

Ultra-structural localization by transmission electron microscopy (TEM)

Cells grown on ACLAR® film (Electron Microscopy Sciences, PA) were exposed to various Gd-SWCNTs concentrations for different time periods as described in the cell viability section. At the end of each time point, the cells were fixed in 2.5% electron microscopic grade glutaraldehyde prepared in 0.1M phosphate buffered saline (PBS). Cells

were then processed without staining using standard electron microscopy (EM) techniques. Briefly after the fixation, the fixed cell samples were placed in 2% osmium tetroxide with 0.1M PBS, dehydrated in a graded series of ethyl alcohol, and embedded in Durcupan resin. Tissue blocks of the areas of interest were prepared and 80 nm ultrathin sections were cut with an ultramicrotome (Reichert-Jung UltracutE), which were then placed on formvar-coated slot copper TEM grids. The sections were then viewed with a FEI Tecnai BioTwinG² transmission electron microscope at 80 kV. Digital images were acquired with an AMT XR-60 CCD digital camera system.

Metal content analysis by inductively coupled plasma-optical emission spectroscopy (ICP-OES)

ICP-OES was performed to determine the cellular uptake of the Gd-SWCNTs by quantifying the gadolinium concentration. The samples for ICP-OES were prepared as follows. Approximately 4×10^6 cells were treated with various concentrations of Gd-SWCNT for 12, 24, and 48 h. At the end of the incubation times, cells were thoroughly washed with PBS to remove excess Gd-SWCNTs in the cell media, and then centrifuged at 1000 rpm for 5 min. The cell pellets were digested with 95% nitric acid, and the solution was heated carefully until a solid residue remained. This solid residue was dissolved in 2% HNO₃ and used for the ICP-OES analysis. The ICP-OES was performed using an Optima 5300 ICP-OES spectrometer (Perkin-Elmer Inc, MA).

Magnetic resonance imaging

Magnetic resonance imaging (MRI) phantoms of 4×10^6 NIH/3T3 cells untreated and treated with 1, 10, and 25 $\mu\text{g/mL}$ of Gd-SWCNTs for 12, 24, or 48 h, and dispersed in 7 mL of 0.7% agar were scanned on a 3 T Trio Siemens MRI Scanner ($T = 27^\circ\text{C}$). High resolution T_1 -weighted images ($\text{TR} = 200$ ms, $\text{TE} = 5$ ms) were obtained using an inversion recovery prepared spin echo technique. The field of view (FOV) and matrix sizes were chosen to yield an acquired voxel size, $0.5 \times 0.5 \times 2.5$ mm³, and reconstructed voxel size of $0.25 \times 0.25 \times 2.5$ mm³. A region-of-interest (ROI) analysis of signal intensity (SI) was performed using a circular 0.35 cm² ROI and the software (Syngo) available on the MR unit.

Confocal Raman microscopy

NIH/3T3 cells grown on two-well chambered slides were treated with Gd-SWCNTs (10 $\mu\text{g/mL}$) for 48 h. The cells were then washed with PBS, fixed in ice-cold methanol, and optical images were taken. Next, cells were subjected to dispersive Raman measurements using an excitation laser at 532 nm on a DXR Raman microscope (Thermo Fischer Scientific, Milan, Italy) using a 50 $\times$ objective. The Raman spectra were obtained for a number of points on the cells. Point-by-point Raman spectra were obtained over an area corresponding to single selected cell and mapped. The G-band intensity of the Gd-SWCNTs was collected, and area maps were constructed, mapped, and overlaid with the optical images for their localization.

Statistical analysis

The statistical significance of differences between various treatment groups was determined by using two-way analysis of variance (ANOVA) followed by Mann-Whitney and Kruskal-Wallis test for multiple comparisons between the non-parametric groups. The data is presented as mean $\pm$ standard deviation ($n = 3$) and a p -value of less than 0.05 ($p < 0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

Experiments were designed wherein NIH/3T3 cells were treated at various concentrations of Gd-SWCNTs (1–100 $\mu\text{g/mL}$), and incubated for various time points (up to 48 h). Their

cytotoxicity, cytocompatibility, cellular uptake, and labeling efficiencies were subsequently analyzed, and *in vitro* MRI was performed. Adherent NIH/3T3 cells, which are widely accepted model cell lines for *in vitro* cytotoxicity and cytocompatibility screening, were used in this study.²⁰

Cell culture and viability/cytotoxicity assessment

In this study, we performed two different cell viability assays: LIVE/DEAD cell assay and Trypan blue assay. A recent report suggests that the assessment of SWCNT cytotoxicity could be studied by various optical and fluorescence based methods.²¹ However, the presence of the SWCNTs has been reported to affect the results of some of these methods. Thus, it is recommended to compare at least two different methods of cell viability assessment when working with SWCNTs. Herein, we have used the fluorescence-based LIVE/DEAD, and Trypan blue assay. To assess the *in vitro* toxicity, cells were treated with Gd-SWCNTs at various concentrations (0.1, 0.5, 10, 50, and 100 $\mu\text{g/mL}$) for 12, 24, and 48 h [Fig.1(A)]. The LIVE/DEAD cell analysis showed a dose- and time-dependent decrease in the percentage of viable cells. Gd-SWCNTs up to 10 $\mu\text{g/mL}$ did not show any significant change in cell viability up to 24 h. At 48 h, as the concentration of Gd-SWCNTs increased from 10 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$, cell death increased by 20% and 30%, respectively. Trypan blue assays showed no significant change in cell viability of a 100 $\mu\text{g/mL}$ treatment with Gd-SWCNTs up to 24 h. Gd-SWCNT treatment above 50 $\mu\text{g/mL}$ for 48 h decreased the cell viability by 12% [Fig.1(B)]. These results are consistent with other SWCNTs cytotoxicity studies, albeit in different cell types.^{22,23} The difference in the percentage of live/dead cells beyond 10 $\mu\text{g/mL}$ of Gd-SWCNT is possible due to the fluorescence quenching ability of the carbon nanotubes.²² Trypan blue assay, also called the dye exclusion method, involves manual counting of live and dead cells. This method, though tedious, is considered more reliable than fluorescence-based assays in the carbon nanotube cytotoxicity analysis.²² From previous studies, viability assessment performed using Trypan blue in various cell types suggests that SWCNTs in the concentration range of 1–100 $\mu\text{g/mL}$ show variable cytotoxicity.^{24–26} This difference in cell viability reported above and by other reports could be due to the variable sensitivity of different cell types and other factors that have been shown to affect cytotoxicity such as the type of dispersing agents used, aggregation of SWCNTs, as well as the size (aspect ratio) of the SWCNTs.^{25–27} It should be noted that the above results provide information only about the short-term effect in the presence of the Gd-SWCNTs. Any delayed effect due to the presence of these nanoparticles beyond the 48-h time point still needs to be determined.

LDH activity

Lactate dehydrogenase (LDH), a cytoplasmic marker for membrane integrity, provides an indirect means of assessing cytotoxicity.²⁸ The results of the LDH analysis are presented in Figure 1(C). No significant rise in LDH release was observed for cells incubated with 0.1 and 0.5 $\mu\text{g/mL}$ Gd-SWCNT for 12 or 24 h time points. After 48 h, only cells incubated with 0.5 $\mu\text{g/mL}$ Gd-SWCNT showed a statistically significant difference compared to control (unlabeled cells). For cells incubated with 10 $\mu\text{g/mL}$ of Gd-SWCNTs, significant LDH release was noted only after incubation for 48 h. Cells treated with Gd-SWCNT at 50 and 100 $\mu\text{g/mL}$ concentration did not show significant LDH release after incubation for 12 h. However, at 24 and 48 h time points, significant amount of LDH was released (~25% to 35% compared to untreated controls). Although these results show similar trends compared to previous studies on SWCNTs that show a dose- and time-dependent increase in the LDH release,^{29–31} the amount of LDH released was lower than the earlier reports.^{29,32} LDH release is directly related to cytotoxicity. The lower cytotoxicity values observed with DSPE–PEG solubilized Gd-SWCNTs compared to the previous studies, where SWCNTs were dispersed in other solubilizing agents, suggest that surface functionalization plays a critical

role in biocompatibility, membrane damage, and the cellular uptake process.²⁹ In summary, the LDH studies indicate that Gd-SWCNTs below 50 µg/mL cause minimal membrane destabilization and effects to cellular homeostasis, and corroborate the results of the cell viability/cytotoxicity tests.

Phosphatidyl serine (PS) externalization

Annexin-V detects phosphatidyl serine externalized in the early phase of apoptosis, and PI stains necrotic cells.³³ No significant change was observed in the PS externalization at any of the doses or time points tested with 0.1–10 µg/mL of Gd-SWCNTs (data not shown). An increase (14%) in PS externalization [Fig.2(A), middle panel, quadrant II] was observed at 48 h after the cells were treated with 50 µg/mL of SWCNT as compared to control. These results are in agreement with previous reports,^{34,35} which show that 50 µg/mL of SWCNT incubated for 48 h increased the PS externalization by approximately 15%. At the highest dose of 100 µg/mL Gd-SWCNT tested for 48 h, the percentage of necrotic cells increased [~30%, Fig.2(A), right panel, quadrant IV] significantly as compared to the apoptotic cells [6%, Fig.2(A), right panel, quadrant II; Fig.2(C)]. The results suggest that high concentrations of Gd-SWCNTs might have completely destabilized the membrane permeability of the NIH/3T3 cells, and allowed the PI to enter the cells, leading to increased subpopulations of necrotic cells in quadrant IV. The intact membrane of apoptotic cells, a main feature not observed in necrotic cells, does not allow PI to enter and intercalate into the DNA. Therefore, it can be inferred that apoptosis is the dominant mechanism of cell death for treatment at 50 µg/mL for 24 h, and cellular necrosis is the cause of cell death observed for treatments at higher concentrations (100 µg/mL).

Caspase-3 activation

Caspases (cysteine-requiring aspartate proteases) activation usually leads to an irreversible commitment towards cell death.³⁶ In this study, doses of Gd-SWCNTs below 50 µg/mL did not induce cellular caspase-3 activity [Fig.2(B)]. At 50 µg/mL of Gd-SWCNT treatment, as compared to control, the caspase-3 activity showed 120 and 220% increase at the 24 and 48 h time points, respectively. Increasing the concentration of Gd-SWCNTs to 100 µg/mL increased the caspase-3 activity to 220 and 280% at 24 and 48 h, respectively. These trends are similar to those observed for pre-apoptotic analysis of PS externalization [Fig.2(A)] that show a 15 and 30% increase at 48 h with 50 µg/mL and 100 µg/mL of Gd-SWCNTs, respectively. The Gd-SWCNTs treatment with 100 µg/mL of Gd-SWCNTs for 48 h did not significantly increase the change in caspase-3 activity. Thus, at these high concentrations, the cells, membrane is likely undergoing damage that leads to more cell death by necrosis than apoptosis.

Cell cycle kinetics

Previous reports suggest that 25 µg/mL of SWCNTs for 24 h treatment induced G1 phase arrest of the cells undergoing normal growth and proliferation.^{26,37} The critical step in cell cycle analysis in response to xenobiotic agents depends on the cell cycle stage. In the normal culture conditions, cells grow asynchronously, and the overall effect of any xenobiotic material depends on the number of cells affected at a specific stage of the cell cycle. To understand the effect of Gd-SWCNTs on cell cycle phase, the cells should be synchronized in the same cell cycle phase. Therefore, by serum starvation, the cells were arrested in G1 phase. G1 phase is the critical stage for the control of cellular growth and proliferation. Serum starvation in the control cells resulted in more than 90% of the cells in the G1 phase [Fig.2(C), left panel]. The Gd-SWCNTs treatment (10 and 50 µg/mL for 48 h) had no effect on regulating the G1 phase of the cell cycle [Fig.2(C), middle and right panel]. Cells undergoing apoptosis show fragmented DNA, which is reflected by an increase in the sub-G1 population. The lack of changes in the sub-G1 phase, and G1 phase population after Gd-

SWCNTs (10 and 50 $\mu\text{g/mL}$) treatment indicates that these Gd-SWCNTs do not increase the apoptotic cell population. More than 90% of the cells were in the G1 phase before, and after Gd-SWCNT treatment up to 48 h (Table I). Gd-SWCNT treatment did not affect the duration of different phases of the cell cycle as the percentage of cells in each phase did not change before and after the Gd-SWCNT treatment (Table I). The results indicate that the Gd-SWCNTs at the above treatment conditions do not induce cytotoxicity by arresting the cells in G1 phase. As all the G1 phase cells are not affected by treatment of Gd-SWCNT, it can be inferred that these nanotubes do not alter either the rate of DNA synthesis or other cell cycle kinetics.

Micronuclei

The formation of micronuclei (MN) is generally attributed to chromosomal breakage by genotoxic agents during the metaphase of cell division.^{19,38} Viable cells stained with acridine fluoresce, while micronucleated cells fluoresce orange.¹⁹ The micronuclei formation in Gd-SWCNTs-treated NIH/3T3 cells (50 $\mu\text{g/mL}$ for 48 h) is shown in Figure 3(B) as compared to untreated cells [Fig.3(A)]. It is clearly evident that qualitatively Gd-SWCNTs did not induce micronuclei formation. Similar results were obtained at all the doses, and time points (images not shown). Our results are in agreement with another study,³⁸ and different from other *in vitro* studies.^{39,40} This variability in results across studies could be due to differences in cell types, and SWCNT solubilizing agents. The absence of micronuclei formation due to Gd-SWCNTs could be attributed mainly to their ability to agglomerate, thus possibly hindering their transport all the way to the nucleus to cause genotoxic damage.

Ultrastructural localization of Gd-SWCNTs

Figure 4 shows the TEM images of the ultrastructural localization of Gd-SWCNTs exposed to 10 and 50 $\mu\text{g/mL}$ for 48 h in NIH/3T3 cells. The control cells show intact cellular structure with normal morphology as observed for most fibroblast cells [Fig.4(A)]. The cells appear to be quite active before, and after exposure to 10 and 50 $\mu\text{g/mL}$ of Gd-SWCNTs as evidenced by large number of mitochondria (M) in the cytoplasm (Cy). The cells treated with 50 $\mu\text{g/mL}$ of Gd-SWCNT show an increase in the size of the vacuoles [Fig.4(B–D)] similar to other reports.^{41,42} The cells show no structural alterations in morphology after treatment with 50 $\mu\text{g/mL}$ of Gd-SWCNTs for 48 h [Fig.4(B,C)]. Cells uptake Eu-SWCNTs (analogs of the Gd-SWCNTs), through the process of endocytosis and are localized into the endosomes.⁴³ The same is likely true for the Gd-SWCNTs, and thus, a dynamic equilibrium may exist between uptake and release of the Gd-SWCNTs from cells leading to the presence of steady state concentrations of Gd-SWCNTs within the cells over longer durations.⁴⁴

Confocal Raman microscopy of cells treated with Gd-SWCNTs

The internalization of Gd-SWCNTs into cells was further corroborated by confocal Raman microscopy. Figure 5 shows a representative Raman microscopy image of a NIH/3T3 cell treated with Gd-SWCNTs (10 $\mu\text{g/mL}$ for 48 h). Raman spectra were obtained from a few select points inside and outside the cell as shown in Figure 5(B). Only the region within the cell shows the characteristic Raman peaks of Gd-SWCNTs [Fig.5(C)]. The peaks include a strong G-band at 1598 cm^{-1} , a weak D-band at 1306 cm^{-1} , and radial breathing modes (RBM) between 150 and 300 cm^{-1} , which indicate the presence of Gd-SWCNTs. Figure 5(D) shows a raster image of the cell that provides a Raman map of the area containing Gd-SWCNT focused on the G-band peak. This peak at 1598 cm^{-1} is the tangential Raman mode, and characteristic of the graphitic structure. The area map shows the regions of elevated G-band Raman intensity corresponding to Gd-SWCNT incorporation into the cytoplasmic vesicles. Figure 5(E) shows the overlay of the Raman G-band intensity map

from Figure 5(D) over the optical image [Fig.5(B)], and confirms the presence of Gd-SWCNTs within the cells.

Magnetic cell labeling

Figure 6(A) shows NIH/3T3 cellular uptake of the Gd-SWCNT presented as the average number of Gd^{3+} ions per cell as a function of Gd-SWCNT treatment concentration (1, 10, and 25 $\mu\text{g/mL}$) for 12, 24, and 48 h. Cells incubated at 10 $\mu\text{g/mL}$ Gd-SWCNT concentration showed best average uptake of 10^9 Gd^{3+} ions per cell with no effect on cell viability. The cellular labeling concentration was similar at 24 h with 1 and 10 $\mu\text{g/mL}$ of Gd-SWCNT treatment. The uptake of Gd^{3+} ions decreased for the 10 $\mu\text{g/mL}$ of Gd-SWCNT treatment for 48 h. Increasing the Gd-SWCNT concentration to 25 $\mu\text{g/mL}$ decreased the average number of Gd^{3+} ions from 10^9 ions per cell to 10^8 ions per cell at all the time points. The viability assays showed approximately 10% cytotoxicity or apoptosis with 10 $\mu\text{g/mL}$ of Gd-SWCNT treatment for 48 h, and increased in a dose-dependent manner. Beyond this Gd-SWCNT concentration, the membrane permeability characteristics for a small fraction of cells get altered, as evidenced by LDH release and PS externalization, which triggers the apoptotic pathway and leads to necrosis. This effect may decrease retention of Gd^{3+} ions in the cells. Thus, the reduced Gd^{3+} ion retention in the cells beyond 24 h could be attributed to a number of phenomena associated with the expulsion of Gd-SWCNTs from the cells, membrane permeability characteristics, or some cell death by apoptosis/necrosis. Gadonanotubes have been shown to internalize up to 10^9 Gd^{3+} ions per cell in breast cancer (MCF7),¹¹ and human mesenchymal stem cells.⁴⁵ The uptake results of the Gd-SWCNTs are similar; however, direct comparisons of Gd-SWCNT's uptake characteristics with those of gadonanotubes are not possible due to difference in cell types and labeling protocols. While Gd-SWCNT uptake and labeling efficiency were only assessed on NIH-3T3 cells, other cell studies on Eu-SWCNTs (analogs of the Gd-SWCNTs), show similar uptake and labeling efficiency in NIH-3T3 and other (MCF7, SKBR3) cell lines.⁴³ Thus, we expect the Gd-SWCNTs to show similar uptake characteristics in other cells.

Phantom studies

MRI phantoms of NIH/3T3 cells treated with 1, 10, and 25 $\mu\text{g/mL}$ of Gd-SWCNTs for 12, 24, and 48 h were prepared in 0.7% agar, and scanned on a 3 T Trio Siemens MRI Scanner at room temperature ($T = 27^\circ\text{C}$) to obtain the T_1 -weighted images [Fig.6(B)]. The MR images clearly show that Gd-SWCNT cell labeling substantially increases (up to four-fold) MR contrast as compared to unlabeled cells. The Gd-SWCNTs show a dose-dependent increase in MR signal intensity [Fig.6(C)]. Additionally, Figure 6(C) indicates that cells incubated with 10 and 25 $\mu\text{g/mL}$ of Gd-SWCNT at 24 and 48 h display a lower (26%) MR signal intensity as compared to the 12-h time point. The lower MR signal intensity could be explained by the decrease in retention of Gd-SWCNTs above 10 $\mu\text{g/mL}$ incubation concentration, and 12 h incubation time. The 400,000 Gd-SWCNTs labeled cells were uniformly distributed in the agarose suspension for final volume of ~ 4 mL. Thus, the suspension has 100,000 cells/mL or cm^3 or 62 labeled cells in a 0.625 mm^3 volume; the voxel size of the 3 T scanner. Therefore, the detection threshold using the conditions used in the MRI phantom experiments is ~ 100 cells. The in-plane, acquired spatial resolution of 3 T scanner can easily be lowered to $0.25 \times 0.25\text{ mm}^2$, and at these lower resolutions, the detection threshold could be lowered to a smaller cluster of ~ 15 cells.

The cytotoxicity studies on gadolinium carbon nanostructure complexes till date have mainly focused on assays that assess cell viability or proliferation.^{45–47} In depth cell studies evaluating their effect on the cell anatomy and physiology at toxic or potentially safe concentrations still need to be performed. This study not only provides the toxicity screening of the Gd-SWCNTs using well-accepted cell viability and proliferation assays, such as

LIVE/DEAD cell and Trypan blue exclusion assay, but also provides insights into their effect at toxic and potentially safe concentrations on cell phenotype, cell kinetics, cell membrane, intracellular toxicity mechanisms, and genes. Additionally, the results of this study identify the potentially safe concentrations of Gd-SWCNTs and indicate that the Gd-SWCNTs label cells efficiently at these potentially safe concentrations. Furthermore, the enhanced MRI contrast without any structural damage to the cells bodes well for their further development as magnetic labels for *in vivo* cellular MRI. Current clinical, Gd-based, small molecule chelate compounds are unsuitable for cellular MRI due to relatively low relaxivity (between 4–6 mM⁻¹s⁻¹ at clinical fields 1.5–3 T), and thus can only be detected at micromolar (μM) concentrations.⁴⁸ As high sensitivity MRI CAs, Gd-SWCNTs could overcome the limitations of current *T₁* MRI by allowing their detection at nanomolar (nM) concentrations necessary for cellular or molecular imaging by MRI.⁴⁹ Recently, other Gd³⁺-based experimental MRI CAs have also been shown to be suitable as contrast agents for cellular MRI.^{50–57} The *in vitro* detection threshold by MRI of cells labeled with the Gd-SWCNTs (~62 cells) is comparable or better than cells labeled with these sensitive MRI CAs. Furthermore, the Gd-SWCNTs exhibit near infrared fluorescence,¹⁵ and could integrate the advantages of both MRI and optical imaging to allow the same biological specimen to be imaged at multiple scales, resolution, and depths. Thus, Gd-SWCNTs also show potential for preclinical *in vivo* cellular imaging studies as novel high sensitivity magnetic labels that should allow co-validation of their accumulation by multimodal imaging. The Gd-SWCNTs could also be developed as multifunctional nanoparticles that allow simultaneous cellular therapeutics and diagnostics (theranostics) in a single setting. For instance, the external carbon sheath of Gd-SWCNTs can be covalently or non-covalently functionalized with biological moieties that target specific cells as well as deliver drugs. Alternately, the physical properties of SWCNTs can be exploited for therapeutics. For example, the strong microwave absorption properties of SWCNT could facilitate targeted hyperthermia of Gd-SWCNT-containing cells.⁵⁸

CONCLUSION

In summary, the results indicate that exposure of NIH/3T3 fibroblasts, at Gd-SWCNTs concentrations between 50–100 μg/mL, for 24 or 48 h, show dose- and time-dependent cytotoxicity. Gd-SWCNTs efficiently labeled the cells (100 labeling efficiency) at concentrations below 25 μg/mL without affecting their viability, proliferation, and structure. Cell death observed above 50 μg/mL of Gd-SWCNT was due to apoptosis and necrosis. The prevalence of apoptosis or necrosis as the cell death mechanism is concentration dependent. At 50 μg/mL, apoptosis is the dominant pathway, and at 100 μg/mL, necrosis is the dominant mechanism. Gd-SWCNT-labeled cells show up to four-fold increase in MR contrast compared to unlabeled cells.

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FIGURE 1.

Influence of Gd-SWCNTs on the cellular viability and membrane destabilization. (A) LIVE/DEAD cell viability at the various Gd-SWCNT incubation concentrations (0.1, 0.5, 10, 50, and 100 µg/mL) and time points (12, 24, and 48 h). (B) Trypan blue exclusion assay to assess the cytotoxicity of the Gd-SWCNTs at various incubation concentrations (0.1, 0.5, 10, 50, and 100 µg/mL) and time points (12, 24, and 48 h) on NIH/3T3 cells. (C) Lactate dehydrogenase assay (LDH) to assess the membrane integrity of NIH/3T3 cells treated with various Gd-SWCNTs concentrations (0.1, 0.5, 10, 50, and 100 µg/mL) at 12, 24, and 48 h time points. Data are presented as mean $\pm$ SD ($n = 3$ per group). * $p < 0.05$ with respect to control (unlabeled cells), and @ = $p < 0.05$ with respect to 24-h time point. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 2.

Effect of Gd-SWCNTs on the apoptotic markers and cell kinetics. (A) Phosphatidyl serine externalization analysis by flow cytometry using annexin-V-FITC and propidium iodide labeling of untreated cells (left panel), and cells treated with 50 μ g/mL (middle panel) and 100 μ g/mL (right panel) of Gd-SWCNTs for 48 h. The numbers in each quadrant represents the percentage of PS positive cells counted for 10,000 cells. Quadrants I–IV represent the population percentage of cells either in apoptosis or necrosis (I—PS non-expressing cell, II and III represents the percentage of cells expressing PS, and IV—necrotic cell population). (B) Induction of caspase-3 activity by Gd-SWCNTs in the NIH/3T3 cells. Cells were exposed to various concentrations (0.1–100 μ g/mL) of Gd-SWCNTs up to 48 h. Data are presented as mean $\pm$ SD ($n = 3$ per group, * $p < 0.05$ with respect to control (unlabeled cells), and @ $p < 0.05$ with respect to 24-h time point). (C) Representative cell-cycle histograms obtained by flow cytometry representing NIH/3T3 cells in different cell cycle phase before

(left panel) and after treatment with Gd-SWCNTs for 48 h. Flow cytometry analysis of sub-G1 DNA content (apoptosis) in NIH/3T3 cells is also represented. The cells were treated with Gd-SWCNT concentrations of 10 $\mu\text{g/mL}$ (middle panel) and 50 $\mu\text{g/mL}$ (right panel) for 48 h. For each experiment, cells were stained with propidium iodide and annexin V-FITC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 3.

Representative fluorescence images of NIH/3T3 cells stained with acridine orange for micronuclei analysis (A) before and (B) after treatment with 50 µg/mL Gd-SWCNTs for 48 h. All the images were taken at 10× magnification. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 4.

Transmission electron microscopy (TEM) images of Gd-SWCNTs distributed in the cytoplasm of NIH/3T3 cells. (A) TEM image of the normal morphology of untreated cells at 48 h; Gd-SWCNT (10 $\mu\text{g/ml}$) treated cells at 48 h and the distribution of Gd-SWCNTs in cytoplasm vacuoles (endosomes) at (B) low magnification and (C) high magnification; TEM image of Gd-SWCNT (50 $\mu\text{g/mL}$) treated cells at 48 h showing their distribution in the endosomes at (D) low magnification and (E) high magnification. Cellular sections show the nucleus (N), nuclear membrane (NM), cytoplasm (Cy), vacuole (V), and mitochondria (M). NT refers to endocytosed Gd-SWCNT. Arrows points to the Gd-SWCNTs (NT). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 5.

Raman spectra and G band intensity map of Gd-SWCNTs in NIH/3T3 cells. (A) Bright field image of the cells treated with 10 $\mu\text{g/mL}$ Gd-SWCNTs for 48 h. (B) Raman G-band intensity was acquired at different spots (marked as red points) to detect and confirm the presence of Gd-SWCNTs within a NIH/3T3 cell. (C) Raman spectra of Gd-SWCNTs uptaken by the cells. (D) Area map of the Raman G-band intensity of Gd-SWCNTs in cells. (E) Area map of Raman G-band intensity of Gd-SWCNTs in cells overlaid on an optical micrograph of the same region. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 6.

Cellular uptake and MRI phantoms. (A) Histogram of Gd^{3+} ion uptake per NIH/3T3 cell vs. Gd-SWCNTs (1, 10, and 25 mg/mL) incubation concentrations at 12, 24, and 48 h time points. Data is presented as mean $\pm$ SD ($n = 3$ per group). (B) Representative T_1 -weighted MRI images of untreated and Gd-SWCNT-treated NIH/3T3 cells (4×10^6 cells) dispersed in 0.7% agar. (C) The signal intensity calculated from these images. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I

Cell Cycle Kinetics of NIH/3T3 Cells in Serum-Starved Medium Incubated with Gd-SWCNTs at Concentrations of 10 $\mu\text{g/mL}$ and 50 $\mu\text{g/mL}$

Gd-SWCNT Concentration ($\mu\text{g/mL}$)	24 h				48 h			
	Sub-G1 (%)	G1 (%)	S (%)	M (%)	Sub-G1 (%)	G1 (%)	S (%)	M (%)
0	1.18	96.42	1.27	1.23	1.31	95.09	1.22	2.59
10	1.02	93.83	1.90	3.40	2.32	91.62	2.46	3.72
50	1.25	93.86	2.11	2.90	1.91	89.77	3.37	5.20

Untreated cells were used as controls. The numbers of cells in different phases are expressed as a percentage of total 10,000 cells analyzed by flow cytometry.