Tube length and cell type-dependent cellular responses to ultra-short single-walled carbon nanotube

David A. Donkor, Xiaowu S. Tang

Department of Chemistry, Waterloo Institute for Nanotechnology, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

1. Introduction

A large variety of nanoparticles have been explored as drug carriers for more effective and targeted delivery in biomedicine. Regardless of their material compositions, the sizes of the nanoparticulated drug carriers are known to be of critical importance to delivery efficacy. A vast number of literature reports have presented evidence that size plays an important role in the cellular uptake of liposomes, polymer nanoparticles, artificial viruses, as well as inorganic nanoparticles such as Au, with optimal particle diameter predicted to be between 30 and 50 nm [1–4]. Likewise, size dictates the intracellular localization of nanoparticles [1]. For instance, compartmentalization of CdTe quantum dots into different sub-cellular organelles depends on size as well as cell type [5]. Furthermore, for high aspect ratio 1D (e.g. carbon nanotube, gold nanowire) and 2D (e.g. graphene) nanoparticles, sizes of all dimensions and aspect ratio could strongly influence the nanoparticle-induced cellular responses.

One and two dimensional structures reported in literature often possess a well-defined size along one dimension but much larger size disparity along the other, which complicates the studies on size effects and the exact physical and chemical mechanisms involved, leading to controversial conclusions. For example, single-walled carbon nanotube (SWNT) typically have well-defined diameters around 1.4 nm (HiPco) but a wide length distribution from 50 nm to a few μm. Although the efficient uptake of SWNT by virtually all cell types is widely accepted, several different mechanisms of uptake have been proposed [6–8]. After the internalization of nanotubes, the intracellular distribution appears to vary. While few reported nuclear accumulation [6,9] and one reported reversible nuclear accumulation of PEGylated nanotubes in multiple cell lines [10], most studies showed that nanotubes accumulated in the cytoplasm [11–13]. We believe a narrow length distribution is the key to achieving a more deterministic intracellular trafficking of SWNT and a better understanding of the underlying cellular mechanisms, which are relevant to the rational design of SWNT nanocarriers for drug delivery and as imaging contrast agents. So far, the few studies done to determine the length related characteristics of SWNT involved tubes with length below 50 nm [14–17]. Several reports have shown the generation of ultra-short SWNT (US-SWNT) with length around 50 nm [18–20] and reported their usage as contrast agents, but without detailed investigation of cellular responses. Only one report of US-SWNT could be found showing tube accumulation in the cytoplasm [21]. In the present work, we show that extended acid oxidation combined with sonication, filtration processes involved in SWNT purification and PEGylation can be utilized to effectively shorten SWNT and generate US-SWNT with superior homogeneity in...
length and surface coating. With tight control in length distribution, we were able to generate two US-SWNT systems with average lengths of 30 nm and 50 nm respectively and show their distinctively different characteristics in terms of cellular uptake, intra-cellular location, excretion, and partitioning at cell division. How a parent cell partitions intracellular SWNT into daughter cells is a rarely studied parameter, however, with important theranostic outcomes.

2. Materials and methods

2.1. Preparation and characterization of US-SWNT

Raw HiPCo “veted-mud” (with 1:9 mixture of ethanol and deionized H2O) SWNT was purchased from Unidyne. The purification and characterization was done following protocols as previously published [22]. Briefly, the SWNT were added to 8 mM nitric acid and sonicated for 10 min followed by refluxing at 150 °C for 20, 32 and 44 h. After oxidation, the SWNT solution was filtered using a 100 nm pore size nuclease Track-ETch membrane (Whatman) and washed with deionized (DI) water until neutral pH. SWNT were resuspended in DI water, sonicated for 1 h and centrifuged at 22,000 × g for 8 h. The insoluble pellet was discarded and the supernatant was collected. Raman spectroscopy was performed on all oxidized SWNT with a LabRAM HR system (HORIBA Jobin Yvon) equipped with an upright microscope and a piezo sample stage. For PEGylation of US-SWNT, 6-arm PEGs with primary amine terminus (20,000 MW, Sigma) were grafted on oxidized US-SWNT via amide linkage [23]. Briefly, an oxidized US-SWNT solution was diluted with DI water until an O.D. of 0.4 at 808 nm was obtained. The solution was then bath sonicated with 10 mg mL⁻¹ of the 6-arm PEG for 5 min. Next, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDC (G Biosciences) was added to the solution to reach a concentration of 5 µM and the solution followed sonication for 30 min. Anhydride was then added to reach a final concentration of 20 µM and the solution was stirred for 12 h at room temperature. After the 12 h reaction, the solution was centrifuged at 14,000 rpm for 3 h and the pellets discarded. The supernatant was then centrifuge-filtered multiple times using a 100 kDa molecular weight cutoff Amicon Ultra centrifugal filter (Millipore) to remove excess PEG.

For fluorescence labeling and FA loading of PEGylated US-SWNT, FITC in DMSO and FA were added at 10-times molar excess to the PEGylated US-SWNT in 0.1 M sodium bicarbonate buffer (pH 9). In the case of FA, the 10× EDC was added at the same time. The mixture was then allowed to react overnight at room temperature and protected from light. Then the US-SWNT solution was centrifuge-filtered using a 100 kDa molecular weight cutoff Amicon ultra centrifugal filter. The centrifugal filtration was carried out several times with phosphate buffered saline (PBS, pH 7.2) as the wash buffer. UV–vis absorbance of the filtrate was measured after each wash cycle and the washing was complete when there was no detectable FITC and/or FA absorption. The filtered FITC and/or FA-labeled US_SWNT were then suspended in PBS (pH 7.2) and stored at 4 °C.

2.2. AFM characterization

The physical dimensions and purity of the oxidized and PEGylated SWNT were characterized with AFM using a Veeco Multimode III microscope. Silicon substrates were soaked in SWNT solutions (10 nM) for 30 min and then rinsed in DI water. The substrates were then dried in an oven at 50 °C for 30 min.

2.3. Cell culture and cell transfection

HUVEC cells (HUVEC-CS, ATCC No: CRL-2873) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (ATCC) supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin (Sigma Aldrich). HUVEC cells were cultured in several 75 cm² tissue culture flasks containing 40 nM FA_SWNr_30, SWNT_30, SWNT_100, FA_SWNr_30 or FA_SWNr_100 was then added into the flask containing cells (80 mM PIPES, 1 mM MgCl2, 1 mM EGTA pH 6.7) for 10 min. For staining cell nuclei, the SWNT were incubated in PBS with 5 µM DRAQ5 nuclear dye for 20 min at room temperature and then washed twice with PBS right before imaging. For the time course studies, cells were grown on multiple pieces of cover glasses placed in one 100 mm × 15 mm petri dish. One cover glass was retrieved at each time point, washed with cell medium to remove loosely bound materials, imaged, and discarded. Lysosomes were stained with LysoTracker Red DND-99 (Invitrogen) (75 nM, 30 min, 37 °C).

2.5. Flow cytometry

Cells were cultured in several 75 cm² tissue culture flasks until 60–70% confluence. SWNT_30 or FA_SWNr_50 was then added into the flasks to reach a concentration of 40 nM in cell media and the cells were incubated for 4 h at 37 °C. After incubation, all cells were washed three times with cell media, after which some were trypsinized (ATCC) for flow cytometry and cell counting (4 h cell population). The remainder of the cells were incubated in fresh media (no SWNT) further to generate the day 1 to day 5 cell populations. For flow cytometry, one hundred thousand cells were used each time to generate the fluorescence histogram on a BD FACS Vantage SE flow cytometer.

3. Results and discussion

3.1. Preparation and characterization of PEGylated US-SWNT

Acid oxidation is one of the most commonly used and effective treatments to eliminate contaminants, as well as introduce oxide groups on the ends and defect sites of carbon nanotubes [24]. The oxide groups afford hydrophilicity to nanotubes and act as anchors for further covalent linkage of functional moieties. It is well understood that aggressive acid oxidation can lead to tube shortening, i.e. reduction of average tube length as reported by Forrest et al. [25]. However, the effect of acid oxidation conditions on tube length distribution is often overlooked. Here, we show that an extended period of acid treatment can lead to ultra-short SWNT with superior homogeneity in length and surface modification. Atomic force microscopy (AFM) images of SWNT refluxed in 8 M nitric acid after a period of 20 h, 32 h, and 44 h respectively are shown in Fig. 1. The detailed protocol is presented in the Materials and methods section. It is evident that SWNT generated by 20 h acid oxidation, which is commonly used in literature reported studies, have lengths ranging from 10s of nm to a few µm (Fig. 1A). Fig. 1B and C show that tube lengths are reduced considerably after 32 h and 44 h oxidation and appear more uniform. By measuring hundreds of tubes in AFM images, a histogram was generated to show quantitatively the average tube length and length distribution (Fig. 1D). The lengths of 32 h and 44 h oxidized SWNT are 50 ± 23 nm (average ± standard deviation) and 30 ± 13 nm respectively. Even though the two systems have certain length overlap, main tube populations can be divided at 35 nm. About 77% of 32 h oxidized SWNT are above 35 nm and over 80% of 44 h SWNT are within 10 nm – 35 nm. Further, Raman spectroscopy, in particular the ratio of D-band at 1350 cm⁻¹ to G-band at 1590 cm⁻¹, was used to measure the relative defect density on SWNT induced by acid treatment. Fig. 1E shows the representative Raman spectra of oxidized SWNT on SiO₂ substrates taken in the circular regions shown in Fig. 1B and C. For comparison, Raman spectrum of as-purchased HiPCo SWNT is also shown, where the small D-band and dominant G-band indicate the high structural integrity. SWNT after 32 h oxidation exhibited certain variations in tube defectiveness (Fig. 1E i & ii) even though the variations were considerably less than that of 20 h oxidized tubes (data not shown), with long tubes coincident with low-defect density. Oxidizing for 44 h reduced SWNT uniform in high defect density (Fig. 1E iii) with the D/G ratio almost unity across the whole substrate. The presence of the RBM peaks in the range 100–500 cm⁻¹, which are specific to SWNT caused by the rhythmic movements of carbon atoms in the radial direction, confirmed the preserved tubular characteristics of HUVEC cells, they were fixed with 3% glutaraldehyde (Sigma Aldrich) in Pipes buffer (80 mM Pipes, 1 mM MgCl₂, 1 mM EGTA pH 6.7) for 10 min. For staining cell nuclei, cells were incubated in PBS with 5 µM DRAQ5 nuclear dye for 20 min at room temperature and then washed twice with PBS right before imaging. For the time course studies, cells were grown on multiple pieces of cover glasses placed in one 100 mm × 15 mm petri dish. One cover glass was retrieved at each time point, washed with cell medium to remove loosely bound materials, imaged, and discarded. Lysosomes were stained with LysoTracker Red DND-99 (Invitrogen) (75 nM, 30 min, 37 °C).
Fig. 1. SWNT length distribution and surface defect density are dependent on the duration of acid oxidation. AFM images of SWNT acid oxidized for (A) 20 h, (B) 32 h, and (C) 44 h. Scale bar 500 nm. (D) Tube length distribution of 32 h and 44 h oxidized SWNTs. (E) Raman spectra taken from the color-coded circled regions shown in (B) and (C), compared to the Raman spectrum of as-purchased HiPco tubes (light blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Two PEGylated US-SWNT systems with narrow length distribution. AFM images of PEGylated (A) 32 h and (B) 44 h oxidized SWNT. Scale bar 500 nm. Length distribution of PEGylated 32 h and 44 h oxidized SWNT (C) extracted from AFM images and (D) determined by dynamic light scattering.
all oxidized tubes. In summary, the homogeneity in tube length and surface defect density was significantly improved by extending the oxidation time from 20 h to 32 h and 44 h, and therefore 32 h and 44 h oxidized tubes were used in the subsequent experiments.

By covalent linkage of 6-arm polyethylene glycol (PEG) molecules with amine terminus to carboxylic acid groups on the defect sites of the SWNT, the dispersion stability of the tubes in aqueous media was improved (Fig. S1 in supporting information) [26–28]. PEGylated SWNT was then examined by AFM and dynamic light scattering (DLS) to show the effects of the PEGylation step on the tube length distribution. PEGylated SWNT are shown to be uniform in AFM images (Fig. 2A and B). Histogram generated from AFM images and DLS data show consistent tube lengths of 50 ± 19.5 nm (32 h) (with 73% of PEGylated 32 h SWNT above 35 nm) and 30 ± 10.6 nm (44 h) (with 85% of 44 h PEGylated SWNT below 35 nm) (Fig. 2C and D). The two PEGylated tube systems are referred hereafter as SWNT_30 and SWNT_50 respectively. It is worth noting here that although the relative standard deviation of the oxidized and PEGylated 32 h tubes were similar, all tubes longer than 80 nm were effectively eliminated after the PEGylation process, which we attribute to the precipitation of low-defect tubes induced by salt. No obvious difference was observed before and after PEGylation of the 44 h oxidized tubes, which had uniformly high defect density as shown by Raman spectroscopy. The brief
sonication involved at the PEGylation step was not effective in tube cutting. A Fourier transform infrared (FTIR) analysis of oxidized and PEGylated tubes confirmed the successful conjugation of PEG molecules onto the SWNT (Fig. S2). A carbonyl stretch at 1774 cm\(^{-1}\) was observed on oxidized tubes, while prominent stretches of C–O–C at 1079 cm\(^{-1}\) and C–H at 2920 cm\(^{-1}\) were observed on PEGylated tubes.

3.2. Feasibility of using US-SWNT for active targeting

One of the aims in developing delivery vectors is to achieve targeting, which could either be passive or active. Whereas passive targeting relies on the leaky tumor capillaries and the enhanced permeability and retention (EPR) characteristics of tumors [29], active targeting employs molecular recognition for delivery to specific sites, thus minimizing the uptake by non-targeted cells [29]. Several in vitro and in vivo studies have shown effective active targeting using SWNT carriers [1,30–32], where tube lengths are in the range of 50 nm–1000 nm. However, it is unclear what’s the ideal or minimum SWNT length for active targeting, which could be cell-type dependent. Using our SWNT_30 and SWNT_50 systems, we attempted to answer this question. Cellular uptake of SWNT_30, SWNT_50, and their folic acid (FA)-loaded counterparts by three types of cells (HUVEC, Human hepatoma, and HeLa) were

![Fig. 4. Cellular uptake of SWNT_30 unaffected by folic acid loading. Confocal images of three different cell cultures (HUVEC, hepatoma, HeLa) after incubation for 4 h in 40 nM of (A) SWNT_30 and (B) FA_SWNT_30 showing similar uptake. SWNT_30 and FA_SWNT_30 are labeled with FITC (green), and cell nuclei are stained red using DRAQ5. Insets: images showing green fluorescence only. Scale bar 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](http://dx.doi.org/10.1016/j.biomaterials.2013.12.075)
compared. FA is one of the most researched targeting ligands for cancer therapeutics [33–35] and therefore was used as a model ligand for active targeting. All SWNT were labeled with fluorescein isothiocyanate (FITC) so that confocal fluorescence microscopy and flow cytometry studies on cell cultures could be carried out. It is evident in Fig. 3A that cells incubated with SWNT_50 without FA showed very little internal green fluorescence, indicating little to no uptake of the nanotubes. With FA-loaded SWNT_50 (FA_SWNT_50), however, uptake was enhanced significantly across all three cell types (Fig. 3B). To confirm that the enhancement was due to folate transport, we also did a control experiment where cells were cultured in FA dosed medium prior to the introduction of FA_SWNT_50. As a consequence, uptake of FA_SWNT_50 was largely blocked (Fig. S3). Unlike SWNT_50, the shorter SWNT_30 were internalized spontaneously by all three types of cells without the assistance of FA (Fig. 4). More interestingly, SWNT_30 was found to reside in the nuclei of both hepatoma and HeLa cells as green fluorescence from FITC attached to SWNT_30 was observed to coincide with DRAQ 5 (deep red-fluorescing bisalkylaminoanthraquinone number five) a 1,5-bis[(2-(methylamino)ethyl)amino]-4,8-dihydroxy anthracene-9,10-dione. Draq5 is a nuclear discriminant dye (ex/em wavelengths 647/665–780 nm) with a high affinity for DNA [36]. As well, our results correlate well with the assertion by Kang et al. that tubes less than 50 nm can easily penetrate the plasma membrane and the nuclear envelope [1]. Our data suggests that a tube length of 35 nm and above is needed to minimize spontaneous cellular uptake of longer tubes. For example, in a previous publication, we showed that oxidized tubes (without PEG corona) in the range of 50 nm–1 um were readily internalized by HeLa cells into the cytoplasm [22]. Furthermore, Zeineldin et al. observed nonspecific uptake of SWNT with lengths between 300 and 1000 nm.

Fig. 5. SWNT_30 as gene transfection agent. Confocal fluorescence images of HeLa cells transfected using (A) lipofectamine 2000 and (B) SWNT_30 loaded with pCS2+GFP-N1 plasmid. Insets: merged green fluorescence and white light transmission images. Bottom row: schematic representations of plasmid complexing with lipofectamine 2000 and SWNT_30 respectively, and trafficking into HeLa cells. Scale bar is 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Although the tubes used by Zeineldin et al. were coated with phospholipid PEG (PL-PEG), the authors observed fragmentation of PEG when solubilizing the tubes by ultra-sonication. This led to poor shielding of the tubes, resulting in non-specific uptake and cytoplasmic accumulation [39]. On the other hand, reducing sonication time prevented fragmentation, which resulted in uptake only through active targeting. Therefore, when the native hydrophobic surface of SWNT is well shielded, tube length dictates uptake and intracellular localization, as illustrated in this work.

3.3. SWNT_30 as gene transfection agent

The efficient nuclear penetration of SWNT_30 was exploited for delivery of a 4700 bp plasmid DNA (pCS2+GFP-N1) into the nucleus of HeLa cells. This plasmid expresses a green fluorescent protein (GFP) under the control of the human cytomegalovirus (CMV) promoter. Cells transfected using SWNT_30 and a commercially available transfection agent, Lipofectamine 2000, were examined under a confocal fluorescence microscope and GFP expression in the two cases were compared. As shown in Fig. 5A, cells transfected with pCS2+GFP-N1 using lipofectamine 2000 resulted in little to no expression of GFP. Lipofectamine 2000 is a cationic polymer that condenses DNA and transports it into the cell cytoplasm. Since neither the CMF promoter [40] nor lipofectamine 2000 was able to transport the plasmid into the nucleus, we saw no protein expression, as expected. Alternatively, pCS2+GFP-N1 was first conjugated onto SWNT_30 (without FITC label) through EDC activation of phosphate groups on the DNA and then introduced to cell culture. Within 24 h, GFP expression could be seen throughout the culture (Fig. 5B) indicating uniform cell transfection. Our results suggest that ultra-short PEGylated SWNT (<35 nm), although unsuitable for active targeting, could find potential applications in gene transfection.

3.4. Size effect on SWNT excretion

Further, we probed the difference in cell excretion of SWNT_50 and SWNT_30. HeLa cells were exposed to 40 nM FITC-labeled FA_SWNT_50 and SWNT_30 for 4 h respectively, followed by culturing in fresh media (no SWNT) for three days. Note that cell responses to FA_SWNT_30 and SWNT_30 were identical and therefore SWNT_30 was used here because of the ease of making it. Persistent intracellular fluorescence was observed in FA_SWNT_50 exposed cells (Fig. 6A). In contrast, both confocal images and flow cytometry analysis show a drastic reduction in fluorescence in cells exposed to SWNT_30 after day one. By day two and day three, almost no fluorescence could be observed in the cells (Fig. 6B and C). In conclusion, SWNT_30 were rapidly recycled out of cells while FA_SWNT_50 exhibited prolonged intracellular accumulation. This data suggests that the longer tube length required for active targeting might also prevent the rapid excretion of SWNT carriers by the targeted cells.

3.5. Cell-type dependent excretion of SWNT_30

Besides HeLa cells, hepatoma and HUVEC cells were used to investigate the cell-type dependent excretion of SWNT_30. Here, all three types of cells were exposed to FITC-labeled SWNT_30 for 4 h and subsequently cultured in SWNT-free media for 3–5 days. Similar to HeLa cells, sharp decrease in fluorescence was observed in hepatoma cells on day 1 and 2 (Fig. 7A and B). HUVEC cells on the other hand, retained fluorescence in the cytoplasm even after 5 days (Fig. 7C). This result suggests that hepatoma cells are more efficient in recycling SWNT_30 compared to HeLa cells, which may be due to differences in cell metabolism and cell-cell interaction.

Fig. 6. Intracellular location of US-SWNT and the rate of excretion are tube length dependent. Confocal images of HeLa cells at various time points (4 h, day 1, day 2, day 3) showing (A) persistent residence of SWNT_50 in cytoplasm and (B) intranuclear uptake and effective excretion of SWNT_30. (C) Flow cytometry data of SWNT_30 incubated HeLa cells at 4 h, day 1, day 2, compared to blank (i.e. background fluorescence level of HeLa cells), confirms tube excretion.

Please cite this article in press as: Donkor DA, Tang XS, Tube length and cell type-dependent cellular responses to ultra-short single-walled carbon nanotube, Biomaterials (2014), http://dx.doi.org/10.1016/j.biomaterials.2013.12.075
days of observations (Fig. 7C). To probe the correlation between intracellular fluorescence levels and SWNT traffic, flow cytometry analysis was performed on cells incubated with 5-chloromethylfluorescein diacetate (CMFDA) and SWNT_30 respectively (Fig. 8). CMFDA is a fluorescent dye commonly used to study cell proliferation. It can freely diffuse into the cytoplasm, and be cleaved by cytosolic esterases into a cell membrane-impermeant product, which is then conjugated with glutathione (GSH) to form a fluorescent adduct. This product is retained in living cells through several generations and inherited by daughter cells after cell division [41]. During each round of cell division, partitioning of the dye is equal among daughter cells. Flow cytometry is often used to determine the fluorescence intensity distribution within the cell population, as shown in Fig. 8A, where the fluorescence peak down shift by half at each cell division without broadening. Here, this set of data serves as the reference, showing symmetric partitioning at cell division with no excretion. Flow cytometry data from FITC-labeled SWNT_30 incubated cell cultures were then compared with this reference. Fluorescence distribution of SWNT_30 exposed hepatoma and HeLa cell populations down shifted and broadened significantly after day 1 and almost completely overlapped with the control two days after (Fig. 8B and C), which is consistent with the confocal microscopy observation of rapid SWNT_30 excretion. Fluorescence distribution of SWNT_30 incubated HUVEC cells over 5 days (Fig. 8D), however, were similar to that from CMFDA except considerable broadening. Under normal growth condition, the cell cycle of HUVEC is 27 h [42], thus we expect HUVEC cells to have gone through at least one division within a 27 h period. If SWNT_30 partitioning between daughter cells were symmetric, conforming to a 50:50 split, then the fluorescence histogram should show a peak down shift to half the intensity with an unchanged peak cell count at each cell division, as was the case for CMFDA (Fig. 8A). The broadening of the fluorescence peak (Fig. 8D) therefore indicates that partitioning of SWNT_30 at cell division is asymmetric. We further used two parameters, the arithmetic mean (X), (the average SWNT_30 fluorescence per cell) and the co-efficient of variation (CV = σ/X) to quantify the distributions. The values of X and CVs are plotted in Fig. S5. The CV doubled between 27 and 54 h due to the

Fig. 7. Time course confocal images show that intracellular traffic of SWNT_30 is cell type dependent. (A) HeLa cells and (B) hepatoma show spontaneous nuclear uptake of SWNT_30 (green) at 4 h and efficient excretion over day 1 – 3. (C) Persistent residence of SWNT_30 in HUVEC cells over 5 days. Inset: merged green, red, and white light channel images. SWNT_30 are labeled with FITC, cell nuclei are labeled with DRAQ5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 8. Quantitative measurement of cellular uptake, excretion, and partitioning of CMFDA and SWNT_30 by flow cytometry. (A) Fluorescence level of HeLa cells incubated with CMFDA is observed to down shift by half each day and without apparent peak broadening for 5 days. Fluorescence peaks of (B) hepatoma and (C) HeLa cells incubated with FITC-labeled SWNT_30 are shown to down shift drastically and broaden from day 0 to day 1 and overlap with blanks on day 2. (D) Fluorescence peaks of HUVEC cells incubated with FITC-labeled SWNT_30 are shown to down shift gradually and broaden over 5 days.

Fig. 9. Cell-type dependent lysosomal accumulation of SWNT_30. Confocal images of HeLa and HUVEC cells at various time points (4 h, day 1, day 2) showing SWNT_30 (green) excreted by HeLa cells but sequestered in lysosomes (red) by HUVEC cells. Inset: zoomed in confocal image of an HUVEC cell on day 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
asymmetric division of SWNT_30 (Fig. S5, solid line). In contrast, for symmetric partitioning in the case of CMFDA (Fig. S5, dashed line), X and σ half upon division, resulting in a constant CV. This analysis provides a mechanism for the quantitative evaluation of the partitioning of SWNT_30 in a growing cell population. Our results showed clearly that both intracellular location and excretion of SWNT_30 were cell-type dependent. HUVEC cells were able to retain intracellular SWNT_30 and generational transfer to daughter cells through asymmetric partitioning.

3.6. Lysosomal retention of SWNT_30 by HUVEC cells

To elucidate the site of non-excreted SWNT_30 accumulation in HUVEC cells, a lysosomal assay was performed. To do this, lysosomes of HeLa and HUVEC cells were labeled with LysoTracker after the initial 4 h SWNT_30 exposure. Fig. 9 shows that both HeLa and HUVEC cells compartimentalize nanotubes into their lysosomes. Within two days of observation, the punctate lysosomal structures in HeLa cells had disappeared (Fig. 9), confirming both confocal (Fig. 7) and flow cytometry (Fig. 8) conclusions of SWNT_30 excretion. HUVEC cells, however, showed high levels of punctate lysosomal structures even after two days. These observations indicate that both HeLa and HUVEC cells utilize lysosomal pathways for the recycling of SWNT. However, the pathway was interrupted in HUVEC cells and caused the accumulation of SWNT-containing lysosomes. We suspect that the different intracellular architecture of polarized cells (HUVEC), compared with the non-polarized cells (HeLa and hepatoma), is responsible for the difference in SWNT trafficking [43]. The specific reasons for the interruption of lysosomal pathway in HUVEC cells await future investigation.

4. Conclusion

Highly water-soluble and ultra-short SWNT were successfully created through prolonged oxidation and PEGylation, resulting in two systems (SWNT_50 and SWNT_30) with identical surface characteristics differing only in tube length (50 nm vs. 30 nm). This detailed study on the cellular responses to ultra-short SWNT showed clearly the tube length and cell-type dependent cellular uptake, intracellular localization, and excretion of SWNT, as well as SWNT partitioning at cell division. Our results suggested a cutoff length around 35 nm for PEGylated SWNT to display minimal spontaneous cellular uptake and to be applied to active targeting. SWNT_30 (with 85% tubes below 35 nm) could find alternative applications in gene transfection due to its ability to spontaneously cellular uptake and to be applied to active targeting. SWNT_30 (with 85% tubes below 35 nm) could provide foundation for the rational design of SWNT carriers for targeted drug delivery, as contrast agents, and for new niche applications, where cellular trafficking of SWNT could be of significance in efficacy and toxicity.

Acknowledgements

The authors would like to thank Dr. Mungo Marsden and Dr. John J. Heikkila’s lab for providing the pCS2-GFP-N1 plasmid and related information, MishI Groh for her assistance in flow cytometry and confocal microscopy. This work was supported by a Discovery grant from the Natural Science and Engineering Research Council (NSERC) of Canada, the Leaders Opportunity Fund from the Canada Foundation of Innovation (CFI), and the Ontario Research Foundation (ORF) funding to Dr. Tang.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.12.075.

References


