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Preparation of water soluble carbon nanotubes and assessment of their biological activity in embryonic zebrafish

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Abstract

Carbon nanotubes (CNTs) are currently one of the most important classes of nanomaterials with unique properties sparking off numerous applications in many fields, including electronics, material science and medicine. However, applications of CNTs in medicine and other biological fields are hampered by their insolubility in aqueous media and concerns regarding toxicity. In this study, seven types of CNTs, including two single-walled, one double-walled, and four multi-walled, were evaluated for possible toxicological effects. Soluble CNTs were prepared by treatment with a mixture of acids (D₂SO₄ and DNO₃), washed with Milli-Q water and oven dried. Transmission electron microscopy, thermal gravimetric analysis, and other techniques were used to characterize the prepared CNTs. CNT toxicity was assessed using the embryonic zebrafish. Results showed that none of the CNTs studied caused significant adverse developmental effects. These results support the potential safe use of CNTs as components of indwelling medical devices and drug delivery tools.

Keywords

Carbon nanotubes; Deutrated acids; Solubility; Biomedical applications; Zebrafish; nanotoxicity; Teratogenic endpoints

1 Introduction

Since discovered by Iijima in 1991, carbon nanotubes (CNTs) have emerged as one of the most important nanomaterials catching the attention of both industries and researchers in different areas of nanotechnology. The peculiar physical, mechanical and electronic properties of CNTs such as high electrical conductivity, high tensile strength, nanosize diameters and large aspect ratios (Innovative Research and Products, Inc, 2011; Endo et al., 2008) make it the wonder material of the 21st century (Constantine et al., 2010; Li et al., 2010; Vivek, 2011) and shows great promise for various areas of applications such as

molecular electronic devices (Bandaru, 2007), molecular reinforcements in composites (Liu et al., 2005), biomedical engineering (Bianco et al., 2005; Balasubramanian and Burghard 2005), tissue engineering (Harrison and Atala, 2007), drug delivery (Bianco, 2004), nanoprobes and biosensor technology (Star et al., 2003).

Applications of carbon-based nanotechnology in biomedical research are being keenly explored by many researchers (Harrison and Atala, 2007; Li et al., 2010; Watari et al., 2004; Yokoyama et al., 2005; Mattson et al., 2000; Firkowska et al., 2006; Aoki et al., 2006; Correa et al., 2004; Li et al., 2008; Akasaka and Watari, 2005; Macdonald et al., 2005; Kam et al., 2004; Supronowicz et al., 2002). In spite of the versatility of carbon nanotubes, their insolubility in process-friendly solvents which poses difficulty to their processing and manipulation in a facile manner and fears about any potential toxicity has severely hindered their widespread use in the areas of nanomedicine and nanobiotechnology (Wu et al., 2010). The recent developments in chemical modification and functionalization of CNTs has greatly improved the stability, solubility and dispersion of CNTs in water, subsequently opening the path for their handling and processing in physiological environments (Li et al., 2010).

Toxicity of CNTs is a principal concern, with different groups pointing to their resemblance to asbestos fibers as a major issue (Poland et al., 2008). CNT toxicity in various studies has been credited to various factors like size, concentration, duration of exposure, method of exposure, and even the material used to solubilize/disperse the nanotubes (Constantine et al., 2010). Most areas of CNT toxicity are inconsistent and many studies suggest that elements of CNT toxicity are unsubstantiated (Constantine et al., 2010). The discrepancies appear to arise chiefly due to variances in experimental protocol especially the ability to obtain stable aqueous soluble CNTs as well as removing possible contaminants including metal catalyst residues that are associated with the synthesis of CNTs (Shvedova et al., 2003).

The ability to solubilize and separate discrete CNTs from their tight bundles would not only help in their use, but would also help in their purification allowing their integration in more nanobiotechnology applications. In this study we have successfully prepared water soluble CNT of different types and evaluated their toxicity using the zebrafish model. To efficiently investigate the interactions between nanomaterials and the biological system, a sensitive, *in vivo*, rapid throughput model must be used. Cell-culture assays are rapid, high throughput and cost efficient, however they lack the complexity of a whole biological animal. *In vivo* models possess this complexity, but are traditionally low throughput and cost- and labor-intensive. However, zebrafish are the ideal model that possesses the convenience of cell culture, while combining the power of an *in vivo* system. It is also a well established *in vivo* toxicological model (Yang et al., 2009; den Hertog, 2005; Bowman and Zon, 2010; Dodd et al., 2000). Zebrafish share a high degree of homology to the human genome and many cellular and anatomical similarities to vertebrates. A single female can lay several hundred embryos every 3–5 days that are small, develop externally, and are optically clear. An embryo's organs develop within 5 days post fertilization and are genetically tractable. The zebrafish requires a significantly less amount of material to assess nanoparticle toxicity at multiple concentrations with replicates compared to other *in vivo* models, such as mice. By using the embryonic zebrafish model, we have developed a rapid process to investigate how

nanomaterials induce biological responses (Harper et al., 2011; Truong et al., 2011; Truong et al., 2010; Truong et al., In press; Usenko et al., 2007; Usenko et al., 2008). By using this model, our data will position us to understand what physicochemical properties of carbon nanotubes drive the differential biological responses and observe whether these water soluble CNTs are developmentally toxic.

2 MATERIALS AND METHODS

2.1 Materials

Deuterated sulfuric acid D_2SO_4 : with a concentration of 96–98 wt. % in D_2O , isotopic purity of 99.5 atom % D, deuterated nitric acid (DNO_3 : with a concentration of 65 wt. % in D_2O and isotopic purity of 99 atom % D) were purchased from Sigma-Aldrich and were used as received. The nanotubes used, single walled carbon nanotubes (SWCNTs), double walled carbon nanotubes (DWCNTs) and multi-walled carbon nanotubes (MWCNTs) were produced by chemical vapour deposition method (CVD) and were obtained from CheapTubes (112 Mercury Drive Brattleboro, VT 05301 USA) and NanoLab, Inc. (179 Bear Hill Road, Waltham, MA 02451, USA).

2.2 Preparation procedure for soluble carbon nanotubes

Pristine carbon nanotubes (CNTs) up to 5 mg/mL concentration were dispersed by ultrasonication (100 W, 42 KHz, Branson 3510 ultrasonication bath, maximum power) in a mixture of D_2SO_4 and DNO_3 (3:1 v/v) (Ramanathan et al., 2008) for 4 hours at temperature from ambient to 50 °C to obtain a well homogenised colloidal solution (Warning: the acids are highly corrosive and should be handled with care under a chemical hood). The CNTs suspensions thus prepared were thoroughly washed (8 times) with Milli-Q water (18 M Ω) and separated by centrifugation (Clay Adams compact II centrifuge, 3200 rpm) to obtain a pH > 4.5. The CNTs were then filtered, washed again and dried in a vacuum oven. Stable solutions of CNTs were prepared in Milli-Q water by brief sonication for about 90 seconds.

2.3 Characterisation

Scanning electron microscopy (FEI Quanta 3D Dual Beam SEM) and transmission electron microscopy (Philips CM12 TEM) were used to assess the surface morphology of the nanotubes. The samples were dispersed with methanol and then dropcast on pre-cleaned silicon wafer and spun coated with a thin layer of gold. The electron accelerating voltage and magnification for SEM were 15 KV and 200,000, respectively, while the accelerating voltage and magnification for TEM were 120 KV and 200,000, respectively. Thermal gravimetric analysis (Shimadzu TGA-50 thermogravimetric analyzer) was done to study the mass loss behavior of the CNTs. This was done under flowing Argon gas (20 ml/min) at a heating rate of 5°C/min from ambient temperature to 1000°C. To identify the attachment of the functional groups on the surface of the nanotubes, fourier transform infrared spectroscopy (FTIR, Nicolet 510P FT-IR spectrophotometer) was used. The FTIR spectra were recorded using pellets of CNTs and KBr made by pressing the powder mixture into pellet. While the surface elemental composition and assessment of the chemical environment of the detected elements were analysed with X-ray photoelectron spectroscopy (XPS) with methanol dispersed sample dropcast on pre-cleaned silicon wafer until the

surface is well covered. The CNT stability in Milli-Q water and embryo medium was studied by measuring zeta potential using ZetaPALS ζ -potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY). All samples contained 1 mM KCl.

2.4 Toxicity testing

The carbon nanotubes solutions (at a concentration of 100 $\mu\text{g/mL}$) were vortexed briefly prior to making a 50 $\mu\text{g/mL}$ working solution using embryo medium (EM). Five-fold serial dilutions were prepared using a Caliper liquid handler. Adult Tropical 5D zebrafish were housed and reared at Oregon State University Sinnhuber Aquatic Research Laboratory. Embryos were collected and staged from group-spawned zebrafish (Kimmel et al., 1995). To increase bioavailability, the embryonic chorion was removed at four hours post fertilization (hpf) as described by Truong et al, 2011. Embryos were rested for 30 minutes prior to initiating CNT exposure. Dechorionated embryos were transferred into individual wells of a 96-well plate with 100 μL of prepared CNT solution. Exposure plates were sealed and wrapped with aluminum foil to prevent evaporation and minimize light exposure. Embryos were exposed to five concentrations of CNT solutions and a negative control ($n=16$, two replicates) with the highest concentration at 50 $\mu\text{g/mL}$ down to 0.08 $\mu\text{g/mL}$. The static CNT exposure continued until 120 hpf. At 120 hpf, each embryo was euthanized with MS 222 and assessed for mortality and morphological malformations according to previously published protocol (Truong et al, 2001).

3 RESULTS AND DISCUSSION

3.1 Characterization

Seven types of CNTs (Table 1), including two single walled (SWCNT) with different lengths, one double walled (DWCNT), and four multi-walled (MWCNT) with different lengths and diameters, were evaluated. After treatment with a mixture of deuterated sulfuric and nitric acids (D_2SO_4 and DNO_3), thoroughly washed with water and oven dried, the CNTs exhibited solubility and were stable in water at concentrations up to 100 $\mu\text{g/mL}$. As shown in Table 1, all the CNTs studied were negatively charged and stable in Milli-Q water with zeta potential ranging from -46.07 mV to -61.55 mV and showed similar stability in embryo medium with zeta potential ranging from -33.77 mV to -39.61 mV (Greenwood and Kendall, 1999).

The hydrophobicity of unmodified CNT leads to suspension instabilities that result in settling within minutes or an hour depending on the type of modification or surfactant used. Figure 1A showed a well homogenised colloidal solution of the CNT prepared using the described method after 6 months of storage under ambient conditions, with no settling. This ensures that the CNT solutions are stable in homogenous dispersion form throughout the duration of the toxicity testing and that nanotubes aggregation is minimal. The SEM and TEM images obtained of the prepared CNTs (Fig 1B & 1C- for SWCNT, other results are not shown here) demonstrated retention of structural integrity of the CNTs and free of amorphous carbon impurity. This was most likely due to the washing and drying process used to make the samples, rendering them virtually free of contaminants.

Results of TGA show an average maximum oxidation temperature of 610 ± 11 °C and residual percent of 8.2 ± 3.9 % for all the nanotubes studied. Figure 2 showed a maximum oxidation temperature at 615°C for pristine SWCNT with a residual percent of 10.5 % and a percent weight loss of 3 % between ambient temperature and 106°C which was attributed to vapourization of water molecules. A maximum oxidation temperature at 600°C with a residual percent of 5.7 % was observed for the dehydrated acids treated SWCNT. Weight loss of 6.5 % in the temperature range less than 112°C was attributed to vaporization of water molecules while 22 % weight loss at temperature between 145°C to 350°C with mid point at 257°C is attributed to pyrolysis of the carboxylated carbon residue resulted from carboxyl (COO^-) groups grafted on the nanotubes sidewalls through covalent bonds (Men et al., 2008; Osorio et al., 2008). This agrees well with the X-ray photoelectron spectroscopy (XPS) results about the proportion of the oxygenated component of the prepared CNTs and also agreed with the possible functional groups grafted on the CNTs as revealed by the FT-IR results.

As shown in Figure 3, the percent elemental composition for both pristine and treated SWCNT showed pristine SWCNTs contain 96.3% carbon and 3.7% Oxygen atom (attributed to $-\text{OH}$ group of water content), while acid treated SWCNTs contains about 78% carbon and 21% oxygen attributed to carboxylic group and a small amount of water and 1% sulfur. The deconvoluted C 1s XPS spectra revealed a high degree of carboxylated carbons in the acid treated CNTs with none in pristine CNTs. These compositions may be the explanation for the high solubility achieved with the treated SWCNTs. The results show no significant change in chemistry between samples, only a difference in concentrations of sulfur and oxygen and agree with FT-IR results, which revealed the functional groups introduced onto the nanotubes following the treatment.

All the nanotubes studied showed similar IR characteristic. Representative FT-IR spectra of pristine and functionalized CNTs are shown in Figure 4. Infrared spectroscopy measures the quantity of radiation absorbed versus its frequency. When CNTs are subjected to an infrared radiation, the difference of charged state between carbon atoms induces the formation of an electric dipole; the appearance of these dipoles generates signals that are detected (Loiseau, 2006). IR absorption spectrum of D-acid treated CNT display $-\text{OH}$ stretching vibrations band at 3460 cm^{-1} , a characteristic of $-\text{OH}$ group attributed to carboxylic group and trace amounts of water. The bands at 1727 cm^{-1} and 1641 cm^{-1} were attributed to the presence of carboxylic $\text{C}=\text{O}$ and $\text{C}-\text{O}$ stretch respectively. The observed IR absorption peaks from the acid treated CNTs indicate the introduction of carboxyl groups due to surface oxidation (Socrates et al., 1994). Most of these absorption bands were not observed in the untreated sample save for the $-\text{OH}$ stretching vibrations band displayed at 3328 cm^{-1} . However, the intensity of the peak is much lower than the one observed in the acid treated sample spectrum; therefore we can infer that it is due to the presence of trace amounts of water in the sample. This agrees with the results obtained from the TGA and XPS analysis.

3.2 Toxicity

To assess the bioactivity of the seven different types of CNTs (two single-walled, one double-walled, and four multi-walled), the embryonic zebrafish model was used. Embryos

are exposed to 5 concentrations (0.08, 0.16, 0.4, 2, 10, and 50 $\mu\text{g/mL}$) of the CNTs from 6 to 120 hpf to assess for developmental toxicity. At 24 hpf, embryos are evaluated for mortality and developmental progression, since at this life stage, not all morphological structures are present. By 120 hpf, 18 endpoints are assessed (17 morphological and 1 behavioral) to determine if static exposure to CNTs throughout development was adversely affecting the development of the embryos. As Figure 5 illustrates, regardless of the length (1–5 or 5–30 μm) of the SWCNTs, there was no significant toxicity observed. When 15 ± 5 nm MWCNT were modified to have an increased length from 1–5 μm to 5–20 μm , no adverse response was induced, but when the length was maintained at 1–5 μm and the diameter increased from 15 ± 5 nm to 30 ± 15 nm, a mild increase in toxicity was observed. MWCNT with a length of 5–20 μm induced less toxicity at a diameter of 30 ± 15 nm than those with 15 ± 5 nm. Regardless of the length, thicker MWCNTs were more toxic. Yamashita observed that long (5 – 10 μm) and thick MWCNTs caused DNA damage and severe inflammatory effects in the lung of mice, but not the short and thin ones {Yamashita, 2010 #703}. These researchers also examined that the long and thick MWCNTs induced the strongest DNA damage while similar SWCNTs caused little effects. The lack of effect from SWCNTs is consistent with what was observed in this study. A study by Fenoglio et al found the opposite trend, where thin MWCNTs was more toxic compared to the thicker ones in both their *in vitro* (cytotoxicity) and *in vivo* (LDH activity and total proteins) assays {Fenoglio, 2011 #702}. Wang et al reported that a higher toxicity towards alveolar macrophages for short (1–5 μm) CNTs with 40–100 nm diameters than those with 10 – 20 nm {Wang, 2009 #705}. The difference in the role of diameter in toxicity may be due to a number of reasons such as metallic content, presence of surface functionalities or defects. Another potential reason for the different conclusions may be a consequence of the diameter of the CNTs affecting the curvature and modifying the interactions (to cells or proteins) {Fubini, 2010 #706}. Or the thicker MWCNTs exhibit a larger surface area which is exposed and allows for more interactions {Fenoglio, 2011 #702; Waters, 2009 #344}. Collectively, our results and these three studies suggest that the nanotube diameter plays a role in the toxicological assessments of CNTs.

Of the three types of CNTs tested, the DWCNTs induced mortality at a dose dependent manner, and caused snout malformations. The observation that DWCNTs induces snout malformations and mortality is consistent with a study using nasal cells. DWCNTs caused cytotoxicity to nasal cells at concentrations of 0.5 to 50 $\mu\text{g/mL}$ and at 25 $\mu\text{g/mL}$; there was an increase of reactive oxygen species {de Gabory, 2011 #707}. Not only are the DWCNTs cytotoxic to nasal cells, they are capable of activating Nlrp3 inflammasome and causing inflammation similar to that caused by asbestos {Meunier, 2012 #708}. With the first target for nanoparticles being the nasal cavity, these results are concerning and demonstrate the health hazards DWCNTs is capable of.

A sample blank was assessed to see if the methodology used to create water soluble CNTs had inherent toxicity. Although a low level of mortality (<20%) was observed after exposure to the sample blank, the low percentage of incidence is considered background in the zebrafish developmental toxicity assay. The lack of adverse biological response in the sample blanks demonstrates the methodology use was not toxic. It should be noted that we

could not use pristine CNTs in the toxicity test because they form aggregation rapidly because we will not be differential if the lack of a biological response is due to bioavailability or the CNTs themselves. The acid washes caused the surface modification to the CNTs, resulting in them being water soluble and dispersed. Other studies have observed that dispersed CNTs were more toxic than non-dispersed (Sayes et al., 2006). As these results demonstrated, the preparation method did not cause any toxicity and in general, these seven different types of water soluble CNTs were not toxic, but the diameter of CNTs is a parameter that may influence toxicity.

4 Conclusion

Various factors can be responsible for numerous results published in the literature on CNT toxicity and a number of the observations may not be a direct effect of CNTs. Toxicity studies have suggested that, besides the nano-dimensions of CNTs, many other factors may play roles in their toxicity (Simeonova, 2009). Raw nanotubes usually contain significant impurities, such as metal catalysts, which have been shown to contribute to increased toxicity through induction of oxidative stress (Donaldson et al., 2006). Purification procedures, such as strong acid treatment is expected to eliminate the impurities thereby making the CNTs less toxic. Aggregation is another possible factor that can cause false signal when doing toxicity studies of CNT as the functionalization of CNT helps to disperse and water solubilize the tubes and appears to reduce their toxicity (Sayes et al., 2006). It is therefore important to remove any potential contributor(s) to toxicity effects in order to effectively understand the direct effects due to CNT. In this study we have prepared and characterized water soluble CNTs of different types, the solutions obtained were stable for our observation period of 6 months without any settling which means no aggregation. This has enabled us to effectively study the toxicity effects of the prepared CNTs using the zebrafish model. The results obtained showed that the CNTs studied caused no significant adverse effects on development. This bodes well for the application of CNTs as components of indwelling medical devices such as tissue growth scaffolds, monitoring devices, and drug delivery tools.

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Figure 1.

(A) Solution of the prepared CNT after washing, drying and re-suspension in water. (B) An SEM image of the prepared SWCNT taken with 15KV accelerating voltage and 200, 000 magnifications, and (C) TEM image the prepared SWCNT taken with operating voltage of 120KV and magnification of 200,000; demonstrating retention of structural integrity of the nanotubes.

Figure 2.

Thermogravimetric analysis curves of pristine and treated SWCNT (after washing and drying) showing (A) percent weight loss as a function of temperature and (B), mass change per °C temperature ($d\text{Mass (mg)}/d\text{Temperature (}^{\circ}\text{C)}$).

Figure 3.

X-ray photoelectron spectroscopy (XPS) data demonstrates the elemental composition of SWCNTs before and after the acid treatment, indicating that only minor changes (attributable to surface fictionalization with carboxylic groups) arise following treatment.

Figure 4.
FTIR spectra of untreated and acids treated CNTs (after washing and drying)

Figure 5.

Mortality and adverse effects induced by seven different types of water soluble CNTs. Dechorionated embryos were exposed to the CNTs from 6–120 hpf and 4 endpoints were evaluated at 24 hpf, and 18 at 120 hpf. Endpoints evaluated are defined as follows: MO24 = mortality observed at 24 hpf; DP24 = developmental progression at 24 hpf; SM24 = spontaneous movement at 24 hpf; NC24 = notochord malformation at 24 hpf. Endpoints evaluated at 120 hpf were: MORT = cumulative mortality; YSE = yolk sac edema; AXIS = axis defects; EYE = eye defects; SNOU = snout defect; JAW = jaw defect, OTIC = otic (ear) defect; PE = pericardial edema; BRAI = brain defect; SOMI = somite defect; PFIN and CFIN = pectoral and caudal fin defect; PIG = pigmentation abnormalities; CIRC = circulation defects; TRUN = trunk defect; SWIM = swim bladder abnormalities; NC = notochord defect at 120 hpf and TR = touch response abnormality.

Table 1
Description of the nanotubes and their zeta potential in Milli-Q water and zebrafish embryo medium.

S/N	Type	Outer Diameter (nm)	Length (μm)	Zeta Potential (mV) in Milli-Q water	Zeta Potential (mV) in Culture medium
1	SWCNT	1-2	1-5	-46.45 ± 2.52	-39.61 ± 0.38
2	SWCNT	1-2	5-30	-53.34 ± 1.60	-37.30 ± 0.44
3	DWCNT	4 $\pm$ 1	1-5	-49.81 ± 1.97	-37.44 ± 1.67
4	MWCNT	15 $\pm$ 5	1-5	-46.07 ± 1.37	-33.77 ± 1.07
5	MWCNT	15 $\pm$ 5	5-20	-59.33 ± 2.82	-37.05 ± 0.71
6	MWCNT	30 $\pm$ 15	1-5	-47.43 ± 1.65	-35.11 ± 1.30
7	MWCNT	30 $\pm$ 15	5-20	-61.55 ± 4.38	-37.76 ± 1.60