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 (D_2SO_4 and DNO_3), washed with Milli-Q water and oven dried. Transmission electron microscopy, thermal gravimetric analysis, and other techniques were used to characterise 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; CNTs; deutrated acids; solubility; biomedical applications; zebrafish; nanotoxicity; teratogenic endpoints; embryonic.

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1 Introduction

Since their discovery 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 and Prabhakar, 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-Duarte 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 CNTs, 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 functionalisation 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 fibres 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 solubilise/disperse the nanotubes (Constantine and Prabhakar, 2010). Most areas of CNT toxicity are inconsistent and many studies suggest that elements of CNT toxicity are unsubstantiated (Constantine and Prabhakar, 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 solubilise 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 would be an advantage. 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 labour-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 three to five days that are small, develop externally, and are optically clear. An embryo's organs develop within five days post fertilisation 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, 2010, 2012; Usenko et al., 2007, 2008). By using this model, our data will position us to understand what physicochemical properties of CNTs drive the differential biological responses and observe whether water soluble CNTs are developmentally toxic.

2 Materials and methods

2.1 Materials

Deuterated sulphuric acid D_2SO_4: with a concentration of 96–98 wt.% in D_2O, isotopic purity of 99.5 atom % D, deutrated 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 CNTs

Pristine CNTs up to 5 mg/mL concentration were dispersed by ultrasonication (100 W, 42 KHz, Branson 3510 ultrsonication bath, maximum power) in a mixture of D_2SO_4 and DNO_3 (3:1 v/v) (Ramanathan et al., 2008) for four 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 (eight times) with Milli-Q water (18 MΩ) 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 analyser) was done to study the mass loss 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 1,000°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 analyser (Brookhaven Instruments Corporation, Holtsville, NY). All samples contained 1 mM KCl.

2.4 Toxicity testing

The CNTs solutions (at a concentration of 100 µg/mL) were vortexed briefly prior to making a 50 µg/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 fertilisation (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 aluminium foil to prevent evaporation and minimise 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 µg/mL down to 0.08 µg/mL. The static CNT exposure continued until 120 hpf. At 120 hpf, each embryo was euthanised with MS 222 and assessed for mortality and morphological malformations according to previously published protocol (Truong et al., 2011).

3 Results and discussion

3.1 Characterisation

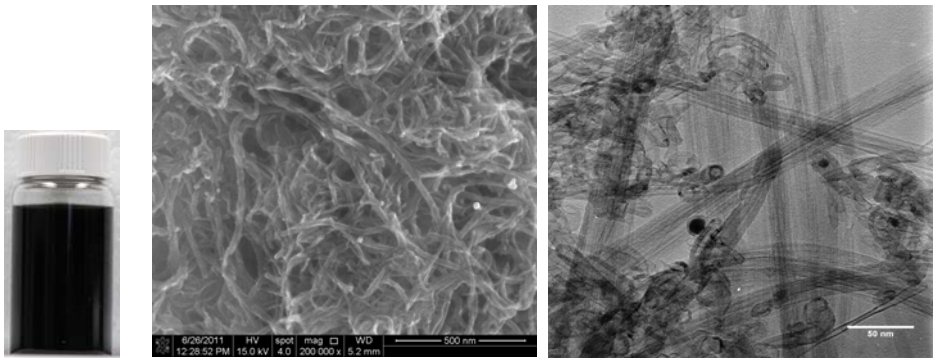
Seven types of CNTs (Table 1), including two single-walled (SWCNT) with different lengths, one double-walled (DWCNT), and four multi-walled (MWCNT) with different lengths and diameters, were evaluated. After treatment with a mixture of deuterated sulphuric and nitric acids (D_2SO_4 and DNO_3), thoroughly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

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 Mili-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±1	1–5	–49.81 ± 1.97	–37.44 ± 1.67
4	MWCNT	15±5	1–5	–46.07 ± 1.37	–33.77 ± 1.07
5	MWCNT	15±5	5–20	–59.33 ± 2.82	–37.05 ± 0.71
6	MWCNT	30±15	1–5	–47.43 ± 1.65	–35.11 ± 1.30
7	MWCNT	30±15	5–20	–61.55 ± 4.38	–37.76 ± 1.60

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 1(a) showed a well homogenised colloidal solution of the CNT prepared using the described method after six 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 [Figures 1(b) and 1(c) 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.

Figure 1 (a) Solution of the prepared CNT after washing, drying and re-suspension in water (b) a SEM image of the prepared SWCNT taken with 15 kV accelerating voltage and 200,000 magnifications, and (c) TEM image the prepared SWCNT taken with operating voltage of 120 kV and magnification of 200,000; demonstrating retention of structural integrity of the nanotubes

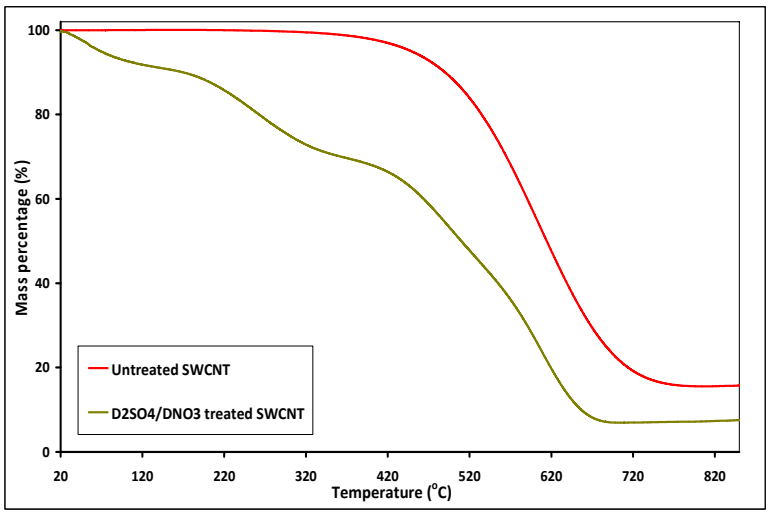


(a) (b) (c)

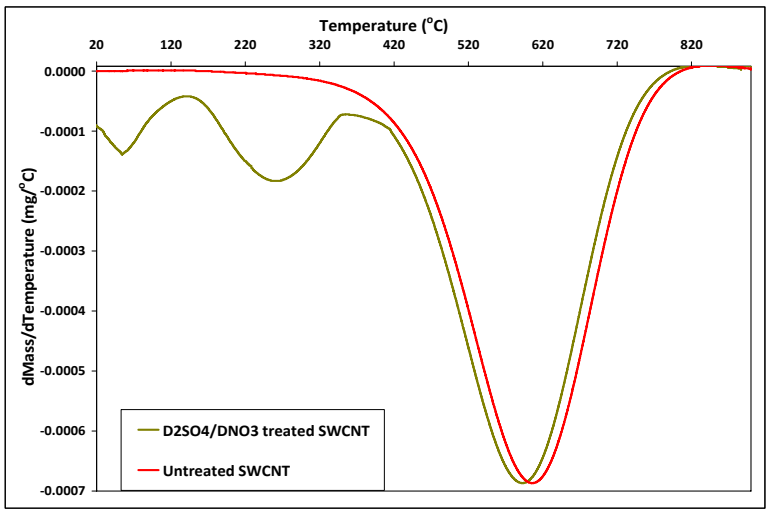
Results of TGA show an average maximum oxidation temperature of 610 ± 11°C and residual percent of 8.2 ± 3.9% for all the nanotubes studied. Figure 2 showed a maximum oxidation temperature at 615°C for pristine SWCNT with a residual percent of 10.5 and a percent weight loss of 3% between ambient temperature and 106°C which was attributed

to vaporisation of water molecules. A maximum oxidation temperature at 600°C with a residual percent of 5.7 was observed for the deutrated acids treated SWCNT. Weight loss of 6.5% in the temperature range less than 112°C was attributed to vaporisation 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 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 FTIR results.

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 (dMass (mg)/dTemperature (°C) (see online version for colours)



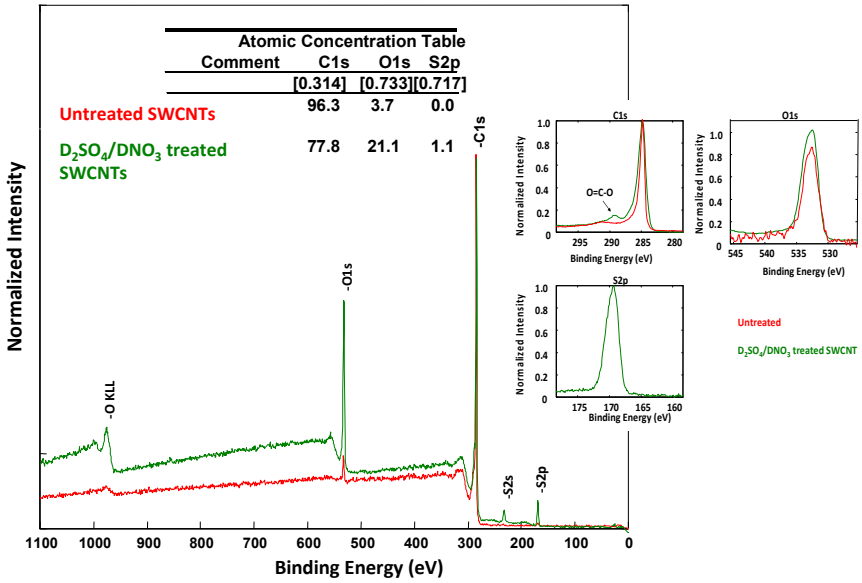
(a)



(b)

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 –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%ph. 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 sulphur and oxygen and agree with FT-IR results, which revealed the functional groups introduced onto the nanotubes following the treatment.

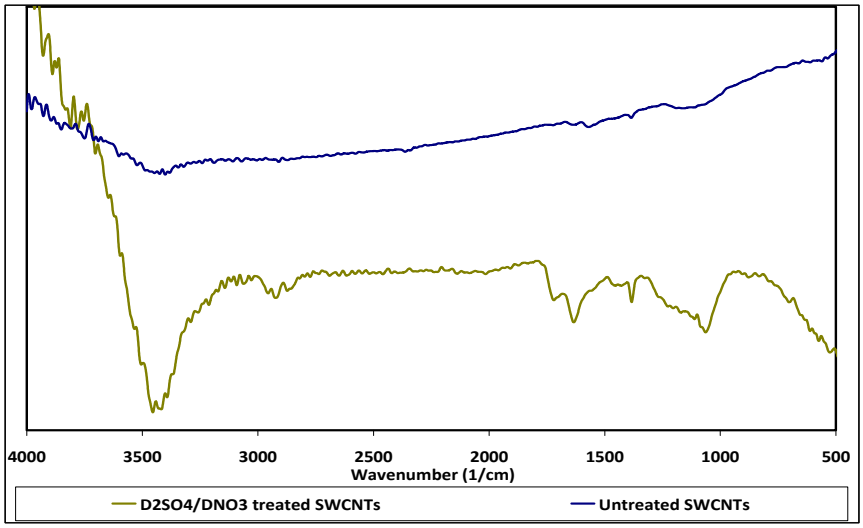
Figure 3 XPS data demonstrates the elemental composition of SWCNTs before and after the acid treatment, indicating that only minor changes (attributable to surface fictionalisation with carboxylic groups) arise following treatment (see online version for colours)



All the nanotubes studied showed similar IR characteristic. Representative FTIR spectra of pristine and functionalised 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 –OH stretching vibrations band at 3,460 cm^{-1}, a characteristic of –OH group attributed to carboxylic group and trace amounts of water. The bands at 1,727 cm^{-1} and 1,641 cm^{-1} were attributed to the presence of carboxylic C = O and C – O stretch respectively. The observed IR absorption peaks from the acid treated CNTs indicate the introduction of carboxyl groups due to surface oxidation (Socrates, 1994). Most of these absorption bands were not observed in the untreated sample safe for the –OH stretching vibrations band displayed at 3,328 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.

Figure 4 FTIR spectra of untreated and acids treated CNTs (after washing and drying) (see online version for colours)

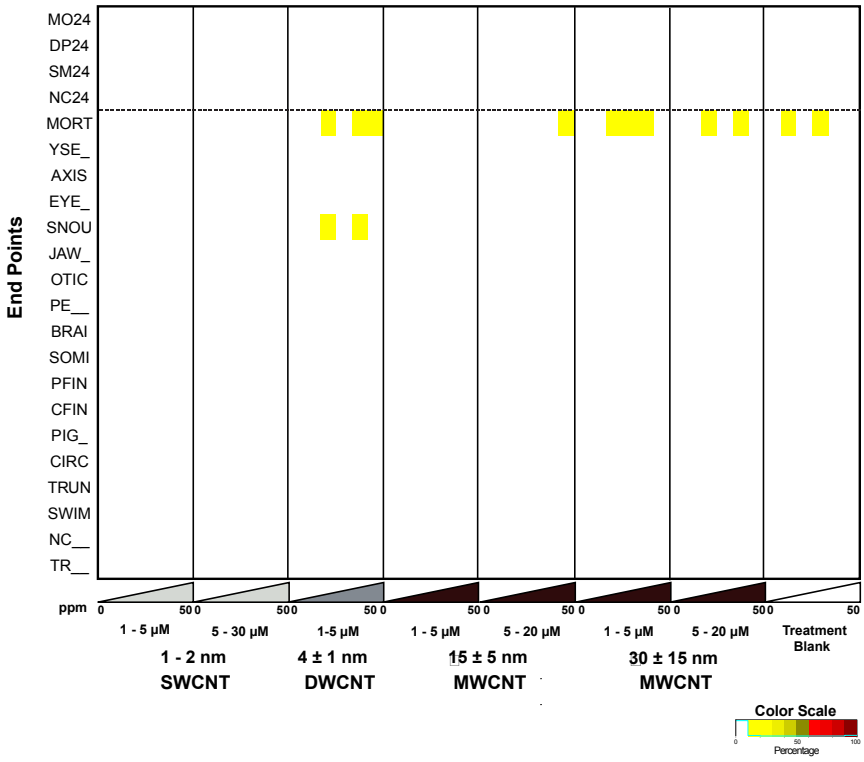


3.2 Toxicity

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 five concentrations (0.08, 0.16, 0.4, 2, 10, and 50 µg/mL) of the CNTs from 6–120 hpf to assess for developmental toxicity. At 24 hpf, embryos are evaluated for mortality and developmental progression, since at this life stage, no morphological structures are present. By 120 hpf, 18 endpoints are assessed (17 morphological and 1 behavioural) 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 moderate 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 et al., 2010). 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 were more toxic compared to the thicker ones in both their in vitro (cytoxicity) and *in vivo* (LDH activity and total proteins) assays (Fenoglio et al., 2011). Wang et al. (2009) 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. 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 et al., 2010). Or the thicker MWCNTs exhibit a larger surface area which is exposed and allows for more interactions (Waters et al., 2009; Fenoglio et al., 2011). Collectively, our results and these three studies suggest that the nanotube diameter plays a role in the toxicological assessments of CNTs.

Figure 5 Mortality and adverse effects induced by seven different types of water soluble CNTs (see online version for colours)



Notes: Dechorionated embryos were exposed to the CNTs from 6–120 hpf and four 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 cadual 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.

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 0.5–50 μg/mL and at 25 μg/mL; there was an increase of reactive oxygen species (De Gabory et al., 2011). 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 et al., 2012). 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 Conclusions

Various factors can be responsible for disparate 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 functionalisation of CNT helps to disperse and water solubilise 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 six months without any settling which means immeasurable 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 may bode 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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