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Functionalization density dependence of single-walled carbon nanotubes cytotoxicity in vitro

Christie M. Sayes [a], Feng Liang [a], Jared L. Hudson [a], Joe Mendez [a], Wenhua Guo [b], Jonathan M. Beach [a], Valerie C. Moore [a], Condell D. Doyle [a], Jennifer L. West [c,b], W. Edward Billups [a,b], Kevin D. Ausman [b,*], Vicki L. Colvin [a,b]

[a] *Department of Chemistry, Rice University, 1900 Rice Blvd., MS-60, Houston, TX 77005, USA*
[b] *Center for Biological and Environmental Nanotechnology, Rice University, 6100 Main Street, Houston, TX 77005, USA*
[c] *Department of Bioengineering, Rice University, Houston, TX 77005, USA*

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Abstract

The cytotoxic response of cells in culture is dependant on the degree of functionalization of the single-walled carbon nanotube (SWNT). After characterizing a set of water-dispersible SWNTs, we performed in vitro cytotoxicity screens on cultured human dermal fibroblasts (HDF). The SWNT samples used in this exposure include SWNT-phenyl-SO_3H and SWNT-phenyl-SO_3Na (six samples with carbon/-phenyl-SO_3X ratios of 18, 41, and 80), SWNT-phenyl-$(COOH)_2$ (one sample with carbon/-phenyl-$(COOH)_2$ ratio of 23), and underivatized SWNT stabilized in 1% Pluronic F108. We have found that as the degree of sidewall functionalization increases, the SWNT sample becomes less cytotoxic. Further, sidewall functionalized SWNT samples are substantially less cytotoxic than surfactant stabilized SWNTs. Even though cell death did not exceed 50% for cells dosed with sidewall functionalized SWNTs, optical and atomic force microscopies show direct contact between cellular membranes and water-dispersible SWNTs; i.e. the SWNTs in aqueous suspension precipitate out and selectively deposit on the membrane.

Keywords: SWNT; Single-walled carbon nanotubes; Cytotoxicity; Functionalized nanotubes; Fluid AFM

1. Introduction

Single-walled carbon nanotubes (SWNTs) are becoming increasingly studied, not only for their possible applications in the electronics, optics, and mechanical materials, but also in biological applications, such as imaging and drug delivery (Cherukuri et al., 2004). Because of this, it is imperative to examine the toxicity of these carbon-based nanostructures. Previous toxicological evaluations of single-walled carbon nanotubes have been conducted, both in cell culture and in vivo. One example, using a SWNT surfactant stabilized system where the Fe content was significantly high, reported an elevated cytotoxic response (Shi Kam et al., 2004). Warheit et al. (2004), observed an increase in inflammatory and PMN response in the lung cavities of rats. While these studies report potential negative implications of SWNTs, they did not use a SWNT sample easily dispersed in water via covalently bound functional groups. Since, it is the water-dispersible carbon nanotubes that

* Corresponding author. Tel.: +1 713 348 8212; fax: +1 713 348 8218.
E-mail addresses: csayes@rice.edu (C.M. Sayes), ausman@rice.edu (K.D. Ausman).

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are of considerable interest for biological applications, and a full toxicological evaluation can only be performed with nanomaterials that are disperible in the aqueous phase, we have investigated the cytotoxic response of human dermal fibroblasts (HDF) to a variety of water-dispersible single-walled carbon nanotubes.

In previous studies, we evaluated the differential cytotoxicity of water-suspendable fullerenes on HDFs in culture (Sayes et al., 2004). We concluded that as the degree of functionalization on the surface of the fullerene cage increases, the cytotoxicity of the fullerene decreased significantly. The simple act of functionalizing the fullerene with either carboxyl or hydroxyl groups decreased the cytotoxic response over seven orders of magnitudes for human dermal fibroblasts, human liver carcinoma cells, neuronal human astrocyte cell lines. In this study, we investigate the cytotoxic effect caused by dosing HDFs with varying concentrations of different water-dispersible SWNTs in an effort to similarly determine a differential cytotoxicity as a function of derivatization.

Here, we examine the cytotoxicity of three different water-dispersible SWNT samples in human dermal fibroblast cell cultures. Using a traditional cytotoxicity/viability staining assay, we determined the biological response of HDFs in culture dosed with varying concentrations of sidewall functionalized nanotubes and surfactant stabilized nanotubes. Characterization, including spectroscopy and microscopy, describe each sample used in the study. There are four water-dispersible SWNT samples used in this study: SWNT-phenyl-SO_3H (**1**), SWNT-phenyl-$(COOH)_2$ (**2**), SWNT in 1% Pluronic F108 (**3**), and SWNT-phenyl-SO_3Na (**4**). Cells were also exposed to a 1% Pluronic F108 solution as a control. The density of functionalization on the sidewalls of the SWNT is reported as the ratio of SWNT carbon atoms to addends (carbon/-phenyl-SO_3X). Finally, fluid atomic force microscopy (AFM) provided a tool to image the interaction of water-dispersible SWNTs and an artificial phosphocoline membrane at the nanometer level, modeling the interaction of the tubes with cell membranes.

2. Materials and methods

All chemicals were purchased through Sigma–Aldrich at highest purity unless otherwise stated and experiments were performed minimally in triplicate. Data are presented as mean ± standard deviation, and an analysis of variance (ANOVA) followed by a Dunnett's test was used to determine significance. The single factor ANOVA test was applied specifically to the samples used in a particular study, as well as each dilution of the sample being tested. Statistical significance was established as P and $\alpha < 0.05$. Statistical tests were performed with Excel software (Analysis ToolPak for Microsoft® Excel 2000) and from literature (Dunnett, 1964).

2.1. Water-dispersible single-walled nanotubes preparation

Three different water-dispersible single-walled nanotube samples (SWNT) were used in this study. Compounds **1** and **4** were synthesized by the method described below. These samples were functionalized at various carbon/-phenyl-SO_3X ratios (18, 41, and 80) to evaluate the dependence of cytotoxicity on the functionality of SWNTs.

2 was synthesized by dispersing unfunctionalized SWNT (0.169 g, 14 meq C) in oleum (200 mL, 20% free SO_3) with magnetic stirring (3 h) (Hudson et al., 2004). Sodium nitrite (1.93 g, 28 mmol) was added followed by 5-aminoisophthalic acid (5.07 g, 28 mmol) and azobisisobutyronitrile (AIBN) (0.460 g, 2.8 mmol). The reaction was stirred at 80 °C for 1 h, then carefully poured over ice. The suspension was then filtered through a polycarbonate membrane (1 μm). The filter cake was washed with water and acetone, and then dried (233 mg).

3 had no deliberate sidewall functionality, but instead was a SWNT sample dispersed in water using a 1% Pluronic F108 solution. Moore et al. (2003), describes the method of synthesis for this sample.

2.2. Synthesis of SWNT-phenyl-SO_3H and SWNT-phenyl-SO_3Na

The SWNTs used in this investigation were produced at Rice University by the HiPco process (Bronikowski et al., 2001) and purified as described previously (Xu et al., 2005). SWNTs with residual metal less than 1 wt.% were obtained after purification.

1 was prepared using a two-step process. First, SWNTs (40 mg, 3.33 mmol of carbon) and benzene (100 mL) were added to a 250 mL three-necked round bottom flask equipped with a homogenizer (Peng et al., 2003; Ying et al., 2003). The contents were homogenized for 10 min before benzoyl peroxide (807 mg, 3.33 mmol for the most functionalized level; 202 mg, 0.833 mmol for the medium-functionalized level; 25 mg, 0.104 mmol for the least functionalized level) was added, and heated under argon at 80 °C for 2 h with homogenizing. After cooling, the contents of the flask were diluted with 100 mL of benzene, filtered over a PTFE membrane (0.2 μm), and washed extensively with chloroform to produce phenylated SWNTs.

Second, the phenylated SWNTs (20 mg) were dispersed in oleum (20 mL, H_2SO_4, 20% as free SO_3) and heated to 80 °C for 4 h under argon atmosphere to produce SWNT-phenyl-SO_3H. The suspension was carefully poured into 100 mL of ice water, filtered over a polycarbonate membrane (0.22 μm), and washed extensively with water to produce **1**.

4 was prepared by dispersing **1** (20 mg) in 1 M NaOH (30 mL), and heating to 80 °C under argon overnight to produce **4**. The contents were diluted with 100 mL of water, filtered over

a polycarbonate membrane (0.22 μm), and washed extensively with water.

2.3. Characterization of water-dispersible SWNTs

3 was characterized in previous studies (Moore et al., 2003; Hudson et al., 2004). Samples were characterized using spectroscopy and microscopy. Characterization of **1**, **2**, and **4** are reported here. Degree of functionalization was determined both qualitatively using Raman spectroscopy and quantitatively using thermogravimetric analysis (TGA) and X-ray photoelectron spectroscopy (XPS). Dispersion characteristics, as well as concentrations, were determined with cryo-transmission electron microscopy (cryo-TEM).

2.4. Differential cytotoxicity of water-dispersible SWNTs, in vitro

Human dermal fibroblasts were purchased from Cambrex Biosciences and cultured in Dulbecco's Modification of Eagles Media (DMEM) (McKeeham and Ham, 1977; Anderson et al., 1996). Cells were grown to 70% confluency before exposure to each SWNT sample; each culture plate was incubated in the dark at 37 °C/5% CO_2 for 48 h. Passage numbers 2–10 were used in this study. The concentrations of each SWNT species delivered were between 10^{-4} and 10^{3} ppm.

The viability/cytotoxicity of human dermal fibroblasts exposed to various water-dispersible SWNT samples was measured using calcein AM and ethidium homodimer stains (Molecular Probes). Cells were exposed to nanotube samples at varying concentrations (3 μg/mL–30 mg/mL) for 48 h. As a control, cells were exposed to a 1% Pluronic F108 solution. Using fluorescence microscopy, images of healthy normal cells and compromised unhealthy cells were collected. Each experiment was performed in triplicate. Although we observed an expected dose–response relationship, i.e. as concentration of SWNT suspension increased, cell viability decreased; cell death did not exceed 50% for compounds **1**, **2**, and **4**, providing a lower bound for their LC_{50} values, the concentration at which 50% of cells in culture die, of 2 mg/mL. Stained cells were imaged using fluorescence microscopy (Zeiss Axiovert) and statistical analysis was performed using a single factor ANOVA test followed by Dunnett's test.

2.5. Mitochondrial activity

The 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay (Sigma) was used to evaluate mitochondrial activity (Mossman, 1983). Cells, grown in 24-well plates, were exposed to SWNTs as described above. After 48 h, 150 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h. Afterwards, 850 μL of the MTT solubilization solution (10% Triton X-100 in 0.1 N HCl in anhydrous isopropanol) was added to each well. The 24-well plate was gently mixed on a gyratory shaker to solubilize the formazan crystals. After solubilization in acidic isopropanol, the product was quantified by measuring absorbance at 570 nm.

2.6. Evidence of SWNT deposition of biomembranes

Images of the interactions between water-dispersible SWNTs and biological membranes were obtained by bright-field optical microscopy and atomic force microscopy. For the AFM studies, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids, 5 mg/mL in chloroform) membranes were prepared by drying down the solution using a N_2 flow followed by a vacuum (10^{-5} Torr). The lipids were then hydrated with 2 mL of deoxygenated DI water, vortexed in the dark, and diluted to a total volume of 200 mL. Fifty microliters of the resulting solution was incubated for 20 min over freshly cleaved mica, rinsed with DI water, and transferred to the liquid cell (total volume, 100 μL) of the AFM (Digital Instruments Nanoscope IV) without further treatment. For these experiments, pure water was used as the medium for imaging. Before inoculation of SWNTs to the solutions, the lipids were found to form micron-sized islands on the mica surface (∼5 nm high), which is consistent with the formation of a stable lipid bilayer.

3. Results

Fig. 1 shows the dose-response relationship of three different water-dispersible single-walled carbon nanotube samples, as well as the structural differences in the various samples. SWNT-phenyl-SO_3H and SWNT-phenyl-$(COOH)_2$, covalently bound sidewall functional groups, are less cytotoxic than the SWNT in 1% Pluronic F108, which is stabilized in a micellar solution without covalent functionalization. Cells exposed to a 1% Pluronic F108 solution control exhibited only a 10% decrease in viability (supplemental data available); unexposed controlled cells only had 2–3% decrease in viability. Cell death (% dead) was measured by number of cells fluorescing read divided by the total number of cells. The results from the ANOVA tests of control and experimental groups revealed that samples **1** and **2** are non-cytotoxic to HDF cells, and sample **3** is cytotoxic; those results are statistically significant at $P < 0.000807$. Results from Dunnett's test are shown in Fig. 1.

Phenylated SWNTs, precursors for **1** (SWNT-phenyl-SO_3H) and **4** (SWNT-phenyl-SO_3Na) were characterized by Raman spectroscopy and thermogravimetric analysis. Direct evidence of covalent sidewall functionalization was provided by Raman spectroscopy. The Raman spectrum of the starting purified SWNTs shows a small disorder mode (D-band) at 1290 cm^{-1} (Fig. 2A). The spectra of the least, medium and most functionalized samples exhibit progressively increasing disorder modes relative to the large tangential modes (G-band) at

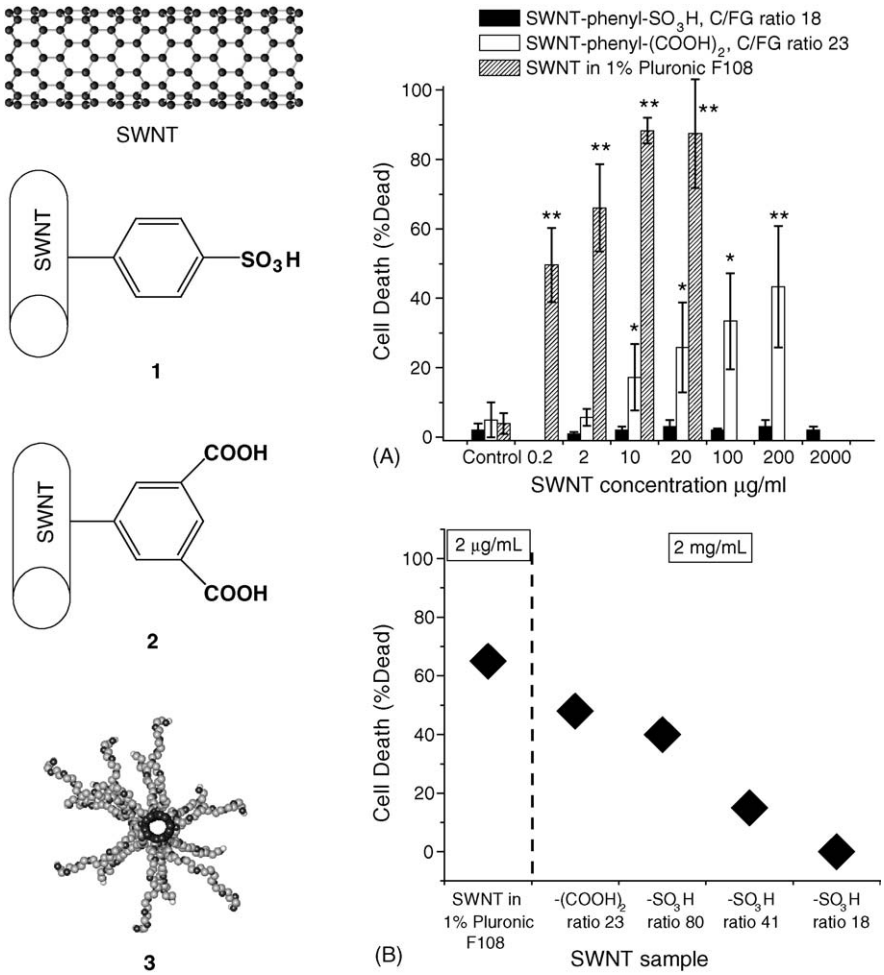

Fig. 1. Differences in the functionality and cellular activity of three different water-soluble single-walled carbon nanotube samples. The water-soluble functional groups of each sample is shown to the left, **1** SWNT-phenyl-SO_3H, **2** SWNT-phenyl-$(COOH)_2$, **3** SWNT in 1% Pluronic F108 (*note*: structures of models are not drawn to scale; **3** represents the surfactant wrapped around the tube, as viewed from the end of the tube). The dose–response curve is shown in part (A): (■) SWNT-phenyl-SO_3H; (■) SWNT-phenyl-$(COOH)_2$; (■) SWNT in 1% Pluronic F108, where C/FG stands for carbon to functional group ratio. Results are combined from three independent exposures. Groups significantly different from the control group (by ANOVA $P < 0.000807$ followed by Dunnett's test) are shown by ($^{*}P < 0.05$) or ($^{**}P < 0.01$). Part (B) plots the relative toxicities of various samples at stated concentrations.

$\sim$1590 cm^{-1}. The degree of functionalization has been determined by thermogravimetric analysis from 80 to 800 °C under an atmosphere of argon. The weight loss for the least, medium and most functionalized SWNTs was 17.0%, 22.5%, 33.5%, respectively. Considering that the weight loss of the starting purified SWNTs was 10.5% under the same conditions, the degree of functionalization (carbon/phenyl group ratio) for the three samples was determined to be 80, 41 and 18 for the least, medium and most functionalized SWNTs, respectively. The synthesis of **1** and **4** alter only the phenyl groups and do not change the degree of functionalization). Further characterization of **1** and **4** was carried out by X-ray photoelectron spectroscopy and cryo-transmission electron microscopy. Fig. 2A, B, and C show the XPS data of the most functionalized **1**. The atomic percentage was 83.05 C_{1s}, 2.82 S_{2p}, and 14.13 O_{1s}, indicating that monosulfonation had occurred. For the most functionalized **4** (SWNT-phenyl-SO_3Na), the atomic percentage was 84.35 C_{1s}, 2 S_{2p}, 11.77 O_{1s}, and 2 Na_{1s}, indicating that the sulfur to sodium ratio was approximately 1:1 and the -phenyl-SO_3H groups were changed to -phenyl-SO_3Na. The cryo-transmission electron micrograph (Fig. 2D) shows dimensions and dispersion of the most functionalized **1** in water. The tubes, on average, are 1 nm in diameter, 400 nm in length, and individually suspended. Lastly,

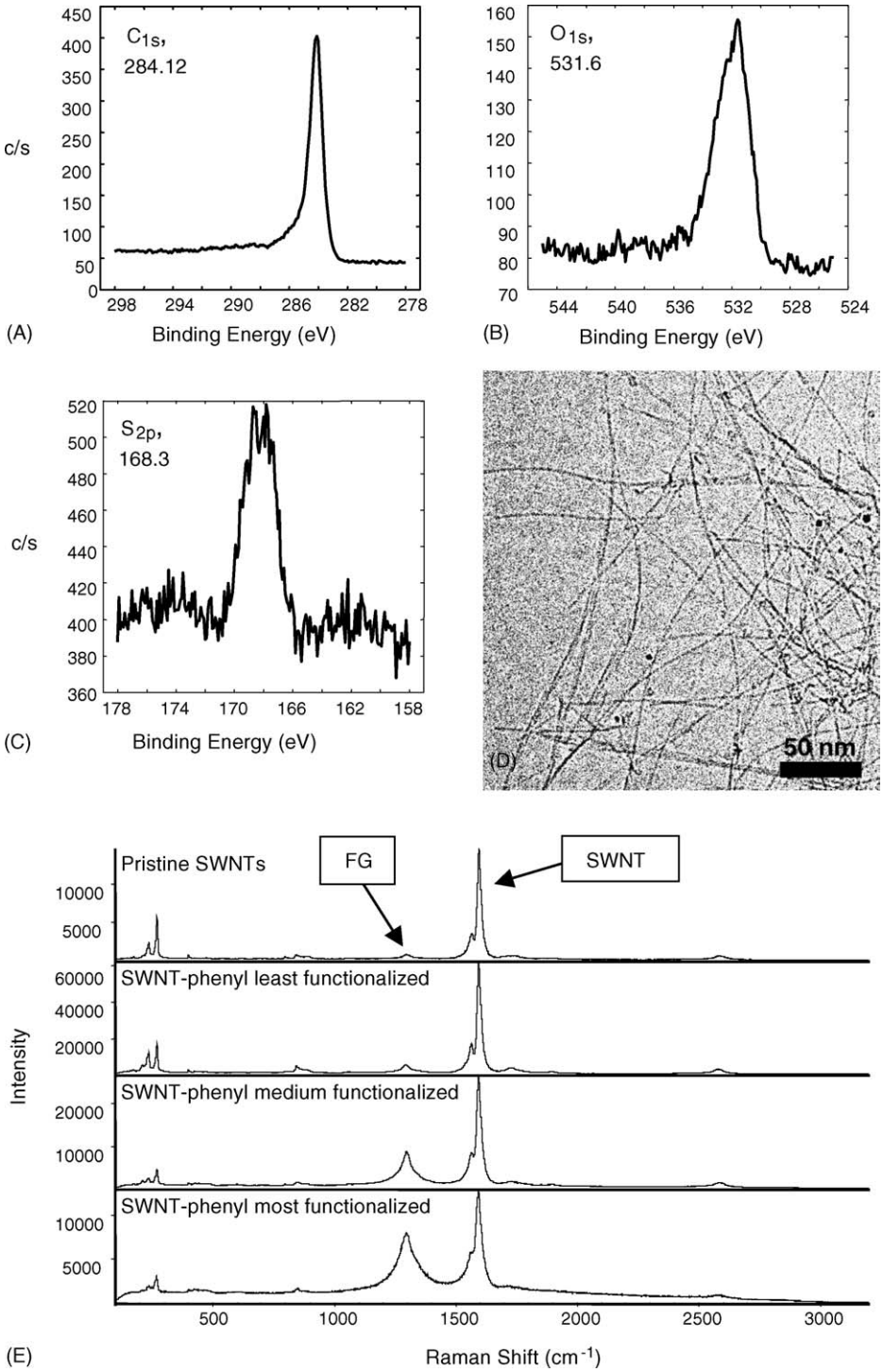

Fig. 2. Characterization of SWNT-phenyl-SO_3H. The characterization data of the nanoparticle sample not only verifies that the sample contains SWNT, but also confirms the absence of catalytic impurities or residual solvents. The XPS spectra of the most functionalized SWNT-phenyl-SO_3H sample is shown here at.%: (A) C_{1s} 83.05; (B) O_{1s} 14.13; (C) S_{2p} 2.82. Other possible contaminants, such as chlorine or iron as found in samples from other reports (Shi Kam et al., 2004), were below the detection limit of the instrument. (D) The cryo-transmission electron micrograph of SWNT-phenyl-SO_3H dispersed in water. This micrograph shows the SWNT suspension in its native state, just before inoculation into cell culture. (E) Raman spectroscopy confirms the degree of functionalization.

Fig. 2E shows the carbon nanotube peak unchanged, but the functionalization peak increasing as functionalization density increases.

2 was characterized by Raman spectroscopy, thermogravimetric analysis, and X-ray photoelectron spectroscopy. The Raman spectrum gives evidence for sidewall functionalization of the SWNTs, while TGA and XPS confirm the carbon/-phenyl-$(COOH)_2$ ratio to be 23.

When examining the SWNT samples with different degrees of functionalization, we saw a differential in the cytotoxic response of the HDFs in culture (Fig. 3). As the degree of functionalization changed from carbon/-phenyl-SO_3H ratios of 80, 41, and 18, the cytotoxicity decreased. The LC_{50} values for each of the functionalized sample could not be obtained because 50% cell death could not be reached. Using the MTT assay, we found that mitochondrial activity was unchanged. The cytotoxic responses of SWNT-phenyl-SO_3Na on HDFs are similar to those of SWNT-phenyl-SO_3H. The results from the ANOVA tests of control and experimental groups revealed that most functionalized SWNT-phenyl-SO_3X are non-cytotoxic to HDF cells, but statistically significant ($P < 0.544$); the medium-functionalized SWNT-phenyl-SO_3X are non-cytotoxic to HDF cells, but statistically significant ($P < 0.495$); and least functionalized SWNT-phenyl-SO_3X are non-cytotoxic to HDF cells, but are not statistically significant ($P < 0.403$). Results from Dunnett's test are shown in Fig. 3.

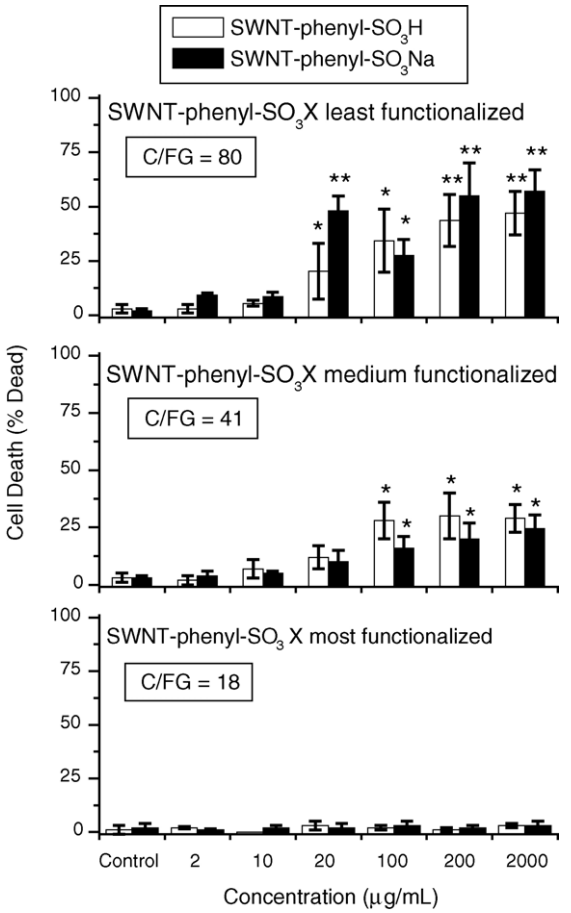

Fig. 3. Similarities in cellular activity of SWNT-phenyl-SO_3H and SWNT-phenyl-SO_3Na. The former is a precursor to the later. Both samples were tested because different SWNT applications requires different starting materials, i.e. SWNT-phenyl-SO_3H or SWNT-phenyl-SO_3Na. The dose–response relationship of the least, medium, and most functionalized (□) SWNT-phenyl-SO_3H and (■) SWNT-phenyl-SO_3Na samples. Results are combined from three independent exposures. Groups significantly different from the control group (by ANOVA $P < 0.403$ followed by Dunnett's test) are shown by ($^{*}P < 0.05$) or ($^{**}P < 0.01$).

After 36 h, there was visible evidence of nanotubes beginning to aggregate and precipitate out of DMEM, confirmed by the Tyndall test. Fig. 4 shows the deposition of nanotubes onto the membrane. Deposition was confirmed on model membranes using atomic force microscopy, where tubes, both individual and aggregates, deposit onto phosphocoline membranes. The optical microscope image in Fig. 3 is of cultured HDFs after exposure to water-dispersible SWNTs for 2 days. The image shows the aggregation and deposition of the nanotubes on the cellular membrane. Further analyses using AFM imaging, reveals that the nanotubes will preferentially precipitate out of the aqueous solution and deposit onto a 1,2-dioleoyl-*sn*-glycero-3-phosphocholine membrane.

4. Discussion

The water-dispersible SWNT forms studied here span the two distinct primary methods for suspending nanotubes in aqueous systems. Samples **1** and **2** are covalent modifications of the tubes themselves, resulting in solubilizing functionality irreversibly attached under even extreme biological conditions. These stable samples are suspended in water without the use of surfactants, but suffer the disadvantage of chemical modifications of the underlying aromatic nanotube structure, which is the source of many properties that make SWNTs attractive for applications.

Sample **3**, on the other hand, contains pristine underivitized SWNTs solubilized by surfactant coatings. It is known that SWNTs in 1% Pluronic F108 prepared in surfactant solution by this method results in individually suspended SWNTs in stabilized micellar surfactant assemblies (Moore et al., 2003). Stabilization of SWNTs

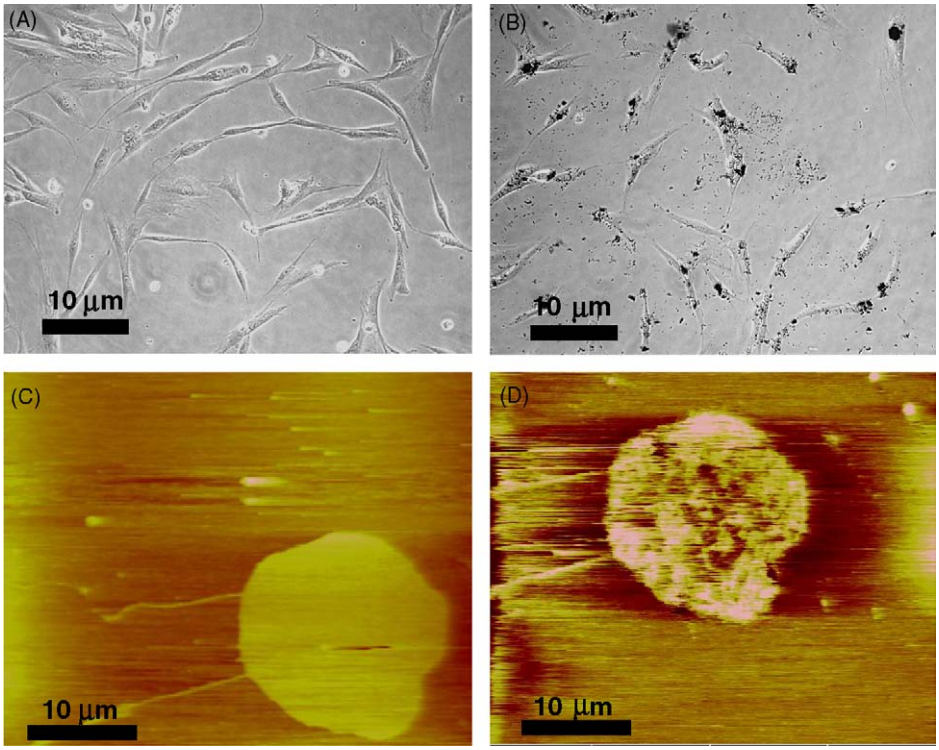

Fig. 4. SWNT-phenyl-SO_3H preferentially deposit on the DOPC membrane. Optical micrographs of (A) healthy HDFs before exposure and (B) HDFs after exposed to SWNT-phenyl-SO_3H for 48 h. Atomic force micrographs of (C) intact DOPC membrane and (D) DOPC membranes exposed to SWNT-phenyl-SO_3H for 2 h.

in aqueous systems by surfactant has long been preformed including Triton X100 and sodium dodecylbenzene sulfonate (SDBS) (Bandow et al., 1997; Duesberg et al., 1998; Burghard et al., 1999; Casavant et al., 2003). These samples retain the inherent properties of underivitized SWNTs, but unlike samples **1** and **2**, their surfactant coating is reversibility, non-covalently attached.

The sidewall functionalized nanotubes were analyzed for degree of functionalization, dispersion in water, and cytotoxic response. Raman spectroscopy and thermogravimetric analysis show degree of functionalization on the surface of the tube. The degree of functionalization decreases from carbon/-phenyl-SO_3H ratios of 80, 41, and 18 for the samples tested. X-ray photoelectron spectroscopy was used to determine the elemental contents for both **1** and **4**. Cells dosed with both samples exhibited the same cytotoxic response. We lowered the HDF cytotoxic response of SWNT by covalent functionalization. The SWNT in 1% Pluronic, which was more dilute than **1**, **2**, or **4** by three orders of magnitude, was more cytotoxic than the sidewall functionalized SWNTs. This increase in cytotoxic response is partly due to excess Pluronic F108 in the aqueous solution. Controlled exposure of 1% Pluronic to HDFs produced a 10% decrease in viability, where HDFs exposed to no additives decreased in viability only 2–3%. In addition to cytotoxic response, the metabolic activity of HDFs in culture was unchanged over the SWNT-phenyl-SO3H concentration range. The reduction of MTT by an oxidizing agent was observed to be independent of SWNT-phenyl-SO3 concentration, ruling out significant interference in the MTT test by the nanotube sample. Functionalization of SWNTs may limit some properties of SWNTs that are key to applications such as its fluorescence, but does increase the biocompatibility of nanotubes with cells in culture.

For the MTT and differential cytotoxicity screens for samples **1**, **2**, and **3**, data was taken for both 24 and 48 h time points, showing no difference in cell death or mitochondrial activity; i.e. the same assay results were seen at both time points. Liquid AFM imaging was performed continuously for 2 h.

Using fluid AFM, we observed two phenomenon involving **1**, a covalently modified derivative, and phosphocoline membranes. First, SWNTs preferentially deposit on DOPC membranes, not on the mica substrate. Second, over a 2 h time period, the water-dispersible SWNT sample begins to aggregate on the DOPC membrane. Two driving forces may facilitate the deposition

of SWNTs on phosphocoline membranes. We speculate that ionic interactions provide a means for nanotube deposition on DOPC membranes; i.e. the –OH group from the sulfonate functional group of SWNT-phenyl-SO_3H, when suspended in water, can dissociate and leave a negative charge of the surface of the nanotube. The ampiphilic molecule, DOPC, is a zwitterion, thus can attract a charged nanotube initiating the observed deposition. These AFM experiments do not distinguish surface deposition and membrane intercalations. The lipid bilayer that composes the biomembranes provides a lipophilic environment, which in turn could stabilize nanotubes suspended in an aqueous environment.

Though the cytotoxicity studies show only very limited impact of cell viability and cell density, these AFM studies suggest relatively significant interactions of SWNTs with biomembranes. This interaction provides further evidence that the bio-nano interface can be developed for drug delivery and diagnostic applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2005.08.011.

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