Research Article

The Effect of DNA and Sodium Cholate Dispersed Single-Walled Carbon Nanotubes on the Green Algae *Chlamydomonas reinhardtii*

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Increasing use of single-walled carbon nanotubes (SWCNTs) will lead to their increased release into the environment. Previous work has shown negative effects of SWCNT on growth and survival of model organisms. The aim of the current study was to determine the effect of SWCNT well-dispersed by either DNA or sodium cholate (SC) on the unicellular green algae *Chlamydomonas reinhardtii* in stagnant water conditions. Growth measurements were taken up to ten days for algae treated with varied levels of DNA:SWCNT or SC:SWCNT or controls, and chlorophyll content after 10 days was determined. Results show no effect on either growth or chlorophyll content of algae at any concentration or duration. This is in contradiction to prior work showing toxicity of SWCNT to environmental model organisms.

1. Introduction

The increased use of manufactured nanomaterials has led to a need for a better understanding of their potential effects on natural systems. Among these products are single-walled carbon nanotubes, which can be represented by sheets of cylindrically rolled graphene, originally described in 1993 by Iijima et al. [1–3]. The widespread commercialization of single-walled carbon nanotubes (SWCNTs) is due to their enormous potential in industrial, biomedical, and electronic sensing applications. This potential is derived from their unique physical, electronic, and optical properties [4–8]. The environmental release and transport of SWCNT at all stages of their production and use will lead to environmental concerns [9, 10]. Therefore, it is necessary to study their interaction with organisms in the environment.

Previous work has shown that carbon nanomaterials have toxic effects on model organisms. Graphite, C60 fullerene,

SWCNT, and multiwalled carbon nanotubes (MWCNT) have been shown to be toxic to various bacterial types and *Daphnia magna* [11–13]. These previous works make it clear that some preparations of SWCNT may have toxic environmental effects.

The unicellular green alga *Chlamydomonas reinhardtii* has been developed as a model for the effects of nanomaterials on photosynthetic microorganisms and indirect assessment of ecosystem-level effects [14]. Work using this model organism has shown toxicity and uptake of silver nanoparticles, quantum dots, and titanium dioxide nanoparticles [15–17]. Additionally, *C. reinhardtii* has been used in finding toxic effects of SWCNT poorly suspended in solution with no surfactant [18]. This work showed a negative effect on growth and photosynthetic output of these algae due to $2\,\mu g/mL$ of SWCNT aggregates in solution. Some previous work has shown reduced toxicity, but not lack of toxicity, from a filtered soluble fraction of SWCNT and from soluble multiwalled

carbon nanotubes (MWCNT) [19, 20]. No work, however, has previously determined the effects of well-dispersed SWCNT on a similar model organism.

This study aimed to determine the effects of well-dispersed SWCNT on the model species *Chlamydomonas reinhardtii*. SWCNTs well-dispersed with DNA or sodium cholate (SC) were utilized, mimicking the environmental release of some SWCNT [10]. The most mobile fraction of SWCNT released into the environment is well-dispersed [21]. Most previous environmental toxicity studies have shown toxic effects of poorly dispersed SWCNT. Here, the system was designed to mimic a stagnant algal growth environment that is similar to many natural systems. This design is ideal for well-dispersed SWCNT; however, nondispersed SWCNT would not interact with the environment in such a system due to sedimentation. Additionally, nondispersed SWCNTs are known to become more well-dispersed over time through interaction with natural organic matter [22, 23]. Deoxyribonucleic acid (DNA) associates with SWCNTs and disperses them individually in solution [24], while sodium cholate (SC) is a biocompatible surfactant which disperses SWCNT well in solution [25, 26]. Herein, nontoxic SWCNTs are obtained by dispersing them with DNA and SC and the constructs were further characterized by near-infrared (NIR) spectroscopy.

2. Materials and Methods

2.1. Preparation of SWCNT. Single-walled carbon nanotube preparations dispersed by either salmon testes genomic DNA or SC (DNA:SWCNT or SC:SWCNT) were made similarly to previously described work [24–27]. Identical preparations have previously been characterized by atomic force microscopy, transmission electron microscopy, and Raman microscopy [28–30]. 2 mg CoMoCat SWCNT of mixed chirality (Sigma-Aldrich; St. Louis, MO) was mixed with 2 mg salmon testes genomic DNA or SC in 2 mL 1x phosphate-buffered saline (PBS). This mixture was sonicated on ice for two hours with a Virsonic XL2020 ultrasonic liquid processor (Misonix; Farmingdale, NY) equipped with a 3.2 mm microtip at approximately 20% power. The resulting solution was centrifuged at 16,000 ×g for 90 minutes to remove insoluble SWCNT. Filtering caused SC:SWCNT to precipitate from solution thus this was used as prepared, unfiltered. For DNA:SWCNT samples, the solution was then filtered through a 100,000 molecular weight centrifugal filter (Millipore; Billerica, MA) three times at 4000 RPM for 15 minutes each to remove DNA that did not associate with SWCNTs. The recovered DNA:SWCNT solution was brought to a volume of 500 μL with 1x PBS, while SC:SWCNT samples remained in 2 mL. Additionally, SWCNTs were sonicated under the same conditions in the absence of DNA or SC to determine their dispersion. Half of this sample was initially centrifuged under the same conditions to study their dispersion. Both fractions of nondispersed SWCNT were further analyzed. The concentration of the sample was obtained by gravimetric analysis of 10 μL of the solution dried in a desiccator and weighed on an Ohaus Discovery Microbalance (Ohaus; Parsippany, NJ). As determined by gravimetric analysis, initial concentrations of prepared disperse SWCNT

solutions ranged from 10 μg/μL to 30 μg/μL. Therefore, the treatment concentration was that of the DNA:SWCNT or SC:SWCNT solution as a whole and not just that of SWCNT alone.

2.2. Characterization of DNA:SWCNT and SC:SWCNT. Each SWCNT preparation was diluted to a 1% solution with Milli-Q water (Millipore; Billerica, MA). This solution was added to a 10 mm path length cuvette (Sterna Cells; Atascadero, CA) and analyzed on a NanoSpectralyzer 1 (NS1) (Applied NanoFluorescence; Houston, TX). The absorbance spectrum was collected from 400 to 1350 nm. Additionally, integrated photoluminescence spectra were obtained using excitation wavelengths of 638 nm, 690 nm, and 784 nm. The resulting spectra were analyzed with ANFSoft (Applied NanoFluorescence; Houston, TX). This software also calculated the SWCNT diameter distribution from the absorbance and integrated fluorescence measurements. Fluorescence efficiency measurements, which are fluorescence power collected across the emission spectrum for a single excitation wavelength divided by the absorbance spectrum, are reported as a relative measure of SWCNT dispersion in solution [31, 32].

2.3. Algal Growth Conditions. *Chlamydomonas reinhardtii* wild-type strain cc-1690 was obtained from the Chlamydomonas Resource Center and cultured similarly to as previously described [33]. Briefly, initial growth was performed on 1.5% Sueoka's high salt medium (HSM) agar. This was used to seed small liquid cultures of HSM for outgrowth, which was kept in a continuous culture of 100 mL HSM. Every 1-2 weeks, the existing culture was diluted to 10% in HSM to allow continuous outgrowth. Culture conditions were sustained at 22–25°C with continuous ambient light and slight daily mixing before measurements to mimic a stagnant growth environment.

2.4. SWCNT Treatments. Treatments of *C. reinhardtii* in liquid culture with SWCNT preparations or controls were performed in a total volume of 10 mL in 50 mL conical tubes with loosened lids. Treatments were performed with DNA:SWCNT or SC:SWCNT with proper controls but not nondispersed SWCNT as they sediment and do not interact with live organisms in large-volume solutions. At day zero, HSM (9 mL) was seeded with 1 mL stock algal culture and the appropriate treatment was added. Treatments were a single addition of one of the following: 0, 0.1, 1, 2, 10, or 100 μg/mL DNA:SWCNT or SC:SWCNT and 100 μg/mL sonicated DNA or SC. The final treatment of DNA or SC alone sonicated in PBS served as a control for both the dispersal agents and PBS vehicle. Contamination and absorbance controls consisted of 10 mL of HSM or HSM with 1 μg/mL DNA:SWCNT or SC:SWCNT. There was no normalized optical density for either the high salt medium, 1 μg/mL DNA:SWCNT, or 1 μg/mL SC:SWCNT only controls, meaning no contamination of media or SWCNT sample was present.

2.5. Growth Measurements. Every 24 hours, from day one to day ten, each sample was mixed and 300 μL was removed.

TABLE 1: Relative dispersion of SWCNT preparations.

Fluorescence efficiency (nW/cm)	638 nm	690 nm	784 nm
SWCNT	−0.00299	−0.00062	−0.00126
SC:SWCNT	0.67997	0.40681	0.77064
DNA:SWCNT	1.09507	0.75541	1.6632

Fluorescence efficiency as calculated by ANFSoft (Applied Nanofluorescence; Houston, TX) as total fluorescence output divided by total absorbance on the principle that more well-dispersed SWCNTs have greater fluorescence output. Measurements are relative with greater positive values signifying greater dispersion in solution.

The optical density of this solution was measured at 750 nm with a Synergy 2 microplate reader and the software Gen5 1.06 (Biotek US; Winooski, VT). The optical density at 750 nm was also measured for prepared absorbance controls of HSM with each individual treatment. The optical density of algae was normalized by subtracting control from sample optical density. On the tenth day, optical density of chlorophyll β and chlorophyll α at 663 nm and 645 nm, respectively, was obtained. A portion of each sample was centrifuged and resuspended in methanol. This was centrifuged, and optical density of chlorophyll extracted in the liquid phase was measured at 663 nm and 645 nm as previously described. At the end of treatments, algae were placed onto microscope slides with a cover slip and imaged using a Leica ATC 2000 light microscope (Leica Microsystems; Wetzlar, Germany) and a TCA 5.0 microscope camera (Ample Scientific; Norcross, GA).

3. Results and Discussion

3.1. SWCNT Preparation and Characterization. Either DNA or SC was used to obtain well-dispersed SWCNT as previously described with poorly dispersed SWCNT obtained by sonication with no surfactant. Optical characterization of SWCNT preparations was performed to determine SWCNT dispersion in solution, chiral composition, and diameter. Efficiency of fluorescence output from SWCNT prepared with no surfactant was essentially zero and was much lower than that of the SC:SWCNT and DNA:SWCNT (Table 1). Additionally, fluorescence efficiency of DNA:SWCNT was greater than that of SC:SWCNT, suggesting DNA is more effective at dispersing individual SWCNTs than SC, which was expected [12, 24, 26]. Fluorescence efficiency is a relative measure of dispersion with the principle being that more well-dispersed SWCNTs have greater fluorescence output for a given concentration, which is measured by absorbance [31, 32]. By virtue of photoluminescence from both DNA:SWCNT and SC:SWCNT, fluorescence efficiency measurements suggest that the SWCNTs are well-dispersed in solution as expected.

Absorption (Figures 1(a) and 1(b)) and NIR fluorescence measurements (Figures 1(c) and 1(d)) were taken as previously described [34, 35]. SWCNTs sonicated in the absence of surfactant and subsequently centrifuged had an absorbance of essentially zero, further indicating that these were poorly dispersed. Additionally, SWCNT with no surfactant and not centrifuged showed no clear nanotube absorbance or fluorescence emission peaks, indicating that surfactant suspension and centrifugation remove most bundled SWCNT
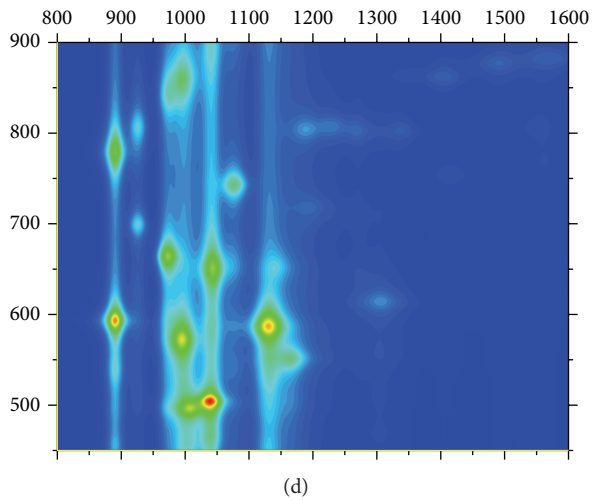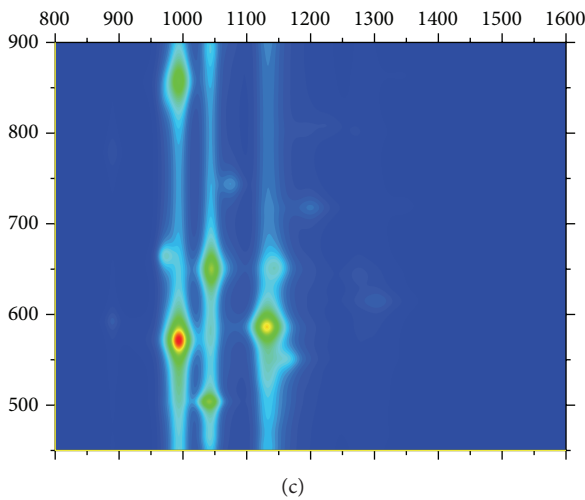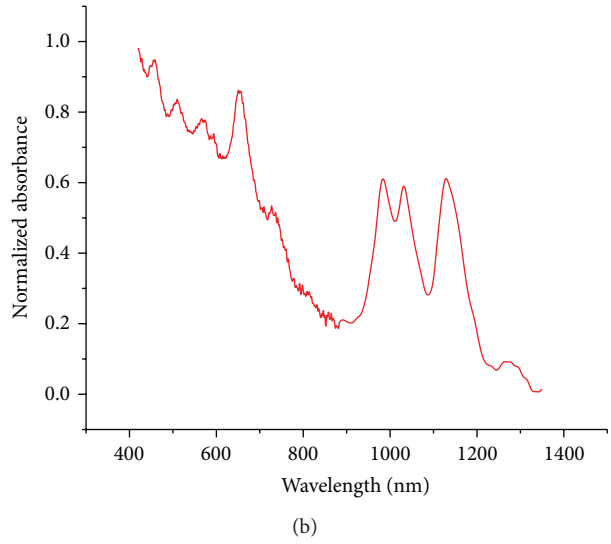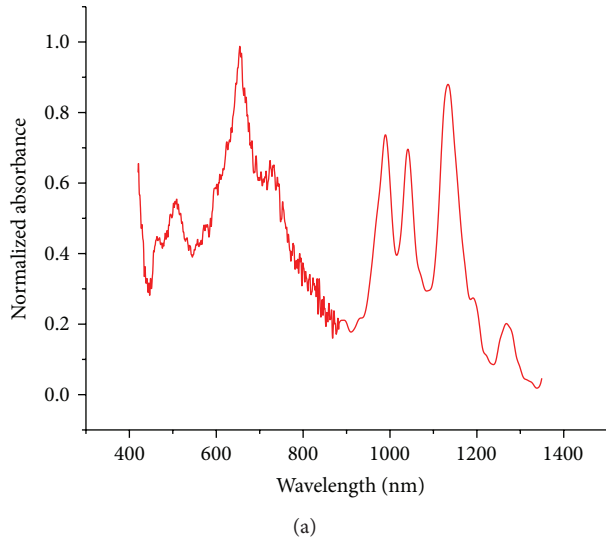
and most amorphous carbon. Based on an integrated fluorescence emission spectrum, the sample of mixed chirality was composed of largely (6,5), (8,4), and (7,5) SWCNTs, as is expected with CoMoCat SWCNT (Figure 1(e)). The DNA:SWCNT sample had an average SWCNT diameter of 0.83 nm, ranging from 0.6 to 1.4 nm (Figure 1(g)). The integrated fluorescence emission spectrum of this sample showed a majority of SWCNTs being of (6,5), (8,4), (7,5), (6,4), and (7,3) chirality (Figure 1(f)). The SC:SWCNT sample had an average diameter of 0.864 nm, with SWCNT being in the same range as DNA:SWCNT (Figure 1(h)).

3.2. Effects of SWCNT and Dispersion on Algal Growth. Results of *C. reinhardtii* growth with DNA:SWCNT and SC:SWCNT treatment of concentrations from 0.1 μg/mL to 100 μg/mL show no effects of DNA:SWCNT or SC:SWCNT on the optical density of the algae (Figure 2). For all cultures, a plateau in algal growth was reached in the sixth day. There was also no difference with treatment of 100 μg/mL sonicated DNA or SC in PBS alone.

After ten days of growth, absorbance of chlorophyll β showed no difference between either DNA:SWCNT or SC:SWCNT and their controls (Figure 3(a)). Additionally, there was no difference between DNA and SC dispersed SWCNT at any treatment concentration. Chlorophyll α absorbance at 645 nm showed similar results (Figure 3(b)). There was no difference between controls and treatments of DNA:SWCNT and SC:SWCNT at any treatment level.

Our finding of no toxicity from SWCNT is in contrast to previously reported data showing negative growth effects on algae for up to five days using 2 μg/mL SWCNT with no dispersant [18]. Nondispersed SWCNTs have also been shown to be toxic to bacteria [11]. Furthermore, a LC_{50} of 2.4 μg/mL has been obtained with *Daphnia magna*. This level and almost 50 times greater caused no growth effects in the current study. The stagnant growth conditions used in this study were not conducive to using nondispersed SWCNT due to hydrophobic interaction causing sedimentation; however, the previously reported studies are a comparative benchmark for their toxic effects. The use of well-dispersed SWCNT is environmentally relevant as well-dispersed SWCNTs are the most mobile fraction in the environment and agglomerated SWCNTs are known to associate with organic matter and become more well-dispersed over time [21–23]. The studies reported in this work suggest that there is no negative effect of well-dispersed SWCNT on algal growth.

From this work, it seems that strong dispersion of SWCNT in DNA or SC or the nontoxic nature of DNA and

(a)

(b)

(c)

(d)

Distribution of (n, m) species

Zigzag

Distribution of (n, m) species
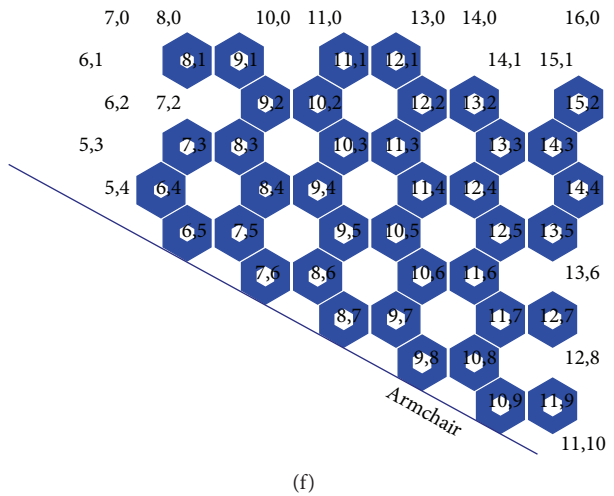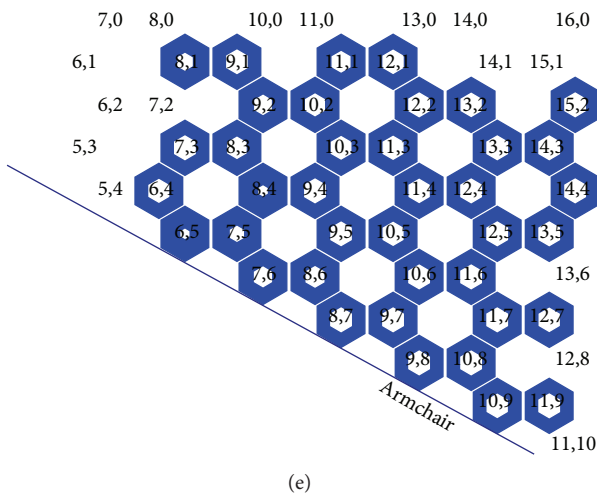
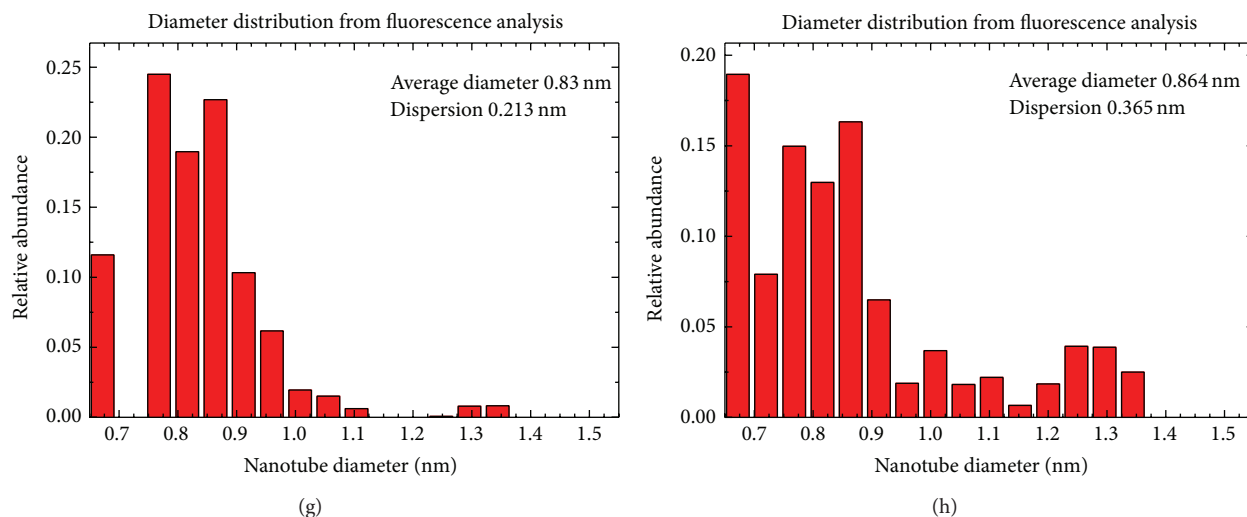Zigzag

(e)

(f)

FIGURE 1: Continued.

(g)

(h)

Figure 1: Characterization of DNA:SWCNT and SC:SWCNT. (a) and (b) Normalized absorbance of DNA:SWCNT and SC:SWCNT, respectively. (c) and (d) Synthesized fluorescence intensity plot of DNA:SWCNT and SC:SWCNT output, respectively. y-axis is fluorescence excitation wavelength (nm) and x-axis is fluorescence emission wavelength (nm). (e) and (f) Calculated chirality distribution of DNA:SWCNT and SC:SWCNT, respectively. (g) and (h) SWCNT diameter distribution of DNA:SWCNT and SC:SWCNT samples, respectively.
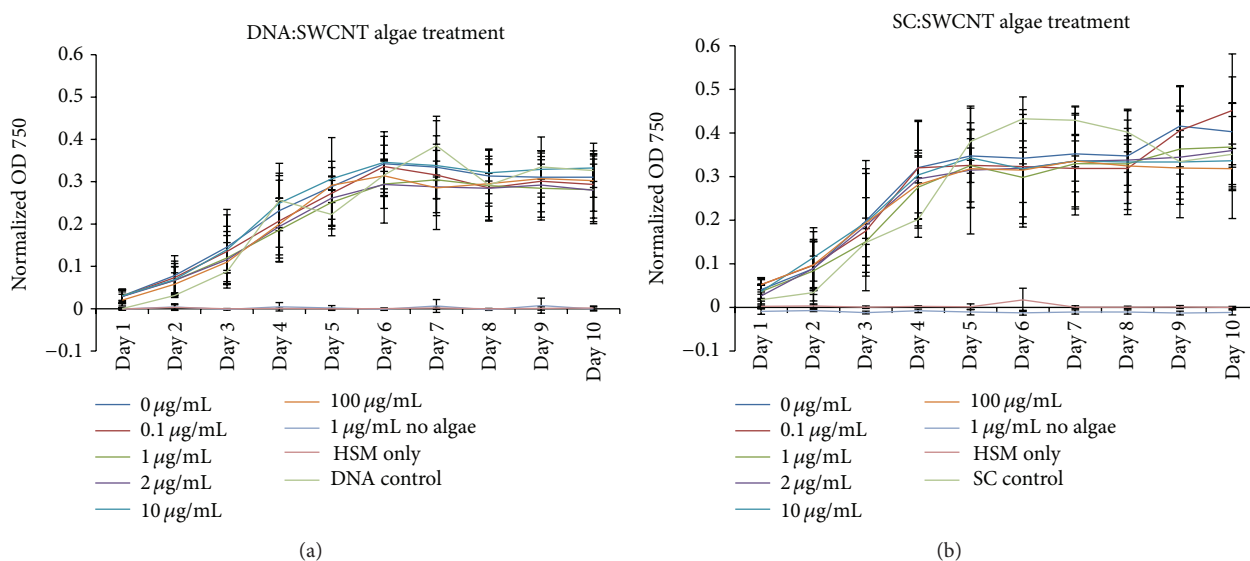

(a)

(b)

Figure 2: Ten-day growth curve of *Chlamydomonas reinhardtii* with SWCNT treatments or controls. (a) Treatment with DNA:SWCNT and (b) treatment with SC:SWCNT. Treatments were 0, 0.1, 1, 2, 10, or 100 μg/mL, DNA:SWCNT only control, media only control (HSM), or 100 μg/mL surfactant only control. Error bars for each point represent (+/−) standard deviation.

SC themselves creates SWCNTs that are not toxic. This is supported by previous work reporting that SWCNT toxic effects on the green algae *Pseudokirchneriella subcapitata* were lost with higher concentrations of the surfactant Gum Arabic [36]. In another study, Schwab et al. found that carbon nanotubes only partially dispersed by natural organic matter had no direct toxic effect but that agglomerated nanotubes have indirect effects [37]. Therefore, it is likely that very well-dispersed and nonagglomerated SWCNTs do not have any toxic effect, as is described herein.

In the current study, well-dispersed SWCNT did not have toxic effects on the model organism used. This is further supported by the fact that, phenotypically, the algae were not different than algae with no treatment (Figure 4). Results of chlorophyll content measurements show no difference due to DNA:SWCNT or SC:SWCNT at any level. The lack of toxic SWCNT effects is likely due to the well-dispersed nature of DNA:SWCNT and SC:SWCNT or the nontoxic SWCNT coating. Work with SWCNT well-dispersed in various nontoxic surfactants on rat liver cells and human lung
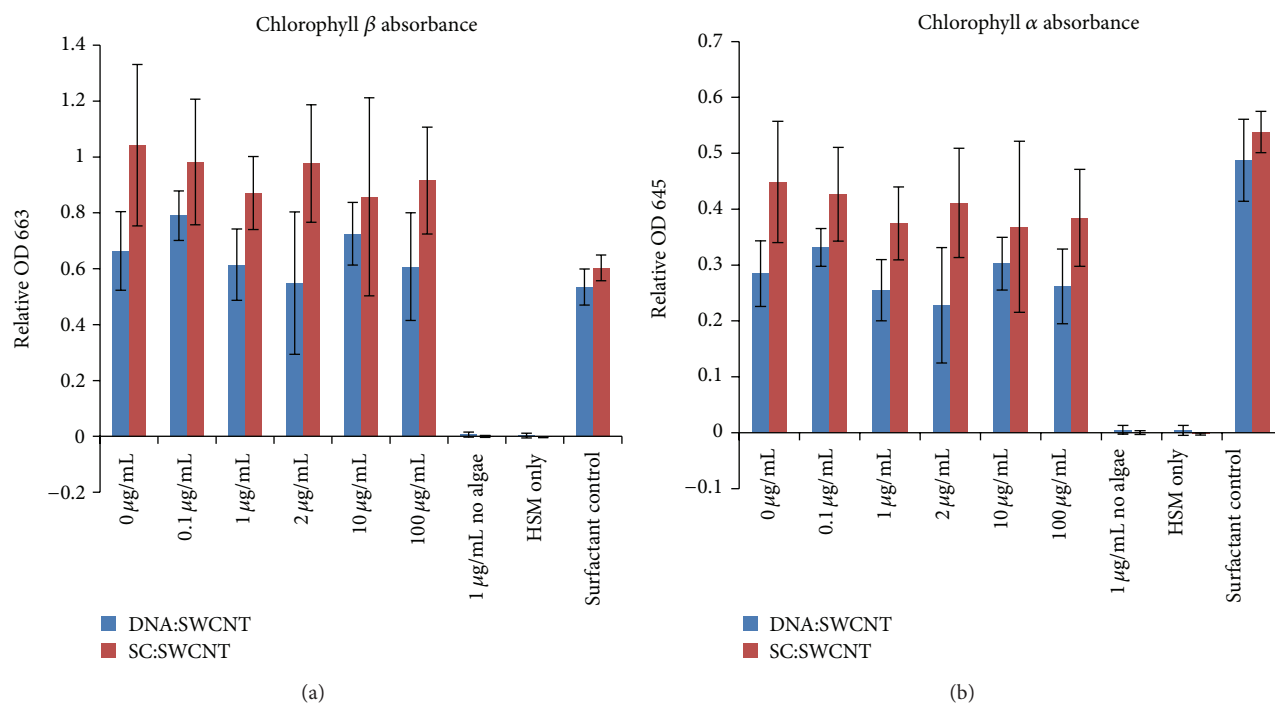
(a)

(b)

Figure 3: Extracted chlorophyll content for DNA:SWCNT and SC:SWCNT treatments. (a) Chlorophyll β content at OD 663 and (b) chlorophyll α content at OD 645. Treatments are 0, 0.1, 1, 2, 10, or 100 μg/mL, DNA:SWCNT or SC:SWCNT only control, media only control (HSM), or 100 μg/mL DNA or SC control. Error bars for each point represent (+/−) standard deviation.

(a)   (b)   (c)
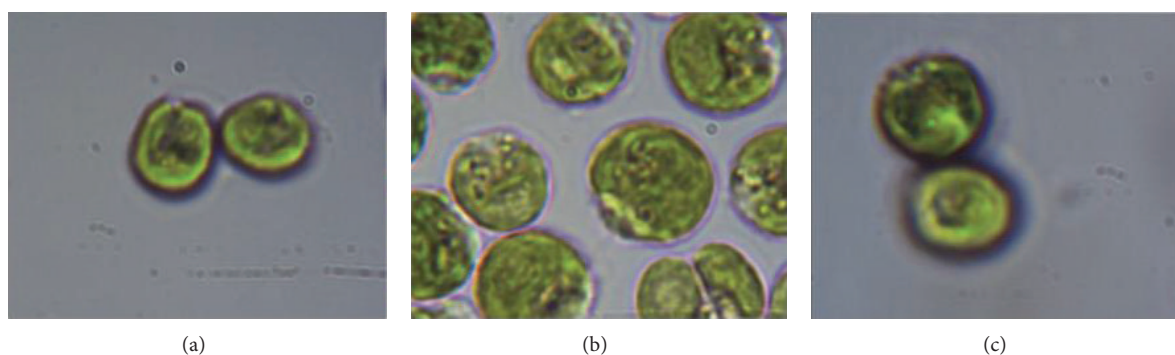
Figure 4: Micrograph of algae grown with SWCNT. Representative image of *Chlamydomonas reinhardtii* after ten days in culture with (a) no treatment, (b) 100 μg/mL DNA:SWCNT, and (c) 100 μg/mL SC:SWCNT with magnification at 1000x.

fibroblasts also showed no toxic effects [38, 39]. Furthermore, DNA:SWCNT and SC:SWCNT showed no toxic effect on human astrocytoma cells [40]. Therefore, it is possible that strong dispersion of SWCNTs with nontoxic coatings negates their toxicity.

4. Conclusions

This study presents findings that well-dispersed SWCNTs with DNA or SC do not show effects on growth, chlorophyll content, or phenotype of *Chlamydomonas reinhardtii* at concentrations of 0.1–100 μg/mL. This is in contrast to previous work that has shown that SWCNT can be toxic to algae,

bacteria, or other model organisms. However, this supports other research that suggests that there is no toxic effect of well-dispersed SWCNT on model organisms or mammalian cells. Thus, increasingly widespread use of SWCNT may not have toxic environmental impacts as well-dispersed SWCNTs are more mobile than the poorly dispersed fraction and well-dispersed SWCNT with nontoxic coatings may have minimal environmental consequences.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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