

# In Vitro Neurotoxicity Resulting from Exposure of Cultured Neural Cells to Several Types of Nanoparticles

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**ABSTRACT:** Laboratory and industrial production of various nanoparticles, single-walled nanotubes (SWNTs), fullerene (C60), cadmium selenide (CdSe) quantum dots, carbon black (CB), and dye-doped silica nanospheres (NSs), has greatly increased in the past 15 years. However, little research has been done to analyze the toxicity of these materials. With recent studies showing that nano-substances can cross the blood–brain barrier, we examined the neurotoxicity of these manufactured nanoparticles. By employing the rat PC-12 neuronal-like cell line as the basis for our studies, we were able to evaluate the toxicity caused by these five nanoparticles. The level of toxicity was measured by testing for cell viability using the lactate dehydrogenase (LDH) cell viability assay, morphological analysis of changes in cellular structures, and Western blot analyses of  $\alpha$ II-spectrin breakdown products (SBDP) as cell death indicators. Our results showed cytotoxicity in nondifferentiated PC-12 cells exposed to CB (10–100  $\mu$ g/mL), SWNTs (10–100  $\mu$ g/mL), C60 (100  $\mu$ g/mL), CdSe (10  $\mu$ g/mL), CB (500  $\mu$ g/mL), and dye-doped silicon NSs (10  $\mu$ g/mL). Exposure to higher concentrations (100  $\mu$ g/mL) of SWNTs, CB, and C60 increased the formation of SBDP150/145, as well as cell membrane contraction and the formation of cytosolic vacuoles. The incorporations of the nanoparticles into cell cytoplasm were observed using the fluorescent dye-doped NSs in both nondifferentiated and nerve growth factor (NGF)-differentiated PC-12 cells. When PC-12 cells are differentiated, they appeared to be even more sensitive to cytotoxicity of nanoparticles such as CB 10 nm (10–100  $\mu$ g/mL), CB 100 nm (10–100  $\mu$ g/mL), and CdSe (1–10  $\mu$ g/mL).

**KEYWORDS:** Nanoparticles, neurotoxicity, calpain, cell death

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## Introduction

Recent increases in the production of nanoengineered materials because of their unique qualities has led the U.S. National Science Foundation to predict that by 2015 \$1 trillion of nanoengineered materials will be marketed.<sup>1</sup> Already an estimated 700 types of nanoparticles, objects with at least one dimension of 100 nm or less, are currently sold in the United States.<sup>2</sup> For perspective, 1 nm spans about 10 hydrogen atoms, while a human hair is 70,000–80,000 nm thick. Because matter at this scale often behaves very differently from traditional bulk materials due to the fact that it is on the same scale as cellular machinery and because it is affected by laws of quantum physics in ways that larger bulk materials are not, they exhibit unique properties such as high reactivity, stability, and interesting optical effects. Although the science behind this phenomenon

is still under investigation, the commercial potential is already being exploited.

For example, nanosized versions of existing drugs are being developed.<sup>3</sup> The US Food and Drug Administration (FDA) approved marketing Abraxane, a nanoscale drug delivery system for metastatic breast cancer treatment.<sup>4</sup> This version encapsulates a potent anticancer drug in a water-soluble protein carrier. Other researchers are working on customizing treatments, such as nanoparticles that match the unique genetic profile of a patient's cancer cells in order to seek out and destroy them.<sup>5–7</sup> More than 60 drugs and drug delivery systems based on nanotechnology, and more than 90 medical devices or diagnostic tests, such as amphiphiles that regenerate injured nerve cells,<sup>8</sup> are already being tested. Neuroscience applications of nanotechnology involve investigating molecular, cellular, and physiological processes and research aimed at limiting and reversing neuropathological diseases.

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Despite the growth in the number of nano-products, companies and university laboratories that make nanoparticles have yet to fully characterize their toxicities or their molecular mechanisms. Until recently, it had been assumed that macro-size material and its nanosized equivalent had similar toxicological profiles. However, a small but growing body of research suggests the very properties that make nanomaterials useful, may include unwanted biological activity.<sup>9–13</sup>

Due to their size, nanoparticles can enter humans through the skin or lungs, and may even reach the brain. They also appear to easily pass through cell membranes into the cells where they may cause harm. Recently, researchers reported brain damage in a largemouth bass that swam in an aquarium laced with common carbon-based nanoparticles known as buckyballs or fullerenes.<sup>14,15</sup> In another study, rats inhaled fine carbon particles, which were later found to have entered their brain. They were believed to have been transported there via the olfactory nerve.<sup>16</sup> Although there is no conclusive evidence that these and other findings are neurotoxic, they do however raise significant concerns. To produce products without arousing undue alarm and further government interference, nanomaterial-based companies must effectively deal with the concerns about the effects of particles on the body. As of yet, no government agency has established safety guidelines specific to nanoparticles due to the paucity of research on the short- and long-term effects of nanoparticles on human and environmental health. However, the issue has not escaped their attention to the point that several government agencies are currently addressing this issue by increasing research funding on the toxicity and characteristics of nano-substances.<sup>17–20</sup>

The purpose of this study was to investigate the potential uptake and neurotoxicity of several types of nanoparticles in a neural cell line – rat PC-12 cells. This study focused on the following five different types of manufactured nanoparticles: carbon black (CB), single-walled nanotubes (SWNTs), fullerene, cadmium selenide (CdSe) quantum dots, and dye-doped silica nanospheres (NSs), as very little research has been conducted to analyze the toxicity of these materials.

## Materials and methods

SWNTs, fullerene (C60), CdSe quantum dots, and CB (5 and 100 nm) were obtained from Sigma. Green dye (Oregon Green 488)-doped silica NSs and Red dye (tetramethylrhodamine)-doped silica NSs were obtained from Dr. Waihong Tan (Department of Chemistry, University of Florida), and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) nuclear DNA staining solution was from Vector Co.

### *Cell culture and nanoparticle exposure*

Nondifferentiated rat pheocytoma PC-12 cells obtained from American type culture collection (ATCC) were cultured in Rosewell park memorial institute (RPMI) media supplemented with 5% fetal bovine serum and 10% heat-inactivated horse

serum. To differentiate the PC-12 cells, they were treated with 100 ng/mL nerve growth factor (NGF) for four days. Generally, nanoparticles were placed into the serum-free cell culture media at different concentrations for 24 hours, followed by end-point observations or cell processing.

### *Microscopic observation*

Phase-contrast and fluorescence microscopy examinations were conducted with a LEICA DM500 fluorescence microscope. Cell culture medium was vacuumed from the wells in the 12-well plate and replaced with a 3.7% formalin solution. After one minute, the formalin solution was removed and replaced with a 100% ice-cold methanol solution. After one minute, the methanol solution was removed from each well and replaced with Phosphate-buffered (PBS) solution. After 15 minutes, the PBS solution was removed from each well and replaced with DAPI nuclear DNA staining solution.

The cells in each well then underwent microscopic observation. Phase-contrast microscopy was applied to examine cell morphology. Fluorescence microscopy was used to examine nuclear morphology (DAPI dye). In the case of dye-doped silica NSs, because they naturally fluoresce, a fluorescence microscope was used to determine if the Nanoparticles (NPs) were incorporated into the cells.

### *Cell lysate collection and preparation*

Primary neuronal cells were collected and lysed for 90 minutes at 4°C with a lysis buffer containing 50 mM Tris (pH 7.4), 5 mmol/L Ethylenediaminetetraacetic acid (EDTA), 1% (v/v) Triton X-100, 1 mmol/L Dithiothreitol (DTT), and a Mini-Complete protease inhibitor cocktail tablet (Roche Biochemicals). The lysates were centrifuged at 10,000 g for 5 minutes at 4°C to remove insoluble debris and were then snap-frozen and stored at –80°C until use. Protein concentrations of cell or tissue lysates were determined via Bio-Rad DC Protein Assay (Bio-Rad).

### *SDS-PAGE, electrotransfer, and immunoblot analysis*

Protein-balanced samples were prepared in a sample loading buffer containing 0.25 mol/L Tris (pH 6.8), 0.2 mol/L DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled water. A quantity of 20 mg of protein per lane was loaded and then routinely resolved by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and separated proteins. These were laterally transferred to polyvinylidene fluoride membranes. Immunoreactive bands were detected by developing with biotin, avidin-conjugated alkaline phosphatase, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate. A 250–14 K rainbow molecular weight marker (RPN800E, GE Healthcare Bio-Sciences) was used to identify

the protein size, and alpha-fodrin ( $\alpha$  II-spectrin) (1:1,000, BML-FG6090, Enzo Life Sciences) was used to measure cell injury. Quantitative evaluation of protein levels was performed via computer-assisted densitometric scanning (NIH ImageJ, version 1.6 software).

#### Lactate dehydrogenase release assay

Promega's cytotoxicity kit was used to monitor lactate dehydrogenase (LDH) release as a colorimetric assay for cell death. LDH is a metabolic enzyme present only in the cellular cytoplasm. However, if the cells are injured or dead, LDH will be released into the cell culture medium. Thus, by monitoring the LDH release, one can quantify cell death directly.

#### Statistical methods

Significant difference between two groups was analyzed with two-sided unpaired Student's *t*-test. Results were considered to be statistically significant at  $P < 0.05$ .

## Results

The in vitro neurotoxicity of SWNTs, fullerene (C60), CdSe quantum dots, CB (10 and 100 nm), and dye-doped silica NSs (Oregon Green 488, or Red dye tetramethylrhodamine) were evaluated using both nondifferentiated rat PC-12 neural cells and NGF-differentiated PC-12 cells. The results are shown below.

#### Cytotoxicity of nanoparticles in nondifferentiated rat PC-12 neural cell line

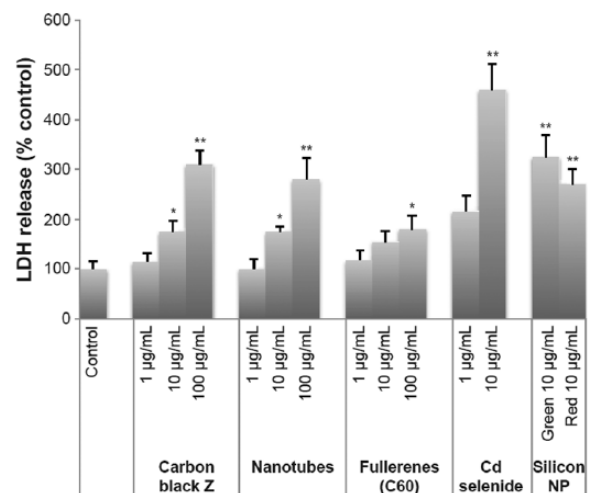
Nondifferentiated rat PC-12 neural cells were exposed to CB (5 nm), SWNTs, fullerenes C60, CdSe, and silica NSs (doped with Green dye = Oregon Green 488, or Red dye = tetramethylrhodamine) at different concentrations. We evaluated the toxicity caused by these five nanoparticles by using the LDH cell viability assay and morphological analysis of changes in cellular structures.

Our results showed that the particles were cytotoxic in nondifferentiated PC-12 cells when they were exposed to CB (10–100  $\mu\text{g}/\text{mL}$ ), SWNTs (10–100  $\mu\text{g}/\text{mL}$ ), fullerene C60 (100  $\mu\text{g}/\text{mL}$ ), CdSe (10  $\mu\text{g}/\text{mL}$ ), and dye-doped silica NSs (10  $\mu\text{g}/\text{mL}$ ; Figure 1).

In addition, bright-field phase-contrast microscopy showed contraction of cell membranes and formation of cytosolic vacuoles following exposure to C60, CB, and SWNTs (at 100  $\mu\text{g}/\text{mL}$  each; Figure 2).

#### Integrity of cytoskeleton protein alphaII-spectrin following NP-treated nPC-12 cells

Immunoblot analyses were examined for alphaII-spectrin breakdown products (SBDP) as an indicator of cell cytoskeleton integrity and cell death. Exposure to higher concentrations



**Figure 1.** Various nanoparticles causing cytotoxicity in nondifferentiated rat PC-12 cells, as measured by LDH release. LDH levels after 24-hour incubation with carbon black Z (5 nm), nanotubes, fullerenes (C60), cadmium selenide (CdSe), and silica nanospheres (doped with Green dye = Oregon Green 488, or Red dye = tetramethylrhodamine). Those that are significantly higher than control are indicated (Student's *t*-test, \*\* $P < 0.01$  or \* $P < 0.05$ ;  $N = 6$ ).

(100  $\mu\text{g}/\text{mL}$ ) of SWNTs, CB, and C60 resulted in increased formation of the calpain protease-mediated alphaII-SBDP of 150 and 145 kDa (SBDP150/145), compared to the loading controls (Figure 3).

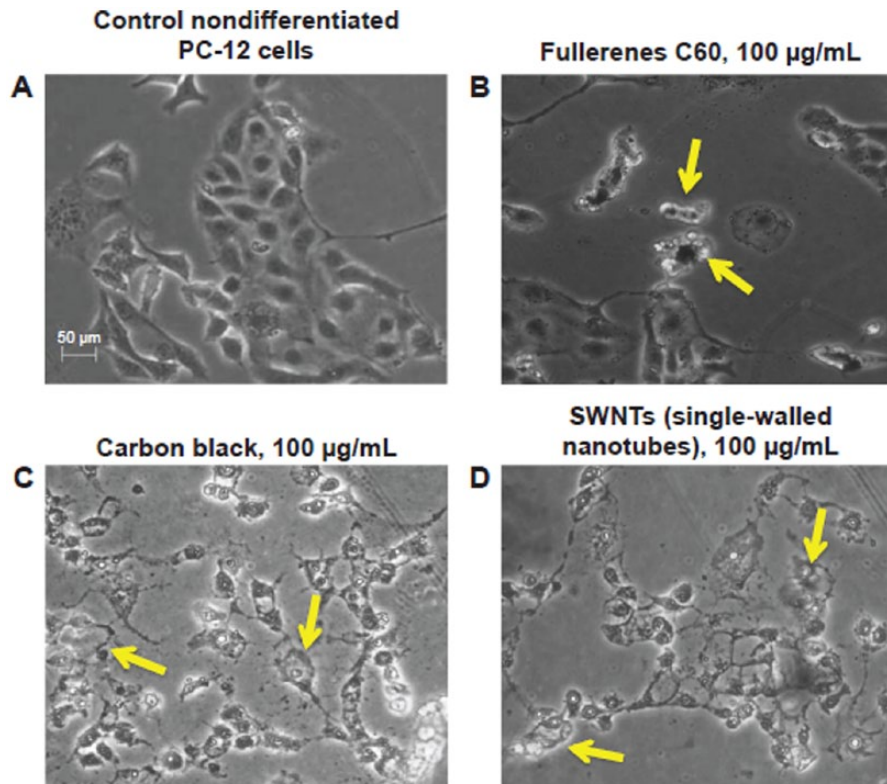
#### Incorporation of fluorescent dye-doped silica NSs into cytoplasm of nondifferentiated PC-12 cells

When nondifferentiated PC-12 cells were exposed to Oregon Green 488 dye-doped silica NSs (10  $\mu\text{g}/\text{mL}$ ) for 24 hours, the fluorescent dye-doped NSs (Oregon Green 488 Excitation 340 nm, Emission 460 nm) are found to be readily incorporated into cytoplasm of these cells (Figure 4). On higher magnification, by combining phase-contrast and Oregon Green fluorescence microscopy, we were able to show that the dye-doped particles were not only found throughout the cytoplasm but also appeared to localize to the perinuclear sites (Figure 5).

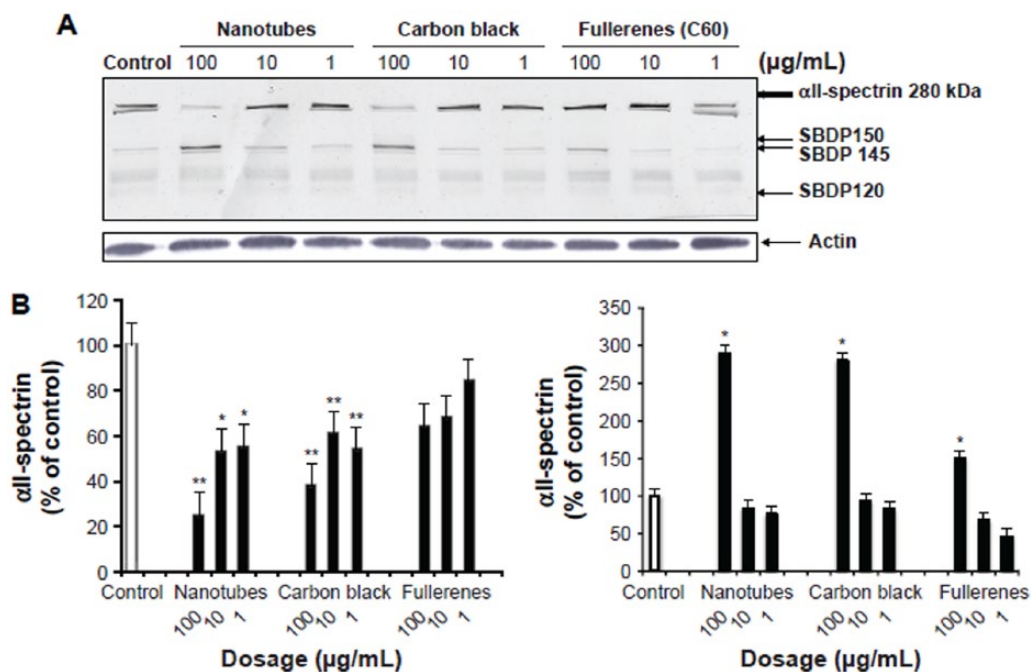
#### Cytotoxic effects of nanoparticles on NGF-differentiated PC-12 cells

Finally, to examine the effects in the more neuronal-like NGF-differentiated PC-12 cells, the cells were exposed to a 24-hour incubation with CB (5 nm (1–10  $\mu\text{g}/\text{mL}$ ) and 100 nm diameter (1–10  $\mu\text{g}/\text{mL}$ )) and CdSe NP (1–10  $\mu\text{g}/\text{mL}$ ). We found that at all concentrations, all three NPs appear to be cytotoxic to NGF-differentiated PC-12 cells, based on LDH release, and apparent dose responses were not observed (Figure 6).

Effects of various nanoparticles on NGF-differentiated PC-12 cells morphology were also examined. Differentiated PC-12 cells show fine and long processes (Figure 7A). CdSe NP (10  $\mu\text{g}/\text{mL}$ ) treated cells appeared to have abnormally shorten the processes (Figure 7B). Quantitation of the processes



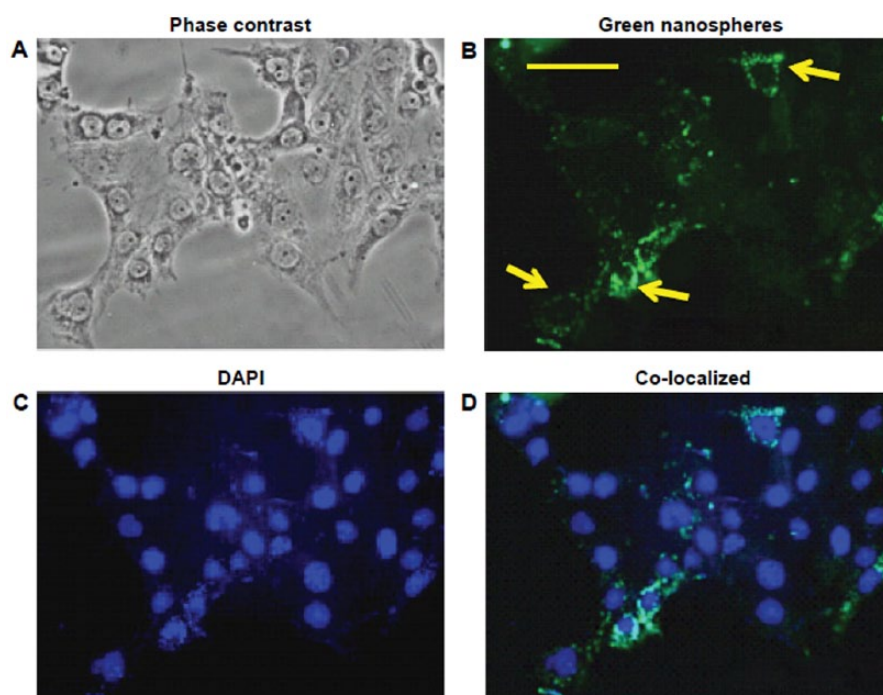
**Figure 2.** Rat nondifferentiated PC-12 cells morphology after exposure to various nanoparticles for 24 hours. (A) Control nondifferentiated PC-12 cells, (B) fullerenes (C60), 100 µg/mL, (C) carbon black, 100 µg/mL, and (D) single-walled nanotubes (SWNTs), 100 µg/mL. Yellow arrows indicate vacuole formation in NP-treated cells. Scale bar = 50 µm.



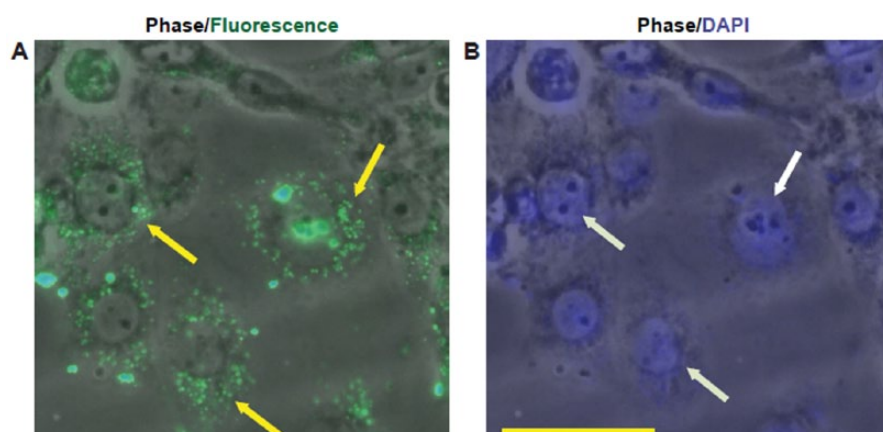
**Figure 3.** αII-spectrin and SBDP patterns in various nanoparticle exposures to nondifferentiated PC-12 cells. (A) Representative immunoblot for αII-spectrin. (B) Quantification of intact αII-spectrin reduction and increased SBDP145. Representative of three blots (Student's *t*-test, \*\**P* < 0.01 or \**P* < 0.05; *N* = 3).

indicated their significant difference between the control and the CdSE-treated samples (Figure 7C). The differentiated

PC-12 cells were also exposed to Tetramethylrhodamine (TMR)-dye-doped NSs (10 µg/mL) for 24 hours. By



**Figure 4.** Fluorescence images of nondifferentiated PC-12 cells exposed to Oregon Green 488 dye-doped silica nanospheres (10  $\mu\text{g}/\text{mL}$ ) for 24 hours. (A) Phase-contrast microscopy, (B) fluorescence microscopy (green), (C) DAPI nuclear stain (blue), and (D) merged image of NP fluorescence and DAPI. Yellow arrows indicate vacuole formation in NP-treated cells. Scale bar = 50  $\mu\text{m}$ .



**Figure 5.** Incorporation of fluorescent dye-doped silica nanospheres into cytoplasm of nondifferentiated PC-12 cells. (A) Combined phase-contrast and fluorescence microscopy (Excitation 340 nm, Emission 460 nm). Oregon Green 488 doped silica (10  $\mu\text{g}/\text{mL}$ ) found throughout the cytoplasm and perinuclear zone (green spots, shown with yellow arrows). (B) Corresponding phase contrast with nuclear DAPI staining showing location of cell nuclei (white arrow). Scale bar = 50  $\mu\text{m}$ .

combining bright-field phase-contrast views with fluorescent microscopy, we were able to show that TMR-NS nanoparticles are uptaken into the cytoplasmic compartment (Figure 8A and B). Under higher magnification, we found that NPs have a distinct perinuclear localization (Figure 8C and D).

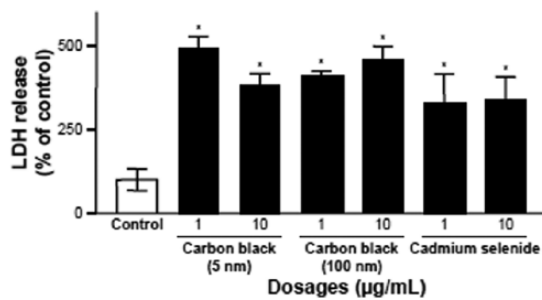
## Discussion

There is a rising interest in using nanomaterials and nanoparticles in both medical and nonmedical research and applications in recent years. Because recent studies showed that nano-substances can cross the blood-brain barrier, we wanted to examine what the potential uptake and neurotoxicity of

manufactured nanoparticles were. In this study, we tested fullerene (C60), CdSe quantum dots, CB, and dye-doped silica NSs in a rat PC-12 neuronal-like cell line in both their undifferentiated and differentiated forms. We evaluated the toxicity caused by these five nanoparticles using the LDH cell viability assay, morphological analysis of changes in cellular structures, and immunoblot analyses of  $\alpha\text{II-SBDP}$  as an indicator of cell death.

Our results showed cytotoxicity in nondifferentiated PC-12 cells exposed to CB (10–100  $\mu\text{g}/\text{mL}$ ), SWNTs (10–100  $\mu\text{g}/\text{mL}$ ), C60 (100  $\mu\text{g}/\text{mL}$ ), CdSe (10  $\mu\text{g}/\text{mL}$ ), and CB (10  $\mu\text{g}/\text{mL}$ ), and dye-doped silicon NSs (10  $\mu\text{g}/\text{mL}$ )

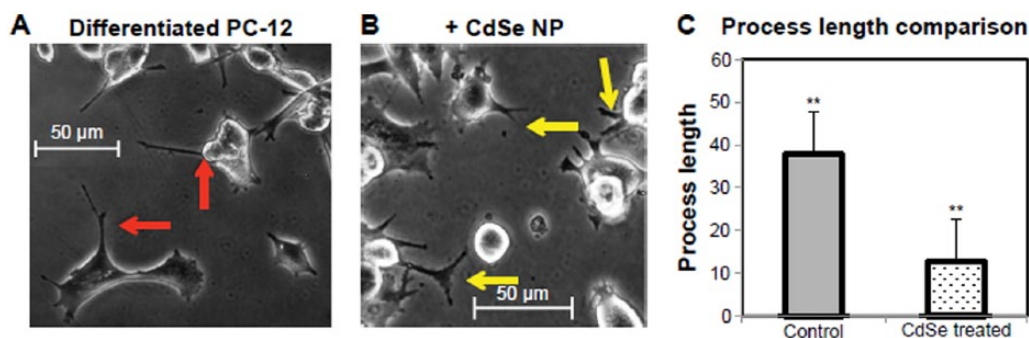
(Figure 1). Bright-field microscopy shows cell membrane contraction and cytosolic vacuole formation following exposure to several of the NPs examined (Figure 2). In addition, as exposure was increased to higher concentrations (100  $\mu\text{g}/\text{mL}$ ) of SWNTs, CB, and fullerene (C60), there was an increased formation of  $\alpha\text{II}$ -spectrin SBDP150/145 (Figure 3), indicative of cell cytoskeletal injury. The fluorescent dye-doped NSs appeared to be readily incorporated into cytoplasm of both the nondifferentiated and



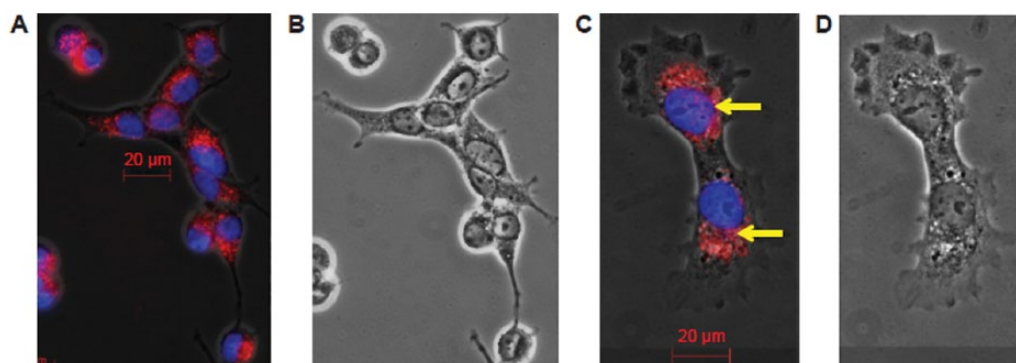
**Figure 6.** Cytotoxic effects of various nanoparticles on NGF-differentiated PC-12 cells. LDH release levels after 24-hour incubation with carbon black (5 and 100 nm diameter) and cadmium selenide NPs were significantly higher (at both 1 and 10  $\mu\text{g}/\text{mL}$ ) than control NGF-differentiated PC-12 cells (Student's *t*-test, \* $P < 0.01$ ).

NGF-differentiated PC-12 cells (Figures 4, 5, and 8). Finally, NGF-differentiated PC-12 cells appear to be more sensitive to cytotoxicity induced by several of the nanoparticles CB 10 nm (1–10  $\mu\text{g}/\text{mL}$ ), CB 100 nm (1–10  $\mu\text{g}/\text{mL}$ ), and CdSe (1–10  $\mu\text{g}/\text{mL}$ ), based on LDH release and cell morphology (Figures 6 and 7).

This study's hypothesis was confirmed: nanoparticles at high enough concentrations are toxic to cells that model brain tissue. The higher the concentration of each nanoparticle, the more the cytotoxic it was. As might be expected, several of the nanoparticles were more cytotoxic than others. The two most toxic nanoparticles tested were CdSe and CB. The mechanisms of neurotoxicity induced by these nanoparticles are still under investigation. In previous studies, CdSe nanoparticles showed elevation of cytoplasmic calcium levels and impairment of functional properties of sodium channels in rat primary cultured hippocampal neurons.<sup>21</sup> CB nanoparticles showed increased glutamate and Adenosine triphosphate (ATP) release by activating connexin and pannexin hemichannels in cultured astrocytes.<sup>22</sup> Our results indicated that the nanoparticles of nanotube, CB, and C60 caused in vitro neurotoxicity potential by inducing  $\alpha\text{II}$ -SBDP of SBDP150/145, which have been demonstrated in previous studies as biomarkers for compound-induced neurodegeneration and neurotoxicity in rat.<sup>23–25</sup> While SBDP150/145/120 are useful as



**Figure 7.** Effects of CdSe on NGF-differentiated PC-12 cells morphology. (A) Differentiated PC-12 cells that show fine and long processes (red arrows). (B) Cadmium selenide NP (10  $\mu\text{g}/\text{mL}$ ) treated cells have shortened and abnormal processes (yellow arrows). (C) Process length comparison of the control and CdSe-treated differentiated PC-12 cells. Scale bar = 50  $\mu\text{m}$ .



**Figure 8.** NGF-differentiated PC-12 TMR-dye-doped nanospheres (10  $\mu\text{g}/\text{mL}$ ) for 24 hours. (A, C) Phase contrast overlaid with fluorescent image of TMR-NS (red) and nuclear dye DAPI (blue). (B, D) Phase-contrast microscopy alone (scale bar = 20  $\mu\text{m}$ ). (C, D) Higher magnification shows that NP has perinuclear localization (yellow arrows).

markers for in vitro evaluation of NP-induced neurotoxicity in our current studies, they are potential biomarkers to assess the NP-induced neurotoxicity in vivo.

In this study, NP appears to be toxic to both nondifferentiated and differentiated PC-12 cells but with different sensitivities. In differentiated PC-12 cells, we found that even 1 ng/mL of CB and CdSe NPs were toxic; they were not toxic to nondifferentiated PC-12 cells. These data suggest that NPs might be more toxic to differentiated neuronal cells than their nondifferentiated counterparts since the cells are more like neurons in vivo. Nondifferentiated PC-12 cells are inherently resistant to chemicals and typically require high concentrations to display effects, while NGF-differentiated PC-12 cells have been useful for examining the chemical effects at specific neurodevelopmental stages including initial cell replication, the transition to differentiation, and the expression of specific neurotransmitter phenotypes. Our data indicated that CB and CdSe NP induced more toxic effects in NGF-differentiated PC-12 cells, which was more significant than their nondifferentiated counterparts. The neurocytotoxicity of NGF-differentiated PC-12 cells induced by CB was observed without significant difference, regardless of its particle size (5 and 100 nm).

These results suggest that these unmodified nanoparticles at certain levels are potentially toxic to neural cells. For example, the results suggest that it is important to regulate the concentration of the nanoparticles if they are to be used as a shuttling device for certain medicines. If these toxic issues are not considered, then those medicines that rely on NP could possibly be harmful to a person's neurons. High concentration of metal ions released from the NP may affect cell morphology, viability, and membrane integrity. For example, Ren et al<sup>26</sup> showed that high Zn (2+) released from zinc nanoscale metal organic frameworks (MOFs) significantly changed the membrane integrity of PC-12 cells through regulation of neurotrophin signaling pathway-associated GAP-43 protein. Therefore, this and other findings further highlight the importance of cytotoxic evaluation of NPs before their biomedical applications.

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## Author Contributions

Conceived and designed the experiments: SFL, JW, JG, KKWW. Analyzed the data: SFL, JW, JG, MOA, KKWW. Wrote the first draft of the manuscript: JW, JG, MOA, KKWW. Contributed to the writing of the manuscript: JW, JG, MX, KKWW. Agree with manuscript results and conclusions: SFL, JW, JG, MOA, MX, KKWW. Jointly developed the structure and arguments for the paper: SFL, JW, JG, MOA, MX, KKWW. Made critical revisions and approved final version: MX, KKWW. All authors reviewed and approved of the final manuscript.

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