Role of Reactive Oxygen Species in Nanotoxicity

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Abstract:

Though the emergence of the nanotechnology field has increased the incorporation of nanomaterials into commercially available products, we have limited understanding of how its widespread applications can affect us personally. This project focused on observing the presence of reactive oxygen species (ROS) in mouse fibroblast model cells when exposed to nano-titanium dioxide ($nTiO_2$) utilizing various fluorescent probe assays including 4-((9-acridinecarbonyl) amino)-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO-9-AC) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). ROS can cause oxidative stress in cells, which may lead to cell dysfunction, mutation, or death. Cellular uptake of the nanoparticles was studied using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) analysis to correlate ROS generation with cellular internalization of $nTiO_2$, followed by a commonly used cellular viability assay which showed insignificant difference in cell viability; the elevated uptake and increased ROS levels may have harmful effects on cell function of living cells.

Experimental Procedure:

Anatase titanium dioxide nanoparticles were made by a sol-gel synthesis using isopropyl alcohol and titanium isopropoxide that were stirred and chilled for 30 minutes while a nitric acid catalyst was added; then the mixture was refluxed for 24 hours. Samples were dialyzed over a 5-10 day period followed by aging in an acid digestion bomb for 48 hours in a 200°C oven. Finally, the aged nanoparticle suspension was washed with ethyl alcohol and dried [1]. Nanoparticles were characterized using x-ray diffraction to determine their crystalline structure, dynamic light scattering to determine particle aggregation, and transmission electron microscopy to determine particle size.

ICP-AES was performed to quantify the number of nanoparticles internalized by cells after incubation with $nTiO_2$ at a concentration of 100 μg/ml for 24, 48, and 72 hours. Cells were rinsed three times with PBS and removed from the Petri® dish using trypsin enzyme. The Petri dish was scraped and the trypsin-cell suspension was put in a centrifuge tube with 1 mL of 2:1 H_2SO_4:HNO_3 digest solution in 3 mL H_2O and sonicated for one hour.

For the fluorescent assays, 3T3 mouse fibroblasts were plated in a clear bottom 96-well plate at a density of 10^4-10^5 cells/well and exposed to the fluorescent probe. TEMPO detects superoxide and hydroxyl radical while H_2DCFDA detects superoxide, hydroxyl radical, peroxyl radical, and singlet oxygen. After a one hour incubation period with the fluorescent probe, the probe was removed and the cells were rinsed once with PBS. Cells were then exposed to $nTiO_2$ at various concentrations (12.5, 25, 50, 100, 200, and 400 μg/ml) for 24 and 48 hours. For the positive controls, malachite green—a superoxide and hydroxyl radical generator, and rose bengal—a singlet oxygen generator, were used. After incubation, the $nTiO_2$ and media were removed, cells rinsed twice with PBS, and a uniform volume of PBS added to each well. Fluorescence intensity was then measured with a fluorescent plate reader.

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed in 24 well plates to assess cell viability. 3T3 cells were again incubated with the various concentrations of $nTiO_2$ listed above, for 24, 48, and 72 hours. Following incubation, the media and $nTiO_2$ were removed, cells rinsed three times with PBS, and incubated with MTT in cell media for two hours. The MTT was removed and DMSO was added to each well to dissolve the formazan crystals. After 20 minutes, optical density was measured at λ = 570 nm, and the absorbance was correlated to the percentage of viable cells.

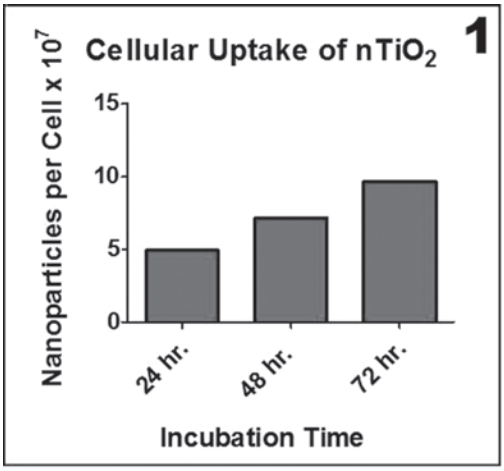
Results and Conclusions:

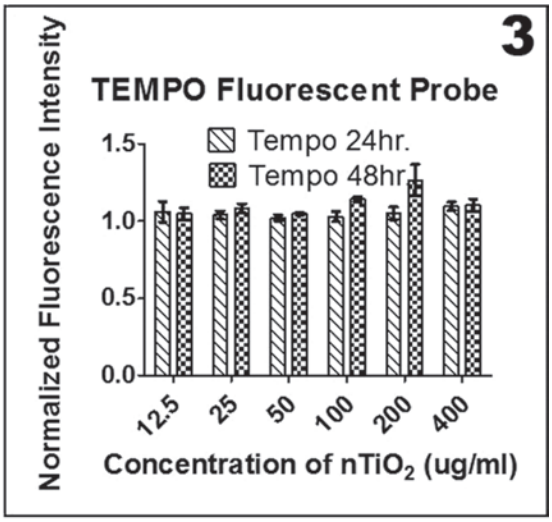
Results from ICP analysis (Figure 1) show that the longer the cells were exposed to $nTiO_2$, the more nanoparticles the cells internalized. We hypothesized that longer incubation time, and more cellular internalization of $nTiO_2$, would lead to more ROS generation.

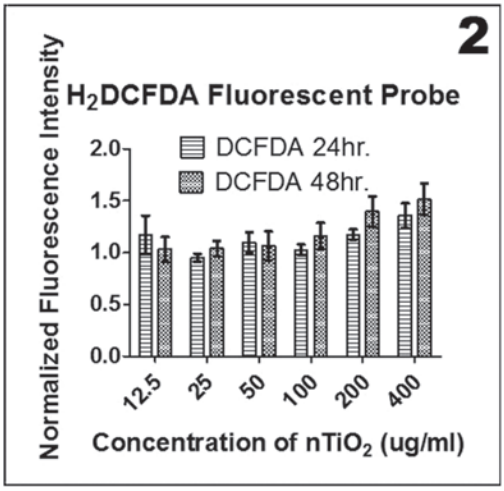
Linear regression analysis of our fluorescent probe assays (Figures 2 and 3) showed that there was a significant

increase in ROS as the concentration of $nTiO_2$ increased, leading us to assume greater oxidative stress on the cell and to hypothesize that this would lead to a decrease in cellular viability. However, statistical analysis (by student t-testing) shows that there is no significant difference between 24 and 48 hour incubation times, meaning that our previous hypothesis about longer incubation correlating to increased ROS production is false. In the future, we would like to extend incubation times past 48 hours, as this might show a more obvious trend.

Results from the cell viability assay (Figure 4) show statistically insignificant change in cell viability. This means that the increased concentration of $nTiO_2$, as well as varying exposure times, is not killing the cells, negating our hypothesis.







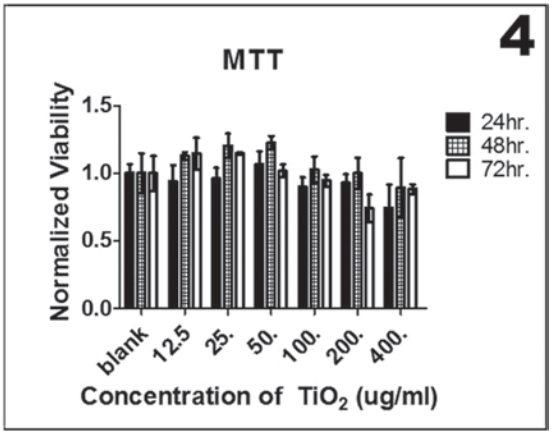


Figure 1: Cellular internalization of $nTiO_2$ increases with incubation time.

Figure 2: Increase in fluorescence intensity indicates an increase in ROS production as $nTiO_2$ concentration increases.

Figure 3: Linear regression analysis shows significant increase in fluorescence intensity as $nTiO_2$ concentration increases.

Figure 4: There is no significant decrease in cell viability over time or as concentration of $nTiO_2$ increases.

Future Work:

We would like to compare these results to those of similar assays performed on the immortalized mast cell line rat basophilic leukemia (RBL) cells. Mast cells play a critical role in immune response, and thus are an interesting, relevant cell for nanotoxicity studies.

Amperometry studies are currently underway to determine the effects of ROS on cell exocytosis. The results of these experiments will allow us to make predictions about overall cell health and function due to the internalization of $nTiO_2$ and increased amounts of ROS generated.

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References:

[1] Titanium Dioxide Nanoparticles: Effect of Sol-Gel pH on Phase Composition, Particle Size, and Particle Growth Mechanism, S. L. Isley and R. L. Penn (2008), Journal of Physical Chemistry C, 112, 4469-4474.